Detection of neurofilament-H in serum as a diagnostic tool to predict injury severity in patients who have suffered mild traumatic brain injury

Clinical article

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Object. In previous studies of traumatic brain injury (TBI), neural biomarkers of injury correlate with injury severity and predict neurological outcome. The object of this paper was to characterize neurofilament-H (NFL-H) as a predictor of injury severity in patients who have suffered mild TBI (mTBI). Thus, the authors hypothesized that phosphorylated NFL-H (pNFL-H) levels are higher in mTBI patients than in healthy controls and identify which subjects experienced a more severe injury such as skull fractures, intracranial hemorrhaging, and/or contusions as detected by CT scans.

Methods. In this prospective clinical study, blood (8 ml) was collected from subjects (n = 34) suffering from mTBI (as defined by the American Congress of Rehabilitation and Glasgow Coma Scale scores between 13 and 15) at Parkland Hospital, Dallas, Texas, on Days 1 and 3 after injury). Additional clinical findings from the CT scans were also used to categorize the TBI patients into those with and those without clinical findings on the scans (CT+ and CT− groups, respectively). The serum levels of pNFL-H were measured using the enzyme-linked immunosorbent assay.

Results. Compared with healthy controls, the mTBI patients exhibited a significant increase in the serum levels of pNFL-H on Days 1 (p = 0.00001) and 3 (p = 0.0001) after TBI. An inverse correlation was observed between pNFL-H serum levels and Glasgow Coma Scale scores, which was significant. Additionally, using receiver operating characteristic curve analysis to compare the mTBI cases with controls to determine sensitivity and specificity, an area under the curve of 100% was achieved for both (p = 0.0001 for both). pNFL-H serum levels were only significantly higher on Day 1 in mTBI patients in the CT+ group (p < 0.008) compared with the CT− group. The area under the curve (82.5%) for the CT+ group versus the CT− group was significant (p = 0.021) with a sensitivity of 87.5% and a specificity of 70%, using a cutoff of 1071 pg/ml of pNFL-H in serum.

Conclusions. This study describes the serum profile of pNFL-H in patients suffering from mTBI with and without CT findings on Days 1 and 3 after injury. These results suggest that detection of pNFL-H may be useful in determining which individuals require CT imaging to assess the severity of their injury.

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Key words • mild traumatic brain injury • neurofilament-H • serum

E ach year in the United States alone, approximately 1.8 million people experience a traumatic brain injury (TBI) event that requires hospitalization. Of these TBIs, about 75% are mild traumatic brain injuries (mTBIs), such as sports-related concussions, motor vehicle collisions, and falls.4

Abbreviations used in this paper: AUC = area under the curve; ELISA = enzyme-linked immunosorbent assay; GCS = Glasgow Coma Scale; GFAP = glial fibrillary acidic protein; MAMBA = Mild and Moderate TBI Biomarker (study); mTBI = mild TBI; NFL-H = neurofilament-H; NSE = neuron-specific enolase; pNFL-H = phosphorylated NFL-H; ROC = receiver operating characteristic; TBI = traumatic brain injury.

After the initial blow to the head, a secondary injury in the brain may persist for longer periods of time.5,15,19,30 This secondary brain injury consists of excitotoxicity, oxidant injury, mitochondrial dysfunction, inflammation, and subsequent cell death that begins almost immediately after the primary injury.6,10,13,32 With respect to mTBI, to predict neurological outcome after mild, moderate, and severe TBI, the detection of fluid-based neural biomarkers such as neuron-specific enolase (NSE), tau, ubiquitin C-terminal hydrolase, spectrin, glial fibrillary acidic protein (GFAP), and S100B in serum have been shown to correlate with a number of outcomes.2,8,11,12,16–18,21,23,24,27,31,34 Another protein, neurofilament, is detectable in se-
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rum at both short and long time points after moderate/severe TBI in humans and correlates with injury severity in both children and adults, and may be useful in assessing mTBI as indicated by recent data collected from animal studies. Neurofilaments are instrumental in maintaining the diameter of both axons and dendrites and are an integral part of forming synapses and neurotransmissions. In people suffering from TBI and subsequent calcium influx, neurofilament-H (NFL-H) is subsequently phosphorylated (pNFL-H), resulting in the accumulation of pools of dysfunctional pNFL-H and a reduction in the integrity of the axons.

A major problem with identifying and treating mTBI is that a large number of individuals in this TBI population are not aware that they had a significant head injury or they might refuse to seek medical attention. By measuring the blood levels of pNFL-H in mTBI victims, we believe that this biomarker will first identify which individuals suffered a head injury and might also lead to the accurate detection of more serious forms of TBI that require medical attention/hospitalization or prolonged rest.

In this prospective clinical study, the aim was to elucidate whether pNFL-H is a sensitive predictor of brain injury in TBI patients at later time points, such as 24 and 72 hours after injury. As a secondary aim of the project, we wanted to determine if pNFL-H is a sensitive biomarker to detect injury severity up to 3 days after injury. Utilization of a highly sensitive/specific biomarker for determining which individuals should undergo CT scanning at later time points is clinically relevant. Since a majority of TBI victims do not present to the emergency department immediately after injury, this biomarker may also be used to decide which individuals should seek medical attention and undergo subsequent CT scanning if the injury occurred a day or two later.

Methods

The purpose of this prospective clinical study (Mild and Moderate TBI Biomarker [MAMBA] study) was to measure the serum levels of pNFL-H in patients with mTBI at Days 1 and 3 after injury. Since S100B and NSE are modest predictors of injury severity within the first 24 hours, we hypothesize that pNFL-H predicts injury severity at time points longer than 24 hours. We propose that detection of this biomarker will predict injury severity and lead to the recommendation for subsequent CT scanning to identify skull fractures, hematomas, and lesions. Prior to initiating any study-specific procedures, the institutional review board at the University of Texas Southwestern Medical Center (Dallas, Texas) approved this study.

Enrollment Procedures

As defined by the American Congress of Rehabilitation, patients with an mTBI consisting of periods of loss of consciousness, loss of memory before or after the event, altered mental status, and/or neurological deficits that are acute or chronic were enrolled into this TBI biomarker study. The Glasgow Coma Scale (GCS) scoring system was also used to identify the mTBI patients. TBI patients with a GCS score between 13 and 15 who were admitted to Parkland Hospital (Dallas, Texas) were identified and screened using our patient database. Both men and women between the ages of 18 and 50 years with an mTBI were screened. Patients with penetrating injuries or those included in an interventional clinical trial were excluded. Full written informed consent was obtained from the mTBI patients (n = 34) who met our inclusion criteria, and these patients were enrolled in this observational fluid-based biomarker study. Demographic information was obtained from the patient’s medical chart and the patient or family. In addition, as determined by a board-certified neuroradiologist, we documented whether the patient had abnormalities on the CT scan. A scan was deemed to be positive if there were evidence of skull fractures, subdural/epidural/subarachnoid hemorrhaging, edema, and/or confusions. Blood was also collected from noninjured healthy control subjects (n = 28) between the ages of 18 and 50 years for comparison between control/baseline subjects and mTBI patients. The subject information and research chart were kept in a locked file cabinet in the research office (located on the campus of the University of Texas Southwestern Medical Center). Only study investigators and coordinators were given access to the subject’s file.

Blood Collection/Storage

Once informed consent was obtained from the patient, blood was collected on Day 1 (18–24 hours) or Day 3 (66–72 hours) after injury. In brief, at each time point 8 ml of blood was collected, and, after clotting, the sample was centrifuged at 4°C for 10 minutes at 1500 rpm. The serum was collected and aliquoted into 1-ml tubes and immediately frozen at −80°C.

Detection of pNFL-H

The serum levels of pNFL-H were measured using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (EMD Millipore). The range of detection is 0.0293 ng/ml to 15 ng/ml. The coefficient of variance of the standard samples is about 5%. In brief, 100 µl of each sample was loaded onto each well. Each sample was tested in triplicate in a 96-well plate. The plate was incubated for 2 hours at room temperature, washed, and 200 µl of the substrate was added for 90 minutes at room temperature. After 90 minutes, the absorbance was detected at 405 nm. The values were compared with the standard curve, and the concentration was determined. Serum samples from a total of 34 mTBI patients and 28 healthy controls were analyzed.

Statistical Analysis

The data collected from mTBI patients were compared with control subjects to determine if there was an increase in serum levels of pNFL-H within this brain injury group. Differences between subjects with (CT+) and without (CT−) clinical findings on CT scans were also analyzed. Data derived from the ELISA test were transformed using log base of 10. The Student t-test analysis was used to demonstrate that both CT− and CT+ groups had elevated serum pNFL-H compared with the control group.

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A 2-way ANOVA was used to compare log-transformed serum pNFL-H levels for CT− and CT+ groups on Days 1 and 3. The receiver operating characteristic (ROC) curve analysis was used to find a serum NFL-H cutoff that would best predict CT− and CT+ groups for Days 1 and 3, separately. The area under the curve (AUC), sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio, and accuracy (cases correctly classified) were provided. Assumptions for the parametric analyses (distribution and variance) were reviewed; when using log base 10 transformations no violations to the assumptions were noted. The IBM SPSS Statistics (version 20, IBM) and GraphPad software (GraphPad Software Inc.) were used to analyze these data. All statistical tests were 2-tailed and significance was set to p < 0.05. The data are presented as a bar/line graph depicting the mean ± SEM.

Results

Patient Demographics/Characteristics

The demographic and clinical characteristics are summarized in Table 1. The mean ± SD values are listed for age. The characteristics documented in Table 1 include age, sex, mechanism of injury, and GCS score in the emergency department. Of the mTBI patients who were admitted to the hospital, 47% of subjects (n = 16) had normal findings on CT scans (CT− group), and intracranial findings were documented on the CT scans of 53% (n = 18; CT+ group). No difference in the ages between controls, the CT− group, and the CT+ group were observed. No significant difference was observed between the CT− and CT+ groups with respect to age, sex, or mechanism of injury. The CT+ group had significantly (p = 0.07) more patients with a GCS score of 13 (n = 10) on arrival to the emergency department than the CT− group (n = 2).

Patients With mTBI Exhibited a Significant Increase in Serum pNFL-H Levels Compared With Noninjured Controls

On Days 1 and 3 after mTBI, blood was collected from 18 and 16 patients who were enrolled in the MAMBA study, respectively. The mean serum pNFL-H levels for the noninjured control subjects were 17.86 ± 4.4 pg/ml, which was approximately 100 times lower than the mean values for the mTBI patients on Days 1 (mean 2290 pg/ml, median 1850 pg/ml, range 120–8000 pg/ml) and 3 (mean 1739, median 360 pg/ml, range 89–6750 pg/ml). When comparing the mean levels of control versus mTBI serum pNFL-H by day (Day 1 and Day 3), the levels of pNFL-H in the mTBI group were significantly higher on Day 1 (p = 0.0001) and Day 3 (p = 0.0001) (Fig. 1). Also, there is a negative correlation (r = −0.49) between GCS score and pNFL-H serum levels. We found that a large number of the mTBI patients with increased levels of pNFL-H in the serum who were admitted to Parkland Hospital had a GCS score of 13 (Fig. 2).

Serum pNFL-H Levels Are Significantly Elevated in mTBI Patients in the CT+ Group

Mean levels for log-transformed serum pNFL-H levels for the CT− and CT+ groups were compared using a 2-way ANOVA. After analysis, compared with the non-
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injured control group, the levels of pNFL-H in the serum of the CT– group were significantly higher on both Days 1 (p < 0.000009) and 3 (p < 0.001) after TBI. With respect to the CT+ group, compared with the control subjects, a significant increase on Days 1 (p < 0.000003) and 3 (p < 0.000001) was observed. The CT– and CT+ groups were significantly different (p < 0.008) on Day 1 after injury. On Days 1 and 3 after injury, the CT– group had mean pNFL-H values of 855.3 ± 328.4 and 669.8 ± 468.2 pg/ml, respectively. The CT+ group had a mean serum pNFL-H value of 4083.7 ± 905 and 2428 ± 809.7 pg/ml on Days 1 and 3 after injury, respectively. Neither day (1 vs 3) within each group (CT– or CT+) was significant; however, the mean values for Day 1 (CT– group, 855.3 pg/ml; CT+ group, 4083.7 pg/ml) were higher than those for Day 3 (CT– group, 669.8 pg/ml; CT+ group, 2427.7 pg/ml) (Fig. 3).

The ROC curve analysis was used to determine if a serum pNFL-H cutoff level could be used to identify injured subjects and categorize individuals into the CT– or CT+ group. When comparing healthy controls with all mTBI subjects, for Day 1, the AUC was 1.0. Using a cutoff value of 110.5 pg/ml, a sensitivity of 100% and specificity of 100% were obtained, which was highly significant (p = 0.0001). On Day 3 after injury in the control versus mTBI comparison, an AUC of 99.5 was observed with a sensitivity of 100% and specificity of 96.43% (p < 0.000003) and a cutoff of 77.5 pg/ml (Fig. 4). For the CT– versus CT+ group, the AUC was 82.5% and was significantly different than 50% (p = 0.000003) and a cutoff of 4083.7 pg/ml (Fig. 3). (Fig. 3).

Days 1 and 3 Serum pNFL-H ROC Curve

The ROC curve analysis was used to determine if a serum pNFL-H cutoff level could be used to identify injured subjects and categorize individuals into the CT– or CT+ group. When comparing healthy controls with all mTBI subjects, for Day 1, the AUC was 1.0. Using a cutoff value of 110.5 pg/ml, a sensitivity of 100% and specificity of 100% were obtained, which was highly significant (p = 0.0001). On Day 3 after injury in the control versus mTBI comparison, an AUC of 99.5 was observed with a sensitivity of 100% and specificity of 96.43% (p < 0.000003) and a cutoff of 77.5 pg/ml (Fig. 4). For the CT– versus CT+ group, the AUC was 82.5% and was significantly different than 50% (p = 0.0021) on Day 1. The cutoff for Day 1 was found to be 1071 pg/ml, resulting in a sensitivity of 87.5% and a specificity of 70%. For Day 3, the AUC was 71.7% and was not significantly different than 50% (p = 0.22) (Fig. 5).
The purpose of this study was to characterize a serum biomarker of brain injury as a potential predictor of injury severity after mTBI during later time points after injury such as 24 and 72 hours. These time points are more clinically relevant since a number of TBI victims will present to the emergency department within 1–3 days after the initial injury. We found that compared with noninjured controls, there was a significant increase of pNFL-H in the serum on both Days 1 and 3 after injury (Fig. 1), suggesting that this biomarker can be used to identify brain injury up to 72 hours after trauma. On Day 1 after injury, there was a significant difference between the patients with and without findings of mTBI on CT. This phenomenon was not observed on Day 3, since the serum levels of pNFL-H decreased by 35% compared with Day 1. Both groups (CT+ and CT− groups) were significantly different from noninjured controls (Fig. 3). In addition, as indicated by the ROC curves that were generated in this study, this is the first study in mTBI patients to demonstrate that pNFL-H is a sensitive and specific biomarker with respect to identifying injury severity in mTBI patients and differentiating such patients based on their CT scan (Figs. 4 and 5).

An important expectation of all biomarkers is that the detection of the protein in bodily fluids is reliable at both the early and subacute phases of brain injury after TBI. Within the TBI field, a plethora of biomarkers have been characterized and have shown promise during the early phases (within minutes to hours) after TBI. A number of these biomarkers such as S100B consistently correlate with the severity of the injury early on but failed to detect TBI during the later stages (days to weeks). In contrast, with great consistency, the levels of pNFL-H in the blood and cerebrospinal fluid during the acute and chronic phase of secondary TBI have correlated with the severity of mild, moderate, and severe TBI as demonstrated in this study and other published reports. Another important finding is that on Day 1 after injury, this biomarker was found to have a high sensitivity/specificity (Fig. 4), which strengthens the argument that this biomarker may prove to accurately identify which individuals require immediate medical attention after mTBI. By utilizing a biomarker that reliably predicts the severity of the TBI up to 72 hours
after injury, a larger window is available to assess individuals, such as athletes, military personnel, or civilians, who have suffered an mTBI and who did not seek medical help immediately. Also, the blood levels of pNFL-H may help decide which individuals may return to play, work, or normal daily activities.

In this study, we also attempted to enroll mTBI patients on Days 7 and 14. As a major limitation, a majority of these patients were discharged by Day 3 or 4 after injury. Only a few patients were enrolled on Days 7 and 14 (data not shown). The participants who were discharged from the hospital later than 3 days were assessed further for injuries to other organ systems and/or extremities. In future studies, to collect blood samples on Days 7 and 14, we will arrange for a number of the mTBI participants to visit University of Texas Southwestern to have blood drawn. Additionally, as a future direction, we will also schedule mTBI patients to have blood drawn on Days 30 and 90 after injury. In addition to the blood draws at the later time points, we will administer neuropsychological testing at all time points (1, 3, 7, 14, 30, and 90 days after injury) to determine if biomarker levels correlate with cognition.

Conclusions

We have found that within 72 hours of injury, pNFL-H is a reliable biomarker to predict injury severity in people affected by mTBI. We further hypothesize that multiple biomarkers used in tandem may help characterize the level of secondary brain injury after TBI. For example, S100B/NSE may be used to describe early levels, and other biomarkers such as pNFL-H could determine the level of subsacute secondary TBI in an attempt to predict cognitive deficits.

Disclosure

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