Analysis of Peptidome Profiling of Serum from Patients with Early Onset Symptoms of Ischemic Stroke

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Background: We sought to identify new serum biomarkers for the early diagnosis of ischemic stroke. *Methods:* We collected 63 serum samples from patients with neurologic disease (45 patients with ischemic stroke, 18 patients with other neurologic disorders, and 56 healthy controls). Serum peptides were extracted using immobilized copper ion chromatography on a robotic platform. Mass spectra were acquired by matrix-assisted laser desorption/ionization-time of flight mass spectrometry using an Autoflex II spectrometer (Bruker Daltonics, Billerica, MA). Statistical analyses were performed with Clinprotools 2.2 software (Bruker Daltonics) and SPSS software (version 15.0; SPSS, Inc., Chicago, IL). *Results:* No peptide biomarker or panel of peptide biomarkers was identified to differentiate between ischemic stroke and other neurologic disease, but ischemic stroke patients were differentiated from healthy controls with a single feature of the peptidome (sensitivity 88.6%; specificity 96.4%). *Conclusions:* Analysis of peptidome profiling of serum could be a useful tool in the search for early diagnostic biomarkers of ischemic stroke. **Key Words:** MALDI—stroke—biomarker—serum peptide profiling.

No specific serum biomarker has been identified that can assist in the early diagnostic process of ischemic stroke (IS). Early detection is crucial, because the therapeutic window in which functional recovery of the ischemic territory is possible ranges from 0 to 3 hours after symptom onset. A serum biomarker could be a useful tool for the early diagnosis of IS because serum is an easily accessible, noninvasive, and widely collected sample. In addition, serum biomarkers are easily measurable by immunoanalysis.

The need to find biomarkers in serum for early stroke diagnosis has been emphasized in recent years. Previous studies[1-3] have found several candidates, particularly proteins related to acute ischemic processes, including S-100b, beta nerve growth factor, von Willebrand factor, monocyte chemotactic protein-1, matrix metalloproteinase-9, D-Dimer, and brain natriuretic peptide. Montaner et al[4] and Laskowitz et al[5] tested panels with these serum biomarkers; however, no panel showed adequate sensitivity (S) and specificity (Sp), and none has been validated to date.

Proteomics is the study of proteome, which is defined as the total set of proteins that are in a cell, organism, or body fluid in a specific moment. Proteome is dynamic and depends on physiopathology of the patient.[6] Human serum contains thousands of peptides, and most are thought to be fragments of larger proteins that have been partially degraded by endogenous, proteolytic enzymes.[7] Comparing peptidome profiling patterns between different groups of patients we can find quantitative differences in the expression of specific peptides. Proteomics could therefore be an important tool to find new biomarkers of diagnosis, prognosis, and treatment assessment.[8]

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Proteomics is possible because of an innovative and powerful technology based on mass spectrometry of high sensitivity matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry.[9] This technology allows for the display of hundreds of small (800-15,000 Da) peptides at the same spectra using only microliters of serum.[7]

The primary pitfalls of this methodology are the lack of preanalytic and analytic standardization[2,10] and poor reproducibility.[11,12] In addition, the impact of sex and age on serum peptidome profiling is rarely taken into account.

In the present study, we used a previously evaluated and reproducible method[13] to analyze serum peptidome to identify peptide biomarkers for the early diagnosis of IS in a case control study. Our goal was to find the panel with the minimum number of peptides capable of discriminating between IS, other acute neurologic disease (OND), and healthy controls (HC) and that had, at the same time, the highest diagnostic S and Sp.

Methods

Patients

We included 45 patients (30 men and 15 women ranging from 38-84 years old) who were diagnosed with IS in the Department of Neurology of our institution. These patients arrived to the emergency department with symptoms of acute neurologic deficit <6 hours after symptom onset and had a National Institutes of Health Stroke Scale (NIHSS) score ≥5. IS was confirmed later by neuroimaging, using either computed tomographic or magnetic resonance imaging scans. Twenty-two patients of 45 received thrombolytic therapy with tissue plasminogen activator (tPA). In all other cases, no treatment was administered because of their spontaneous improvement, admission >3 hours after symptom onset, or because the patien had only mild neurologic deficit.

We included a group of 18 patients (13 men and 5 women ranging from 55-85 years old) that arrived to the emergency department with ONDs diagnosed by a neurologist. These included 2 transient ischemic attacks with symptoms of neurologic deficit of <1 hour and negative neuroimaging tests, 9 intracerebral hemorrhages (8 intracranial and 1 subarachnoid) confirmed by neuroimaging, 1 head injury with no presence of intra- or extraparenchymal hematoma, 1 heart attack, and 5 stroke mimics.

In addition, 56 healthy controls (HCs; 18 men and 38 women ranging from 17-85 years old) were included in the study. The study was approved by the local ethics committee, and written informed consent was obtained from all patients.

Blood Collection and Processing

Blood samples were obtained at different times, including at admission and at 6 and 24 hours after symptom onset. Blood collection was carried out in similar conditions for all samples (patients and HCs) following a specially designed protocol to avoid preanalytic variations. Venous blood samples were collected in Vacutainer tubes without anticoagulant (Becton Dickinson, Franklin Lakes, NJ) and left to clot for 1 hour at room temperature. Samples were then centrifuged for 10 minutes at 1500 *g* at room temperature, and several aliquots were immediately frozen at –80°C until analysis.

Sample Preparation

Serum peptides were extracted in duplicate in a 96-well microplate using magnetic beads (MBs) functionalized with copper (IMAC-Cu; Bruker Daltonics, Billerica, MA). The extraction protocol was modified from that recommended by the manufacturer in order to improve peptide recovery. Briefly, 5 μL of MBs were washed 3 times with 50 μL of binding buffer. The cleaned MBs were then treated with 10 μL of binding buffer and incubated with 5 μL of serum for 10 minutes after careful mixing by pipetting up and down several times. Unbound peptides were removed after placing the microplate on a MB separator. The MBs were then washed 3 times with 100 μL of deionized water. Finally, bound peptides were eluted using 10 μL 30% acetonitrile/0.1% trifluoroacetic acid. All processes was automated in a robotic system (Freedom Evo; Tecan, Männedorf, Switzerland).

MALDI-TOF MS

Peptide eluate was spotted on an Anchorchip target (Bruker Daltonics) using the dried droplet method with 2,6-dihydroxyacetophenone (2,6-DHAP) as matrix. The matrix solution was prepared with 3 mg 2,6-DHAP in 20% acetonitrile/0.1% trifluoroacetic acid. All chemicals and solvents were purchased from Sigma-Aldrich (Saint Louis, MO).

Peptide profiles in the mass range of 800 to 15,000 Da were obtained by mass spectrometry in a MALDI-TOF instrument (Autoflex II; Bruker Daltonics) set in a positive, linear mode with a 120-ns delayed extraction time. External calibration was carried out using a peptide mixture provided by Bruker Daltonics ranging from 3 to 20,000 Da. Each spectrum was acquired after averaging 500 laser shots. Two spectra from each serum preparation were obtained.

Data Analysis

Alignment, normalization, peak detection, and peak area calculation of spectra was carried out using ClinProTools 2.2 software (Bruker Daltonics); mass spectra data (peak mass and peak area) were then exported as a text file and paired with clinical data before statistical analysis.

A logistic regression model was performed to identify the best predictive peaks. Variables were transformed