Identification of Novel Biomarkers of Brain Injury by Integrating Bioinformatics and Mass Spectrometry-based Proteomics

MSACL 2014 EU - Salzburg, Austria
Thursday, September 4th, 2014

Eduardo Martínez-Morillo, PhD
Clinical Biochemist
Specialist in Mass Spectrometry
Introduction

- Brain injury:
  1. Non-traumatic
  2. Traumatic (TBI)

Infection

Stroke
Introduction (II)

Approximately **1.7 million** Americans suffer a **TBI** each year, and of those 75-90% are classified as minor head injury (MHI).

**Stroke** is the most common form of non-TBI, with **795,000** people in the United States suffering a new or recurrent stroke each year.

Differentiating between hemorrhagic and ischemic stroke is critical to determine treatment options.

**Hemorrhagic stroke**
- 13%
- Homeostatic therapy

**Ischemic stroke**
- 87%
- Thrombolytic therapy
Rapid diagnosis and prompt medical attention is important because deterioration of patients is common in the first few hours after symptoms onset.

Several proteins such as S100B, NSE and GFAP have been proposed as diagnostic and prognostic biomarkers of TBI and stroke.
Hypothesis

Proteins specifically expressed at high levels in the brain may be released and detected in the CSF of patients with hemorrhagic stroke.

Biomarkers of cellular death

These proteins may be detected in circulation and act as biomarkers.
**Aims**


2. Develop selected reaction monitoring (SRM) assays for candidate protein biomarkers using brain tissue extracts.

3. Quantify these proteins in CSF samples from patients with hemorrhagic stroke, ischemic stroke and controls.
Protein Selection

Aim 1

**The Human Protein Atlas** (Version: 10.0)

14079

- High expression in brain cell types
- Absent or low expression in other cell types

390

- Human Brain Proteome 2012-09
- High number of observations

76

- Human Plasma Proteome 2012-08
- Zero or low number of observations

The Human Protein Atlas (www.proteinatlas.org)
Peptide Atlas (www.peptideatlas.org)
Protein Selection (II)

THE HUMAN PROTEIN ATLAS
(Version: 10.0)

Aim 1

14079
High expression in brain cell types
Absent or low expression in other cell types

390
Human Brain Proteome 2012-09
High number of observations

Human Plasma Proteome 2012-08
Zero or low number of observations

76

(17298 antibodies)

Protein expression profiles based on immunohistochemistry

The Human Protein Atlas (www.proteinatlas.org)
Peptide Atlas (www.peptideatlas.org)
Protein Selection (III)

Aim 1

14079
- High expression in brain cell types
- Absent or low expression in other cell types

390
- Human Brain Proteome 2012-09
  - High number of observations
- Human Plasma Proteome 2012-08
  - Zero or low number of observations

76

The Human Protein Atlas (www.proteinatlas.org)
Peptide Atlas (www.peptideatlas.org)
Protein Selection (IV)

THE HUMAN PROTEIN ATLAS

Neurofilament, medium polipeptide (NFM)

Example:
NFM

Positive Staining = \textbf{8} out of \textbf{81} cell types

Brain cell types = \textbf{4}

Other cell types = \textbf{4}

Brain Proteome (N Observations = \textbf{942})

Plasma Proteome (N Observations = \textbf{16})

Specific expression in neuronal cells
**Peptide Selection**

**Aim 2**

Peptides previously observed in, at least, one of these three databases were selected:

- **SRM Atlas** ([www.srmatlas.org](http://www.srmatlas.org))
- **GPM database** ([gpmdb.thegpm.org](http://gpmdb.thegpm.org))
- **Scaffold software** ([www.proteomesoftware.com](http://www.proteomesoftware.com))

**BLAST**

Uniqueness was confirmed. Peptides containing M residues or N-terminus C or Q residues were avoided if possible.

Peptides were identified in brain tissue extracts (hippocampus) from 68 out of 76 proteins:

- 205
- 127
Peptide Identification

Three ways:

1) Prediction of retention times using SRRCalc 3.0 (Skyline software).

2) Co-elution of, at least, 6 transitions per peptide (from $y_3$ to $y_{n-1}$).

3) Comparison of the observed fragmentation pattern with the fragmentation pattern displayed in publicly available databases (SRM atlas and GPM database) or in our in-house brain tissue proteome.
# Peptide Identification (II)

**Example:**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SRRCalc 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skyline</td>
<td></td>
</tr>
</tbody>
</table>

**Peptide VQSLQDEVAFLR (from NFM)**

- **Predicted RT:** 28.2 min (24.0 – 32.4)
- **95% CI**
- **Observed RT:** 29.1 min
CSF sample collection

Age-matched CSF samples (n=36) were obtained from the department of Clinical Biochemistry at Hospital Universitario Central de Asturias (Spain)

Aim 3

S100B protein was measured using a fully-automated immunoassay (Roche Diagnostics)
Sample preparation

10 μg TP
Brain tissue extract or CSF sample

A
Denaturation

0.05% RapiGest®

B
Reduction Alkylation

DTT (5 mM)
Iodoacetamide (15 mM)

Trypsin 1/10

C
Digestion

K

D
Heavy peptides

K*

R

R*

SpikeTides L

OMIX C18 tips

Micro-extraction

E

F
Analysis

EASY-nLC 1000 + TSQ Vantage (Thermo Fisher)
Selection of Candidate Protein Biomarkers

First analysis:

CSF samples

- HS
  - n=7
- IS
  - n=7
- Control
  - n=7

68 peptides
Selection of Candidate Protein Biomarkers

First analysis:

CSF samples

- HS, n=7
- IS, n=7
- Control, n=7

68 peptides (1 peptide per protein)
Selection of Candidate Protein Biomarkers

First analysis:

68 peptides (1 peptide per protein)

- 33 peptides were not detected (in any sample)
- 27 peptides were not elevated in hemorrhagic stroke

1st

n=7

HS

IS

Control
Selection of Candidate Protein Biomarkers

CSF samples
- HS
- IS
- Control

First analysis:
68 peptides (1 peptide per protein)

- 33 peptides were not detected (in any sample)
- 27 peptides were not elevated in hemorrhagic stroke

8 final peptides/proteins
Selection of Candidate Protein Biomarkers

First analysis:
- CSF samples
- HS
- IS
- Control
- 68 peptides

Second analysis:
- 8 final peptides/proteins
- SRM method (30 min gradient)
- 8 endogenous peptides
- 8 isotope-labelled peptides

2nd
- n=15
- n=11
- n=10
Selection of Candidate Protein Biomarkers

First analysis:

CSF samples
- HS
- IS
- Control

68 peptides

Second analysis:

8 final peptides/proteins

SRM method (30 min gradient)
- 8 endogenous peptides
- 8 isotope-labelled peptides

Linearity and LOQ were studied

Linearity

1000 fmoles
Isotope-labelled peptides

Serial dilutions 1:2
13 points of calibration

0.24 fmoles
(analysed in triplicate)
Multiplex SRM method with 16 peptides (8 endogenous and 8 isotope-labelled)

Co-elution of six transitions for endogenous and isotope-labelled peptide DNLAQDLATVR

Three transitions per peptide were monitored (two qualifiers and one quantifier)
## Linearity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Transitions</th>
<th>LOQ</th>
<th>CV</th>
<th>Linearity</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSE</td>
<td>IEEELGDEAR</td>
<td>580.7-918.4, 585.7-928.4</td>
<td>0.24</td>
<td>4%</td>
<td>0.24 - 1000</td>
<td>0.24 – 3.9</td>
</tr>
<tr>
<td>GFAP</td>
<td>DNLAQDLATVR</td>
<td>608.3-873.4, 613.3-883.4</td>
<td>0.24</td>
<td>5%</td>
<td>0.24 - 1000</td>
<td>0.24 – 3.9</td>
</tr>
<tr>
<td>α-Inx</td>
<td>ALEAELAALR</td>
<td>528.8-872.4, 533.8-882.4</td>
<td>0.49</td>
<td>9%</td>
<td>0.49 - 1000</td>
<td>0.49 – 7.8</td>
</tr>
<tr>
<td>MBP</td>
<td>GVDAQGTLSK</td>
<td>488.2-819.4, 492.2-827.4</td>
<td>0.24</td>
<td>12%</td>
<td>0.24 - 1000</td>
<td>0.24 – 3.9</td>
</tr>
<tr>
<td>MT3</td>
<td>GGEAAEAEAEK</td>
<td>531.2-747.3, 535.2-755.3</td>
<td>0.98</td>
<td>6%</td>
<td>0.98 - 1000</td>
<td>0.98 – 15.3</td>
</tr>
<tr>
<td>NFM</td>
<td>VQSLQDEVAFLR</td>
<td>702.8-1177.6, 707.8-1187.6</td>
<td>0.49</td>
<td>13%</td>
<td>0.49 - 1000</td>
<td>0.49 – 7.8</td>
</tr>
<tr>
<td>β-Syn</td>
<td>EGVVQGVASVAEK</td>
<td>636.8-760.4, 640.8-768.4</td>
<td>0.24</td>
<td>10%</td>
<td>0.24 - 1000</td>
<td>0.24 – 3.9</td>
</tr>
<tr>
<td>γ-Syn</td>
<td>TVEEAENIATGVVR</td>
<td>837.4-788.4, 842.4-798.4</td>
<td>1.95</td>
<td>7%</td>
<td>1.9 - 1000</td>
<td>1.9 – 31.2</td>
</tr>
</tbody>
</table>

LOQ = Limit of quantification (fmoles); CV = Coefficient of variation (triplicates); R² = Coefficient of determination
Results

*** $p<0.001$

**) $p<0.01$

*) $p<0.05$

n.s.) not significant
Results (II)

*** p<0.001
** p<0.01
* p<0.05
n.s. not significant
Results (III)

*** p<0.001
** p<0.01
* p<0.05
n.s. not significant
Discussion

Four known biomarkers: S100B, NSE, GFAP and MBP

Three novel biomarkers: NFM, α-Inx and β-Syn

Focus on biomarkers of cell death since necrosis, apoptosis and autophagy cell death pathways are activated early after hemorrhage
Biomarkers of axonal degeneration

Neurofilament triplets proteins (NFL, NFM and NFH) and α-Inx are the four major components of the neuronal intermediate filaments.

http://www.nature.com/scitable/topicpage/microtubules-and-filaments-14052932
**ELISA kit for NFM**

Good linearity, sensitivity (LOD: 10 pg/ml) and precision (CV intra-batch ≤ 10%, CV inter-batch ≤ 13%)

Evaluate the selectivity of the ELISA kit: (using size-exclusion HPLC and mass spectrometry)

40 chromatographic fractions (from 18 to 58 min)

Mass spectrometry analysis:
Fractons 18, 23 and 34

Frasions 23-24: 160 kDa

NFM identified only in fraction 23
These results agreed with the results obtained with the mass spectrometry-based assay.
# NFM in serum

<table>
<thead>
<tr>
<th>Disorder</th>
<th>n</th>
<th>NFM (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy individuals</td>
<td>46</td>
<td>0.26-8.57 ng/ml</td>
</tr>
<tr>
<td>Hemorrhagic stroke</td>
<td>7</td>
<td>0.97-42.4 ng/ml</td>
</tr>
<tr>
<td>Traumatic brain injury (TBI)</td>
<td>12</td>
<td>3.48-45.4 ng/ml</td>
</tr>
<tr>
<td>Minor head Injury (MHI)</td>
<td>68</td>
<td>0.21-202.2 ng/ml</td>
</tr>
</tbody>
</table>
We found increased concentrations of NFM protein in CSF and serum of patients with brain injury.

New studies are needed to elucidate the value of this protein in the diagnosis, prognosis and management of patients with brain injury and other neurological diseases with axonal degeneration.
Acknowledgements

Eleftherios P. Diamandis
Charmaine Childs
Francisco V. Alvarez Menendez
Belen Prieto Garcia
Alexander D. Romaschin
Giuseppe Lippi
Gianfranco Cervellin