Brain Research Bulletin 142 (2018) 224–232

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Identification of plasma biomarkers for diffuse axonal injury in rats by iTRAQ-coupled LC–MS/MS and bioinformatics analysis

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ARTICLE INFO

Keywords:
Diffuse axonal injury
Proteomics
iTRAQ
Biomarkers
Rats

ABSTRACT

DAI is a serious and complex brain injury associated with significant morbidity and mortality. The lack of reliable objective diagnostic modalities for DAI delays administration of therapeutic interventions. Hence, identifying reliable biomarkers is urgently needed to enable early DAI diagnosis in the clinic. Herein, we established a rat model of DAI and applied an isobaric tags for a relative and absolute quantification (iTRAQ) coupled with nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS) proteomics approach to screen differentially expressed plasma proteins associated with DAI. A total of 58 proteins were found to be significantly modulated in blood plasma samples of the injury group in at least one time point compared to controls. Bioinformatics analysis of the differentially expressed proteins revealed that the pathogenesis of axonal injury underlying DAI is multi-stage biological process involved. Two significantly changed proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hemopexin (Hpx), were identified as potential diagnostic biomarkers for DAI, and were successfully confirmed by further western blot analysis. This proteomic profiling study not only identified novel plasma biomarkers that may facilitate the development of clinically diagnostic for DAI, but also provided enhanced understanding of the molecular mechanisms underlying DAI.

1. Introduction

Traumatic brain injury (TBI) is one of the most frequently encountered cause of death and disability worldwide (Abu Hamdeh et al., 2017). Diffuse axonal injury (DAI) is a special type of TBI that mainly caused by rotational or angular acceleration/deceleration forces to the head during traffic accidents or other traumas (Abu Hamdeh et al., 2017; Zhang et al., 2018a). Recent epidemiological studies showed that DAI accounts for up to one-third of all patients with TBI, and even more in patients with severe TBI (Vieira et al., 2016; Kokkoz et al., 2017). DAI is also considered the most important factor in determining the poor neurological outcome and long-term cognitive, physical and behavioral impairments of patients after brain trauma (Adams et al., 2011; Lin and Wen, 2013). Thus, DAI is considered to be one of the worst results of TBI (Kokkoz et al., 2017; Adams et al., 1989). The pathological changes of DAI are characterized mainly as widespread injury of axons in the brain corpus callosum, brainstem and neural tracts (Abu Hamdeh et al., 2017). At present, the precise molecular mechanisms responsible for this pathological changes remain elusive, and it is still difficult for clinicians to make a diagnosis and take appropriate therapeutic interventions at the early stage of DAI (Ma et al., 2016a).

Due to the lack of objective laboratory-based modalities, the diagnosis of DAI most relies on the injury history, subjective identification of clinical symptoms and traditional brain imaging examinations, such as computed tomography (CT) and magnetic resonance imaging (MRI) (Furtado et al., 2013; Mallouhi, 2014). However, patients with DAI usually rapidly fall into unconscious after brain trauma and/or present no specific neurological symptoms, especially the patients with mild DAI (Zhang et al., 2018a). Recent pathological studies have demonstrated that axonal injuries can still be found in the white matter of the brain with normal imageological results (Li and Feng, 2009; Zhang

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https://doi.org/10.1016/j.brainresbull.2018.07.015
Received 3 May 2018; Received in revised form 24 July 2018; Accepted 25 July 2018
Available online 02 August 2018
0361-9230/

et al., 2016; Li et al., 2011). Those resulting in a high rate of underdiagnosis, a delayed treatment and finally an increased risk of disability, mortality and poorer prognosis in patients with DAI (Li et al., 2011; Shenton et al., 2012).

The ongoing omics based research on DAI has been continuously identifying novel biomarkers and refining the complexity of the pathophysiological mechanisms of DAI (Zhang et al., 2018a, 2016). In our prior study, several metabolites were identified as candidate biomarkers using an integrated ^{1}H NMR and UPLC-Q-TOF/MS-based metabonomics approach (Zhang et al., 2018a). However, there still exist some limitations in the study, such as only one time point was concerned and the dynamic evolvement process of the identified metabolites was not studied. Moreover, our research group has found four potential protein biomarkers in cerebral tissue by an iTRAQ-based proteomics approach (Zhang et al., 2016). However, due to the limitation of biological samples, clinical application of these results is difficult. In DAI patients, blood may contain various axonal injury related biomolecules, which not only may be serving as reliable DAI biomarkers, but also link to the pathological changes of axons occurred in DAI progression. Thus, blood plasma may be a suitable sample source for DAI biomarkers identifying which has the advantages of minimally invasive and convenient for dynamic monitoring.

Taking note of these, figure out the blood plasma based proteomic deregulation in DAI is important to identify reliable biomarkers that enable early DAI diagnosis and elucidate the mechanisms of axonal injury in DAI. As blood plasma based proteomic has these advantages described above, it has been successfully applied to identify diagnostic biomarkers associated with many diseases such as breast cancer (Suman et al., 2016), myocardial injury (Cheow et al., 2018), and autism (Shen et al., 2017). Therefore, in this study, we established a rat model of DAI, and utilized an isobaric tag for relative and absolute quantitation (iTRAQ) coupled with nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS) proteomic approach to screen differentially expressed plasma proteins in the rat after injury. Bioinformatics analysis of the altered expressed proteins was employed to identify differently expressed proteins associated with the pathophysiological mechanisms of axonal injury underlying DAI. Our study not only identified two differentially expressed proteins may serve as potential plasma biomarkers of DAI, but also may offer us a deeper understanding of the pathophysiological mechanisms underlying DAI.

2. Material and methods

2.1. Chemicals and reagents

All acetonitrile and formic acid used in this study were of high-performance liquid chromatography grade. Acetonitrile was purchased from Tedia (Fairfield, USA). Formic acid was obtained from Sigma Aldrich (St. Louis, MO, USA). Deionized water used in this experiment was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Animals and ethics statement

All experimental procedures for this study were reviewed and approved by the Institutional Animal Research Ethics Committee of C.Q. Medical University (Chongqing, China). All animals procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animals. A total of 70 adult male Sprague-Dawley rats (weighing 275 ± 25 g at the start of the experiment) purchased from the C.Q. Medical University Laboratory Animal Center (Chongqing, China) were used in this study. All animals were housed under standard conditions (12-hour light/dark cycle, 40–70% relative humidity and 21–24 °C), and allowed *ad libitum* access to standard diet and water.

2.3. Models and sample collection

2.3.1. Experimental model of DAI

DAI was performed on animals using an injury model as described in our previous study (Zhang et al., 2016). In brief, 61 rats were randomly selected and used to establish the DAI model. In this study, the severity of DAI depends on the height of the hammer. A height of 150 cm was chosen in our injury model instead of 200 cm by Marmarou et al for obtaining less severe DAI which often are undiagnosed (Marmarou et al., 1994; Foda and Marmarou, 1994). Animals were anesthetized *via* intraperitoneal injections of sodium pentobarbital (100 mg/kg). 12 animals died immediately post-injury with a mortality rate of 19.7%, while the others promptly fell into a coma (5.6 ± 1.2 min). 45 survived animals were randomly selected and assigned to five groups (n = 9 rats/group), and sacrificed at survival periods of 1 h, 6 h, 1 d, 3 d and 7 d, respectively. The remaining four rats were anesthetized with sodium pentobarbital (100 mg/kg) and euthanized by decapitation. Nine sham animals suffered the same surgical procedures of the injured rats, including anesthesia and scalp incision, but were not subjected to injury.

2.3.2. Groups

(1) Control group (n = 9).

(2) Injury group (n = 45, with five subgroups): 1 h group (n = 9), 6 h group (n = 9), 1 d group (n = 9), 3 d group (n = 9) and 7 d group (n = 9).

2.3.3. Sample collection

Heart blood samples from the animals were collected into EDTA-coated blood collection tubes. Plasma was separated from blood cells by centrifugation at 3,000 rpm and 4 °C for 15 min, and then stored at −80 °C until processing for proteomic analysis and iTRAQ-identified candidate proteins verification.

Brain tissue samples from the rats were rapidly removed and 10% phosphate buffered formalin fixed. All the brain tissue samples were used for histopathological examination with Bielschowsky silver staining and hematoxylin-eosin (HE) staining to validate the DAI rats model used in this study (Foda and Marmarou, 1994; Jia et al., 2012).

2.4. iTRAQ sample preparation

In order to minimize biological variation between specimens, three blood plasma samples from each of the six subgroups were equivalently pooled respectively to obtain a final sample volume of 200 µl of blood plasma that was used for proteomic analyses. Plasma proteins were precipitated with 80% acetone overnight at −20 °C, and centrifuged at 13,000 rpm for 20 min at 4 °C. Extracted proteins were solubilized using lysis buffer (pH = 8; 4% CHAPS, 0.001% bromophenol blue, 8 M urea, 0.2% Bio-Lyte and 65 mM DTT). An aliquot of the supernatant was taken for protein concentration determine using the Bradford assay according to the manufacturer's instructions (Abcam Inc., Cambridge, UK; catalog number: ab102535). An aliquot of 100 µg protein from each sample was digested with trypsin solution (protein: trypsin = 50:1 w/w; Promega, Madison, WI, USA) for 12 h at 37 °C, and the tryptic digested peptides were desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA). After that, the eluted peptides were dried in a vacuum concentrator and reconstituted in 0.5 M triethylammonium bicarbonate. The peptides were labeled using an iTRAQ reagent kit (AB SCIEX, Massachusetts, USA) according to the manufacturer's protocol. The six samples were labeled as follows: the control group sample was labeled with iTRAQ tag 113 and the samples from the rats sacrificed at 1 h, 6 h, 1 d, 3 d, 7 d after injury were labeled with iTRAQ tag 114, iTRAQ tag 115, iTRAQ tag 116, iTRAQ tag 118 and iTRAQ tag 121, respectively. The six iTRAQ-labeled samples were then multiplexed and dried in a vacuum concentrator.