Peptidomics Analysis Reveals Serum Biomarkers in Spinal Cord Injury Patients

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ABSTRACT: Spinal cord injury (SCI) is a severe traumatic disease, always resulting in neuronal injury. In this study, we aimed to exhibit a peptidome profile of serum from patients with SCI. A label-free peptidomics strategy was used to analyze the differentially expressed peptides (DEPs). Then, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses was used to evaluate the function of the peptides precursors proteins. Also, the protein–protein interaction networks were mapped using STRING database. Finally, parallel reaction monitoring assays were used to validate the expression of candidate peptides. We identified 217 DEPs including 29 upregulated peptides and 188 downregulated peptides in SCI group. Many pathways such as Platelet activation, Complement and coagulation cascades, Focal adhesion were enriched. Seven peptides including PSPRPSP, RPPGFSP, DKPDMAIEKFDKSCLK, STAVVTNPKE, GHAGAQGPPPQG, SMPPAQQQITS and SKVLPIQDNVK were significantly changed between SCI patients and healthy people. Peptidomics provide a powerful tool to find the variation of SCI. RPPGFSP, DKPDMAIEKFDKSCLK and SMPPAQQQITS may play important roles in SCI. However, the specific function of these peptides and whether they can be used as therapeutic targets for SCI need to be further investigated.

KEY WORDS: spinal cord injury, peptidomics, biomarker, peptides

I. INTRODUCTION

Spinal cord injury (SCI) is a severe traumatic disease in the central nervous system, which accounts for high economic and social costs globally.1–3 Half a million people damage their spinal cord every year and results in permanent dysfunction of movement and sensation, which imposes a heavy economic and social burden.4,5 Although numerous therapies including different kinds of drugs, surgical interventions, physical and hyperbaric oxygen therapy have been used clinically, the treatment efficacy of SCI is still unsatisfactory due to the complex pathologic conditions.6,9 The treatment of SCI is mainly to improve the inflammatory microenvironment, the regeneration and repair of nerve function. However, the widespread lipid peroxidation, cell apoptosis and severe inflammation create a terrible environment for the nerve axonal regeneration.10,11 Therefore, finding novel treatment and therapeutic targets for SCI is urgently needed.

Peptidomics is an emerging branch of proteomics which is based on investigating endogenous peptides in cells, tissues, or body fluid.12 Apart from protein, most peptides have simple structure and consist of 2–10 amino acids. Biologically active peptides are involved in almost all physiological processes, including cell differentiation, immune regulation and even tumor formation.13 Peptidomics focuses on the discovery and quantitation of such endogenous peptides, given their wide range of encoded biological functions and their associated diagnostic and therapeutic potential.14 In recent years, with the technological development of natural peptide purification and improvement of mass-spectrometry, the
peptidome are playing an increasingly important role in the study of biomarkers and therapeutic targets.

Bioactive peptides have been reported to play important roles in some neuropathic diseases. For example, TFP5, a modified 24-aa peptide could significantly reduce the cerebral infarction area of stroke. DEETGE-CAL-Tat peptide plays a neuroprotective role and maintains cognitive function in ischemic brain injury. Also, a peptide named HIBDAP could inhibit the damage of neonates hypoxic-ischemic brain injury. Therefore, peptides target antibodies might be a promising way for the treatment of nerve injury disease. However, as for SCI, there are very few reports for peptide therapies and targets.

In the present study, we aimed to identify differentially expressed peptides (DEPs) of SCI through peptidomics. Through the validation of expression level on certain peptides, we hope to find new therapeutic peptide for SCI.

II. MATERIALS AND METHODS

A. Clinical Samples Collection

The serum samples of three SCI patients and three healthy volunteers from Changzheng Hospital of Naval Medical University were collected for the peptidomics study. Serum of 15 SCI patients and 15 healthy people were collected for validation. All the samples were immediately stored with protease inhibitor (Complete mini EDTA-free, Med Chem Express, USA) in a refrigerator at −80°C before the study. This study was approved by the Ethics Committee of Changzheng Hospital of Naval Medical University, and all participants signed informed consent forms.

B. Sample Preparation

One milliliter of −20°C pre-chilled acetone was added to each 500-μl serum sample to precipitating the proteins for 1 h at 4°C, vortexed every 10 minutes. The samples were centrifuged at 12,000 g for 20 min at 4°C. The supernatant was collected and lyophilized by SpeedVac. Then the sample were re-dissolved in phosphate-buffered saline and ultrafiltered by 10 KD Ultrafiltration Spin Columns (RT-UFC501096-5, Millipore Sigma, Burlington, MA, USA). The HiPPR Detergent Removal Spin Column Kit (88305, Thermo Fisher Scientific, USA) was used to remove the PEG pollution. The flow-through from the filters containing peptide fractions was recycled, desalted, concentrated using C18 ZipTip (Strata-X, 33 μm, 2 g/20 mL, Phenomenex), and finally lyophilized.

C. Liquid Chromatography/Mass Spectrometry (LC-MS/MS) Analysis

The peptides were analyzed by on-line nanospray LC-MS/MS on an Thermo Fisher Scientific Orbitrap Fusion Lumos coupled to an EASY-nano-LC 1200 system (Thermo Fisher Scientific, MA, USA). Each 4 μl of peptide was loaded (analytical column: Acclaim PepMap C18, 75 μm × 25 cm) and separated with a 120 min linear gradient, from 6% B (B: 0.1% formic acid in 80% ACN) to 36% B. The column flow rate was maintained at 400 nL/min with the column temperature of 40°C. The electrospray voltage of 2 kV versus the inlet of the mass spectrometer was used. The mass spectrometer was run under data dependent acquisition mode, and automatically switched between MS and MS/MS mode.

The MS data were searched using the PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, Canada) and UniProt database (Homo sapiens). PEAKS were searched with a fragment ion mass tolerance of 0.02 Da and a parent ion tolerance of 7 ppm. Oxidation (M) and Acetylation (Protein N-term) were specified as the variable modifications. The peptides with −10 lg P-value ≥ 20 were selected.

D. Bioinformatics Analysis

DEP threshold was screened by fold change ≥ 1.5 and p < 0.05. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses were performed for the DEPs precursors proteins. GO analysis was performed to describe genes or proteins attributes in any organism (http://www.geneontology.org). This ontology contains three catalogs: biological processes (BP), cellular components (CC), and molecular functions (MF). Database for annotation, visualization and integrated
discovery (DAVID) (version 6.8; https://david.ncifcrf.gov/) was used for pathway analysis. A p-value < 0.05 under hypergeometric test was considered as statistically significant. The protein–protein interaction networks were mapped using STRING database (https://string-db.org/) and UniProt database (https://www.uniprot.org/).

E. Parallel Reaction Monitoring (PRM)

The peptides abundance differences obtained in the label-free peptidomics study were confirmed by PRM assays. The iRT peptides (Biognosys, Switzerland) were added to the samples according to the manufacturer’s instructions. The PRM assays were run on the thermo fisher scientific orbitrap fusion lumos coupled to an EASY-Nano-LC 1200 system. SpectroDive9 software (Biognosys, Switzerland) was used to run the data analysis using the default parameters.

F. Statistical Analysis

The data analysis was performed using GraphPad prism 8.0 (GraphPad Software, San Diego, CA, USA). The data are expressed as means ± standard deviation. Student’s t-test was performed to assess whether differences between the SCI and control groups (NC) were significant. A p-value < 0.05 was regarded as statistically significant.

III. RESULTS

A. Identification of DEPs

The LC-MS/MS technology were used to identify the peptides from serum between SCI and Normal controls. The workflow of the peptidomics in this study was shown in Fig. 1A. To further investigate the degree of similarity and differences between the SCI and NC groups, PLS-DA was used. The results identified a clear separation of the two groups. Two principal components include PC1 (91.38%) and PC2 (8%) are shown in Fig. 1B. In total, we identified 217 DEPs including 29 upregulated peptides and 188 downregulated peptides in SCI group. The DEPs were shown by heatmap (Fig. 1C).

B. Functional Enrichment Analysis on DEPs

As reported, peptides may play a similar function to their precursor’s proteins. Thus, the putative biological functions of the DEPs were obtained by analyzing on their precursor proteins. GO and KEGG enrichment analysis were used to explore the functions of DEPs. The top 10 counts of the most significant enrichment terms in BP, CC, and MF categories are shown in Figs. 2A–2C. From our results, the top enriched BP terms were platelet degranulation, regulated exocytosis, secretion by cell, vesicle-mediated transport, localization, establishment of localization, transport, immune system process, response to wounding, blood coagulation and so on (Fig. 2A). As for cellular components, the highest enriched sub-categories included platelet alpha granule, secretory granule, secretory granule lumen, extracellular region, endoplasmic reticulum lumen, platelet alpha granule lumen, cytoplasm, endomembrane system and cytoplasmic vesicle (Fig. 2B). Regarding molecular functions, the top three enriched MF terms included protein binding, structural constituent of cytoskeleton, platelet-derived growth factor binding, binding, actin binding, signaling receptor binding, enzyme inhibitor activity, structural molecule activity, endopeptidase inhibitor activity, and growth factor binding (Fig. 2C). The GO analysis reflected the cell secretion, cell transportation, cell location and immune response. For KEGG analysis, Platelet activation, Complement and coagulation cascades, Focal adhesion, Protein digestion and absorption, Phagosome, Pathogenic Escherichia coli infection, Rap1 signaling pathway, ECM-receptor interaction, Staphylococcus aureus infection and Regulation of actin cytoskeleton. These pathways showed a comprehensive inflammation and destruction of SCI.

C. Identified Peptides Potentially Associated with SCI

As we know, multiple peptides often derive from certain same protein precursors. As shown in Fig. 3, ZYX, CAVIN2, TMSB4X, LASP1, and TAGLN2 were the top five number of the peptides. For the intracellular peptides do not always reflect the degradation level of proteins, it is likely that they functioned
The function of the peptides is usually related to the function of their parent proteins. In order to identify important peptides that play key roles in SCI, the functions of DEPs derived precursors proteins were studied using STRING database and UniProt database. The protein–protein interaction (PPI) network of precursor proteins interactions was shown in Fig. 4. We found that collagen family, tubulin family, histone family, and complement proteins formed four subgroups. This may indicate the important roles of these peptides. We subsequently analyzed the top 10 significantly changed peptides derived from their precursor proteins, which was shown in Table 1.

**FIG. 1**: Label-free peptidomics landscape between the SCI patients and healthy controls (NC). (A) Simplified workflow of the label-free peptidomics strategy. (B) Principal component analysis illustrating moderate clustering between the SCI patients and healthy controls. (C) Heatmap of the 217 significantly dysregulated peptides in SCI patients and healthy controls. Red indicates the up-regulated peptides and green represents the down-regulated peptides in SCI patients.
To further validate the expression of the potential peptides, the ten peptides in Table 1 were validated by PRM mass spectrometry method. We found that most of the peptides detected showed the same tendency with the peptidomics results (Fig. 5), such as PSPRPSP, RPPGFSP, DKPDMAEJEKFDKSILK, STTAVVTNPKE, GHAGAQGGPPGPP, SMPPAQQQITS, and SKVLPIQDNVSK.

FIG. 2: GO and KEGG analyses of DEP precursor proteins. (A) GO analysis conducted for the DEPs in terms of the top 20 ranking biological process. (B) GO analysis carried out for the DEPs in terms of the top 20 ranking cellular component. (C) GO analysis performed for the DEPs in terms of the top 20 ranking molecular function. (D) KEGG pathway analysis of the DEPs precursors proteins.

FIG. 3: Precursor proteins of the DEPs. Proteins that matched at least five peptides are listed.

IV. DISCUSSION

There is still a lack of effective means for the recovery of SCI. In the present study, we used label free peptidomics ways to identify DEPs between SCI patients and healthy controls. And we constructed a peptide profile of SCI serum. PRM-MS is a targeted mass-spectrometry method that help to confirm the expression level on certain peptides. It helps to increase detection sensitivity and also reduce reliance on antibody validation.
that response to stress, immune response and cell/platelet activation were significantly changed. As SCI could increase oxidative stress is common sense,\textsuperscript{24,25} indicating our results were consistent with the previous studies. Also, SCI triggers a robust neuroinflammatory response and leads to an increased immune response.\textsuperscript{26} A study showed that exogenous platelet-derived growth factor improves neurovascular unit recovery after SCI.\textsuperscript{27} This may explain why terms about platelet and wound healing enriched. From KEGG pathway, some significantly enriched pathways of precursor proteins associated with cardiomyopathy were identified, such as Arrhythmogenic right ventricular cardiomyopathy, Hypertrophic cardiomyopathy, and Dilated cardiomyopathy. Plenty of studies showed cardiomyopathy could result in oxidative stress, inflammation, and immune


**TABLE 1:** Top 10 significant peptides of protein precursors

<table>
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<tr>
<th>Peptide sequence</th>
<th>Accession</th>
<th>Protein name</th>
<th>Expression model</th>
<th>p-value</th>
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<tr>
<td>PSPRPSP</td>
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<td>PDZD7</td>
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<tr>
<td>RPPGFSP</td>
<td>P01042</td>
<td>VAPB</td>
<td>Downregulation</td>
<td>2.40E-06</td>
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<tr>
<td>DKPDMAEIEKFDKSLK</td>
<td>P62328</td>
<td>CDSN</td>
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<tr>
<td>STTAVTNPKE</td>
<td>P02766</td>
<td>HIST2H2BC</td>
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<tr>
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responses, which is also in the pathological process of SCI.24,31

The top 10 significantly dysregulated peptides were validated by PRM-MS. Among them, seven peptides PSPRPSP, RPPGFSP, DKPDAEIEKFDKSLKLK, STTAVTNPKE, GHAGAQGP-PGPG, SMPPAQQQITS and SKVLPIQDNVS were matched with label free peptidomics. It is reported that endogenous bioactive peptides may play a similar role as their precursor proteins.32 The peptide RPPGFSP is a fragment of Kinoigen-1(KNG1). KNG1 is an important pro-inflammatory and pro-oxidant factor, which can aggravate oxidative stress and mitochondrial dysfunction in cardiomyopathy and brain damage.33 The peptide DKPDAEIEKFDKSLKLK is a part of Thymosin beta-4 (TMSB4X). TMSB4X has physiological functions that are highly relevant to SCI. It can attenuate oxidative stress-induced injury of spinal cord-derived neural stem/progenitor cells through the TLR4/MyD88 pathway.34 Otherwise, SMP-PAQQQITS is a continuous sequence of Talin-1(TLN1). Talin-1 is a cytoskeletal protein that binds integrin, thereby leading to integrin activation and affecting focal adhesions.35 Lagarrigue et al.36 found that talin-1 is the principal direct effector of Rap1 GTPases that regulates platelet integrin activation in hemostasis. This research was consistent with our KEGG pathway analyses.

In this study, we have revealed a peptidome profile of serum from patients with SCI. Also, we found several potential functional peptides in SCI. However, the specific function of these peptides and whether they can be used as therapeutic targets for SCI need to be further investigated.

ACKNOWLEDGMENT

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REFERENCES


