Comprehensive Proteomic Profiling of Patients' Tears Identifies Potential Biomarkers for the Traumatic Vegetative State

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Abstract The vegetative state is a complex condition with unclear mechanisms and limited diagnostic, prognostic, and therapeutic methods. In this study, we aimed to explore the proteomic profile of tears from patients in a traumatic vegetative state and identify potential diagnostic markers using tears—a body fluid that can be collected non-invasively. Using iTRAQ quantitative proteomic technology, in the discovery phase, tear samples collected from 16 patients in a traumatic vegetative state and 16 normal individuals were analyzed. Among 1080 identified tear proteins, 57 were upregulated and 15 were downregulated in the patients compared to the controls. Bioinformatics analysis revealed that the differentially-expressed proteins were mainly involved in the wound response and immune response signaling pathways. Furthermore, we verified the levels of 7 differentially-expressed proteins in tears from 50 traumatic vegetative state patients and 50 normal controls (including the samples used in the discovery

phase) using ELISA. The results showed that this 7-protein panel had a high discrimination ability for traumatic vegetative state (area under the curve = 0.999). In summary, the altered tear proteomic profile identified in this study provides a basis for potential tear protein markers for diagnosis and prognosis of the traumatic vegetative state and also provides novel insights into the mechanisms of traumatic vegetative state.

Keywords Trauma · Vegetative state · Tears · Proteomics · iTRAQ

Introduction

The vegetative state is one of the worst outcomes of acquired brain injury. Patients in a vegetative state open their eyes spontaneously but show no signs of consciousness [1]. The permanent vegetative state is defined as “a clinical condition of unawareness of self and environment in which the patient breathes spontaneously, has a stable circulation, and shows cycles of eye closure and opening which may simulate sleep and waking” [2].

The vegetative state is a complex neurological condition with unclear mechanisms and limited diagnostic, prognostic, and therapeutic methods. The neurophysiological substrates of vegetative state are steadily being unraveled. The vegetative state is mostly caused by injury in the cortex, white matter, and brainstem [3]; secondary injury includes intracranial hemorrhage, infection, and hypoglycemia [4]. Neuroimaging studies have shown a consistent reduction in brain metabolism and reduced basal resting-state activity [5, 6], while unexpectedly high levels of residual cognitive function are present in some cases [7–9]. On the other hand, patients in a vegetative state pose

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problems for diagnosis and prognosis. So far, the diagnosis of vegetative state depends on expert observation of the patient's behavior [10]. Although recent studies have shown the prognostic value of the structure of the resting-state electroencephalogram and resting-state networks [11, 12], there are limited molecular prognostic markers available to predict the chance of recovery or the risk of serious consequences. Therefore, further efforts are needed to investigate the molecular mechanisms underlying the vegetative state and to develop molecular diagnostic/prognostic markers for the vegetative state.

Attempts to discover biomarkers for traumatic brain injuries (TBIs) using proteomics strategies have been made in several studies [13]. However, the invasive nature of collecting cerebrospinal fluid or blood samples limits its potential for clinical application [14, 15]. Alternatively, human tears can be easily collected non-invasively and repeatedly for biochemical analysis. Traditionally, tear fluid has been explored extensively for various eye diseases [16]. Although there are some differences between the plasma and tear proteomes, a significant proportion of blood proteins can be found in tears [17]. Dramatic changes in tear protein profiles have also been reported in systemic diseases, such as autoimmune diseases and cancer [18–21]. In addition, a reflex arc regulates the composition of tear proteins under some circumstances; for example, more epidermal growth factor is found in tears when there is a painful corneal wound [22], suggesting that there is considerable neurophysiological signaling to the Vth nucleus in the brainstem and higher somatic centers. Thus, it is possible to improve our understanding of the mechanism involved in the vegetative state and to develop diagnostic/prognostic markers by investigating the proteome profile of tears from patients in a vegetative state.

In this study, we aimed to investigate quantitative changes in the proteomic profile of tears from patients in a traumatic vegetative state compared to that in healthy controls. This study is the first effort, to the best of our knowledge, to investigate a potential link between the proteomic profile of tears and the traumatic vegetative state.

Methods

Patient Demographics and Sample Collection

Patients with severe, non-penetrating TBI who met the criteria of vegetative state according to the Coma Recovery Scale-Revised (CRS-R) definition were included. Trained experienced doctors performed a CRS-R assessment twice a day for 5 days and used the best response to establish the final diagnosis. In cases of ambiguity or disagreement, the

patients were re-assessed until a consensus was reached. Centrally-acting drugs, neuromuscular blockers, or sedatives were not administered during the 24 h prior to assessment. The exclusion criteria included disabilities related to the central nervous system, pregnancy, medical instability, and ocular diseases. Baseline data, such as age, sex, Glasgow coma scale (GCS) score, Rotterdam CT score, and operation type, were also collected and analyzed. Healthy adults without a history of head injury within 8 weeks matched by gender and age were included as the control group. The study was approved by the Institutional Review Board of Ren Ji Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was given by the control participants and the legal representatives of the traumatic vegetative state patients. In the discovery phase, 16 tear samples were collected from the patients (mean age 40.94; range 31–50 years) and another 16 tear samples were collected from the healthy controls (mean age 42.44; range 35–50 years). In the verification phase, 50 tear samples (including those used in the discovery phase) were collected from patients (mean age 47.5; range 25–80 years) and another 50 tear samples (including those used in the discovery phase) were collected from healthy controls (mean age 47.0; range 22–77 years).

Tears were collected from both eyes using Schirmer's strips (Schirmer test 1, without anesthetic) placed for 5 min in the inferior cul-de-sac [20]. Study participants with no concurrent eye diseases, no recent eye surgery, and non-contact lens wearers were recruited for this study. Special care was taken not to touch the lid margin or the ocular surface. The extent of wetting in each strip was measured and recorded after 5 min. The strips from both eyes were stored in a 1.5-mL Eppendorf tube at -80°C until further analysis.

Experimental Design and Sample Preparation

The Schirmer's strips were cut into small pieces and placed in 100 μL of 50 mmol/L ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) containing protease inhibitor (Halt Protease Inhibitor cocktail, Thermo Scientific, Rockford, IL) for 3 h to elute tear proteins. The total tear protein concentration in each sample was measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) (Fig. 1A). In the discovery phase, 16 tear samples from patients in a traumatic vegetative state and 16 tear samples from healthy controls were used. In iTRAQ (isobaric tags for relative and absolute quantification) 8-plex experiments, 4 samples were randomly selected and pooled to form one arm. In total, there were 4 control arms (labeled iTRAQ 113, 114, 115, and 116) and 4 disease arms (labeled iTRAQ 117, 118, 119, and 121). Samples

Fig. 1 Proteomic analysis of tear samples. **A** Experimental design: in the discovery phase, 16 tear samples from patients and 16 from healthy controls were used. In iTRAQ 8-plex experiments, 4 samples were pooled to form one arm. In total, there were 4 control arms (labeled iTRAQ 113, 114, 115, and 116) and 4 disease arms (labeled iTRAQ 117, 118, 119 and 121). **B** Data analysis: data with coefficient of variation < 30% between two technical runs were retained for further analysis. The proteins with a fold change > 2.0 or < 0.5 and occurred in at least 2 out of 4 biological replicates were considered to be differentially-expressed proteins. **C** Results of protein identification (false discovery rate < 1%).

were dissolved in dissolution buffer (0.5 $\mu\text{mol/L}$ triethylammonium bicarbonate). The protein concentration was measured and 25 μg from each sample were pooled to form one iTRAQ arm (total protein, $4 \times 25 \mu\text{g} = 100 \mu\text{g}$) in iTRAQ 8-plex experiments. Briefly, the protein samples were denatured, alkylated, and digested with trypsin. The digested samples were reconstituted in dissolution buffer (0.5 $\mu\text{mol/L}$ triethylammonium bicarbonate) and labeled with iTRAQ reagents according to the experimental design in Fig. 1A.

2D nanoLC-MS/MS Analysis

For high pH fractionation, the 8-plex pooled samples were reconstituted in mobile phase A (20 mmol/L ammonium formate, pH 10) and injected into a Waters HPLC system with a photodiode array detector (Elstree, UK). Peptides were separated using a reversed phase column (Waters Acquity UPLC BEH C18, 3.5 μm , $3.0 \times 150 \text{ mm}$). A step linear gradient of mobile phase B (80% acetonitrile in 20 mmol/L ammonium formate, pH 10) from 5% to 15% over 20 min, 15%–40% over 20 min, and lastly, 40%–100% over 1 min at a flow rate of 0.4 mL/min was used for this fractionation step.

A total of 10 fractions were collected and reconstituted in 12 μL loading buffer (mobile phase A: 0.1% formic acid, 2% acetonitrile in water), and 2 μL from each fraction was injected into a nanoLC-MS/MS [Ultimate 3000 nanoLC system (Dionex, Thermo Fisher Scientific, MA) coupled with an AB Sciex 5600 TripleTOF (AB Sciex, Framingham, MA)]. The second dimension separation was based on the reversed phase LC with an Acclaim PepMap RSLC C18 column (15 cm $\times$ 75 μm i.d., Dionex). A step linear gradient of mobile phase B (2/98 v/v of water/ACN with 0.1% formic acid) from 5% to 12% over 30 min, 12%–40% for 60 min, and 40%–90% over 20 min at a flow rate of 300 nL/min was used for this analysis. All data were acquired using the information-dependent acquisition (IDA) mode in Analyst TF 1.7 software (AB Sciex, Framingham, MA). Data acquisition parameters were as follows: (1) TOF-MS survey scan: 250 ms; (2) mass range for TOF-MS: 400–1250; (3) product ion scan: 50 ms; (4) mass range for MS/MS: 100–1500; (5) switching criteria: ions greater than m/z 400 and smaller than m/z 1250 with a charge state of 2–5, maximum number of candidate ions to monitor per cycle was 40 spectra and the abundance threshold was > 120 counts; former target ions were excluded for 12 s; (6) collision energy: IDA Advanced “rolling collision energy”.

Data Processing and Statistical Analysis

The data were processed using Proteinpilot software 5.0 (AB Sciex) with database version “uniprot_all_Oct2014”.

Gene Ontology and Pathway Analysis

Gene Ontology classification was performed using both iPathwayGuide and David 6.7 online software (<https://david.ncifcrf.gov/home.jsp>). Pathway analysis was performed using the iPathwayGuide online bioinformatics tool (<https://apps.advaitabio.com>).

ELISA and Receiver Operating Characteristic (ROC) Curve Analysis

In the verification stage, the levels of 7 promising tear proteins [cystatin B (CTSB), protease, serine 1 (PRSS1), S100 calcium-binding protein A7 (S100A7), glutathione S-transferase P (GSTP1), complement factor H (CFH), kininogen 1 (KNG1), and alpha-1-acid glycoprotein 1 (ORM1)] were measured using the ELISA kits for human CTSB, human PRSS1, human CFH, human KNG1, and human ORM1 (Boster Biological Technology, Wuhan, China) and for human S100A7 and human GSTP1 (from Cloud-clone Corp., Houston, TX). Briefly, 5 mm² of Schirmer's strip was used for ELISA assay [23]. The strips were cut into pieces and soaked in 10 μL of 50-mmol/L ammonium bicarbonate to elute the tear proteins. For each protein, the tear solution was diluted appropriately to fit into the linear range of the calibration curve. Absorbance was read at 450 nm. Concentration was determined using a calibration curve. The coefficient of variation (CV%) for inter-assay and intra-assay was $< 10\%$ for all assays. The assay was done in triplicate. ROC curve analysis was performed using MetaboAnalyst 3.0 online software (<http://www.metaboanalyst.ca/>).

Results

Clinical Data

Of the 100 tear samples, 50 were collected from traumatic vegetative state patients and 50 from healthy controls. There was no statistical difference between the patient and control groups with regard to age (discovery phase 40.94 ± 5.30 vs 42.44 ± 4.94 , $P = 0.41$; verification phase: 47.50 ± 14.74 vs 47.00 ± 13.84 , $P = 0.86$) and male gender (discovery phase 14 (70%) vs 14 (70%), $P = 1.00$; verification phase: 33 (70%) vs 35 (66%), $P = 0.83$). The 16 patients in the discovery phase had a CRS-R score of 3.56 ± 1.46 , a pre-operative GCS of 3.88 ± 1.02 , and a Rotterdam CT score of 5.19 ± 0.91 , while the overall CRS-R, pre-operative GCS, and Rotterdam CT scores of the 50 patients in the verification phase were 4.04 ± 2.02 , 4.00 ± 1.05 , and 5.18 ± 0.83 , respectively. The clinical information is given in Table 1.

Table 1 Clinical information and characteristics of participants.

Characteristics	Discovery		Verification (including samples used in discovery phase)	
	Normal controls (<i>n</i> = 16)	Traumatic vegetative state group (<i>n</i> = 16)	Healthy controls (<i>n</i> = 50)	Traumatic vegetative state group (<i>n</i> = 50)
General information				
Male gender	12 (75%)	10 (62.5%)	35 (70%)	33 (66%)
Age, mean $\pm$ SD	42.44 $\pm$ 4.94	40.94 $\pm$ 5.30	47.00 $\pm$ 13.84	47.50 $\pm$ 14.74
Days post-injury, mean $\pm$ SD	–	37.63 $\pm$ 7.43	–	37.86 $\pm$ 7.82
Diagnosis of persistent vegetative state				
CRS-R, mean $\pm$ SD	–	3.56 $\pm$ 1.46	–	4.04 $\pm$ 2.02
Auditory, mean $\pm$ SD	–	0.43 $\pm$ 0.73	–	0.62 $\pm$ 0.81
Visual, mean $\pm$ SD	–	0.43 $\pm$ 0.51	–	0.50 $\pm$ 0.51
Motor, mean $\pm$ SD	–	0.69 $\pm$ 0.87	–	0.76 $\pm$ 0.89
Oromotor/Verbal, mean $\pm$ SD	–	0.00 $\pm$ 0.00	–	0.18 $\pm$ 0.48
Communication, mean $\pm$ SD	–	0.06 $\pm$ 0.25	–	0.18 $\pm$ 0.48
Arousal, mean $\pm$ SD	–	1.88 $\pm$ 0.34	–	1.80 $\pm$ 0.49
Diagnosis of trauma				
Intracerebral hematoma/contusions	–	13 (81.3%)	–	40 (80%)
Acute subdural hematoma	–	14 (87.5%)	–	44 (88%)
Acute epidural hematoma	–	8 (50%)	–	22 (44%)
Skull fracture	–	14 (87.5%)	–	41 (82%)
Subarachnoid hemorrhage	–	14 (87.5%)	–	45 (90%)
Cerebral hernia	–	15 (93.8%)	–	44 (88%)
Rotterdam CT Score				
Sum score	–	5.19 $\pm$ 0.91	–	5.18 $\pm$ 0.83
Basal cisterns	–	1.94 $\pm$ 0.25	–	1.88 $\pm$ 0.33
Midline shift	–	0.88 $\pm$ 0.34	–	0.84 $\pm$ 0.37
Epidural mass lesion	–	0.50 $\pm$ 0.52	–	0.56 $\pm$ 0.50
Intraventricular blood/traumatic sub-arachnoid hemorrhage	–	0.88 $\pm$ 0.34	–	0.90 $\pm$ 0.30
Pre-operative GCS	–	3.88 $\pm$ 1.02	–	4.00 $\pm$ 1.05
Type of operation				
Decompressive craniectomy	–	14 (87.5%)	–	46 (92%)
Epidural hematoma clearing	–	14 (87.5%)	–	30 (60%)
Subdural hematoma clearing	–	13 (81.3%)	–	39 (78%)
Hemorrhagic contusion clearing	–	13 (81.3%)	–	35 (70%)
Lateral ventriculopuncture drainage	–	15 (93.8%)	–	48 (96%)
Treatment without surgery	–	1 (6.3%)	–	2 (4%)

Patients were diagnosed with vegetative state according to the criteria of the Royal College of Physicians (2003). All patients were assessed using the Glasgow Coma Scale (GCS).

Proteomic Profiles of Tears from Patients in a Traumatic Vegetative State

The experimental design and workflow are illustrated in Fig. 1A, B. In this study, 1080 tear proteins were identified with a false-discovery rate (FDR) < 1% and the levels of 1032 were quantifiable (Fig. 1C). After two technical runs 761 proteins had reproducible results (> 80% overlap,

Fig. 1C). We found that the levels of 72 tear proteins (57 up-regulated and 15 down-regulated) were significantly altered when we compared the traumatic vegetative state group with the control group (Fig. 2A). The significant differences were defined as (1) a cutoff ratio of traumatic vegetative state/control > 2.0 or < 0.5, and (2) found in at least two biological replicates (Fig. 1B). The individual and average levels of four biological replicates of these 72

Fig. 2 Proteomic profile of tears from patients in a traumatic vegetative state. **A** Volcano plot of the proteomic profile of tears. A total of 1080 tear proteins were identified with an FDR < 1% and the

levels of 72 were found to be significantly different between patient and control groups. **B** Heat map of the differentially-expressed proteins (red, upregulated; green, downregulated).

differentially-expressed proteins are shown using a heat map in Fig. 2B.

Bioinformatics Analysis of Differentially-Expressed Proteins

The 72 differentially expressed proteins (Fig. 2B) were subjected to bioinformatics analysis to further understand the possible mechanisms underlying the traumatic vegetative state and for better selection of potential biomarkers for diagnosis/prognosis. We first performed Gene Ontology analysis using iPathwayGuide online software [24]. The top 10 Gene Ontology terms in the cellular components,

molecular function, and biological process category are listed in Fig. 3A. In the cellular component category, most of the differentially-expressed proteins were classified as extracellular exosome and vesicle, confirming that most tear proteins are secreted, as expected. To reduce the redundant nature of annotations, we also performed functional annotation clustering using DAVID online software which integrates Kappa statistics and fuzzy heuristic clustering to classify groups of similar annotations based on the degree of common genes between two annotations [25]. As shown in Fig. 3B, the differentially-expressed proteins were mostly involved in wound response (Table 2) and in inflammatory and immune

Fig. 3 Gene Ontology analysis of differentially-expressed proteins. **A** Seventy-two differentially expressed proteins were analyzed and the top 10 Gene Ontology terms in the cellular components,

molecular function, and biological process category are listed. **B** Functional annotation clustering using DAVID to reduce the redundancy of annotations.

Table 2 Gene Ontology analysis revealed 21 proteins involved in response to wounding.

No.	Official gene symbol	Gene name	Up/down regulated in vegetative state patients
1	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	Up
2	S100A12	S100 calcium-binding protein A12	Up
3	S100A8	S100 calcium-binding protein A8	Up
4	AHSG	Alpha-2-HS-glycoprotein	Up
5	CTSB	Cathepsin B	Down
6	C4B	Complement component 4B (Chido blood group)	Up
7	C9	Complement component 9	Up
8	CFH	Complement factor H	Up
9	CFI	Complement factor I	Up
10	FGA	fibrinogen alpha chain	up
11	FGG	Fibrinogen gamma chain	Up
12	FN1	Fibronectin 1	Up
13	ITIH4	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	Up
14	KNG1	Kininogen 1	Up
15	ORM1	Orosomucoid 1	Up
16	ORM2	Orosomucoid 2	Up
17	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	Up
18	SERPINC1	Serpin peptidase inhibitor, clade C (antithrombin), member 1	Up
19	SAA1	Serum amyloid A1	Up
20	SPRR3	Small proline-rich protein 3	Up
21	SOD2	Superoxide dismutase 2, mitochondrial	Up

Ontology term: response to wounding.

response. We also found that peptidase and protease inhibitors may play important roles (annotation cluster 3 in Fig. 3B and supplementary Table S1). Furthermore, pathway analysis showed that the differentially-expressed proteins represent key pathways involved in *Staphylococcus aureus* infection, PI3K-AKT signaling pathway, neuroactive ligand-receptor interaction, complement and coagulation cascades, FOXO signaling pathway, Renin secretion, and metabolic pathways including retinol, sucrose, pyrimidine, purine, histidine, selenocompound, glutathione, β -alanine metabolism, as well as pathway in Huntington's disease (Fig. 4).

Verification of Proteomic Results

Next, we selected 7 of the differentially-expressed proteins based on the fold change and how closely related their functional annotations were to brain function and metabolism (Fig. 5A), to verify the proteomic results. ELISA assays of the 7 tear protein biomarker candidates showed that their levels were significantly different between

traumatic vegetative state patients and healthy controls ($P < 0.01$) (Fig. 5B–H), suggesting that the proteomic results are reliable and these 7 proteins may further serve as diagnostic/prognostic markers.

Evaluation of Tear Protein Biomarkers for Traumatic Vegetative State

As noted above, we selected 7 differentially-expressed tear proteins for discriminating traumatic vegetative state patients from healthy controls. In order to visualize the variations, the normalized ELISA results of the 7 proteins were used to generate a heat map (Fig. 6A). Using the selected tear protein marker candidates, the traumatic vegetative state patients and healthy controls were clustered into two groups with only one exception, suggesting that the combination of these 7 proteins had high ability to discriminate the traumatic vegetative state (AUC = 0.999, Fig. 6B). The accuracy was significantly higher using the 7-protein panel than a single protein marker (AUC ranging from 0.779 to 0.95, Fig. S1A–G).

Fig. 4 Pathway analysis of differentially-expressed proteins. Seventy-three differentially-expressed proteins were subjected to iPathwayGuide online software for pathway analysis. The 30 most related pathways are listed.

Discussion

In this study, we performed comparative analysis of the protein profiles of tears from patients with traumatic vegetative state and healthy controls using quantitative proteomics. In the discovery phase, in order to increase the throughput, 4 tear samples were pooled to form one arm. Nevertheless, there were still four biological replicates (Fig. 1A). The individual variability of the potential tear biomarkers was assessed in the verification phase using ELISA. Two technical replicates were highly reproducible as 761 proteins (~ 83%) were detected in both runs (Fig. 1C). The coverage of the tear proteome was also excellent as > 1000 tear proteins were quantifiable in this study. In total, 1080 proteins (FDR < 1%) were identified and the levels of 72 were significantly different in traumatic vegetative state patients and healthy controls. The differentially-expressed proteins were mostly involved in wound response and immune response. We selected 7 of the differentially-expressed proteins as marker candidates for further verification. The ELISA results correlated well

with the proteomics results. Combination of the 7 proteins had high discrimination for the traumatic vegetative state.

The traumatic vegetative state is an economic burden. Due to a lack of knowledge of its underlying molecular mechanisms, no effective therapeutic methods and diagnostic markers are available. In this study, we explored the tear proteome in traumatic vegetative state patients for the first time. We analyzed the proteomic profile of tears from traumatic vegetative state patients and identified 72 differentially-expressed proteins. The altered levels of tear proteins in traumatic vegetative state patients may provide valuable information to understand the mechanisms underlying that state. As shown in Table 2, increased secretion of proteins involved in wound response was found in their tears, suggesting that the wound-healing pathway is activated in traumatic vegetative state patients.

The wound-healing process consists of four integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling [26]. The first hemostatic phase limits hemorrhage and provides an extracellular matrix for cell migration [27], and platelet activation is its

A

Gene symbol	Description	Up/Down regulated in vegetative state patients	Pathway / Gene ontology term
CTSB	cathepsin B	down	Endopeptidase inhibitor activity, regulation of programmed cell death
PRSS1	protease, serine 1	down	Neuroactive ligand-receptor interaction, pancreatic secretion, protein digestion and absorption
S100A7	S100 calcium binding protein A7	up	Defense response, endopeptidase inhibitor activity, regulation of programmed cell death
GSTP1	glutathione S-transferase pi 1	up	Glutathione metabolism
CFH	complement factor H	up	<i>Staphylococcus aureus</i> infection, Complement and coagulation cascades, endopeptidase inhibitor activity, regulation of programmed cell death
KNG1	kininogen 1	up	Complement and coagulation cascades
ORM1	orosomucoid 1	up	Defense response

B **C** **D** **E** **F** **G** **H**

Fig. 5 Levels of representative proteins in tears from healthy controls and traumatic vegetative state patients. **A** List of 7 selected differentially-expressed proteins. Levels of CTSB (**B**), PRSS1 (**C**), S100A7 (**D**), GSTP1 (**E**), CFH (**F**), KNG1 (**G**), and ORM1 (**H**).

hallmark [28]. In our study, increased levels of fibrinogen gamma and fibrinogen alpha in the platelet activation signaling pathway were detected (Fig. 2B and Table 2),

suggesting that platelet activation occurs in traumatic vegetative state patients. In addition, during this process, platelets secrete chemokines to stabilize the wound and

Fig. 6 Evaluation of tear protein markers for the traumatic vegetative state. **A** Heat map showing the mean level of 7 selected differentially-expressed proteins in normal controls (NOR) and traumatic vegetative state patients. Shades of green to red represent increasing mean values

attract macrophages [29]. Consistent with this, we found that fibronectin 1 was upregulated in the tears of traumatic vegetative state patients (Fig. 2B and Table 2).

Complement cascade activation is an important event in the inflammation phase of wound-healing [30]. As shown in Fig. 2B and Table 2, we found that several key components of complement and coagulation cascades, including KNG1, CD55, CFI, FGG, SERPINC1, CFH, FGA, C9, and C4B, were up-regulated in the tears of traumatic vegetative state patients (Supplementary Fig. S2). Furthermore, increased secretion of S100A8 and S100A12 and dysregulation of their downstream signaling pathways, such as PI3K/AKT and focal adhesion, were also

of a protein. **B** ROC curves of a logistic regression model for distinguishing traumatic vegetative state from healthy controls using the 7 proteins.

detected in tears from traumatic vegetative state patients (Figs. 2B, 4, and Table 2), suggesting that pathways related to the inflammatory process and immune response are activated in traumatic vegetative state patients [31]. Although further studies based on animal models are needed to investigate the exact roles of wound-healing and the immune response pathway in the traumatic vegetative state, changes of these proteins in this pathway could serve as markers to discriminate traumatic vegetative state patients from healthy controls. Furthermore, these pathways could serve as therapeutic targets [32, 33].

Subsequently, we selected 7 differentially-expressed proteins based on the fold-changes and functional annotations

(Fig. 5A) to verify the proteomics results. The ELISA results showed that the levels of these 7 proteins significantly differed between traumatic vegetative state patients and healthy controls ($P < 0.01$) (Fig. 5A–G), suggesting that the proteomics results are reliable and these 7 proteins may further serve as potential diagnostic/prognostic markers.

The persistent traumatic vegetative state is still a diagnostic challenge. The widely-used revised Coma Recovery Scale (CRS-R) is the current gold standard to differentiate persistent vegetative state from minimally-conscious state [34]. Due to the high rate of clinical misdiagnosis, new diagnostic methods are needed. For example, ^{18}F fluorodeoxyglucose positron emission tomography (FDG-PET) and quantitative electroencephalography have proven useful in the determination of a patient's state of consciousness. Particularly, FDG-PET assesses glucose metabolism in the brain. However, these methods are either invasive or expensive. Alternatively, tears can be used as a non-invasive means of diagnosis and this has been demonstrated in many eye diseases and systemic diseases [16, 35]. Our study is the first to show that the tear proteomic profile in traumatic vegetative state patients is markedly different from healthy controls. At this moment, we are unable to answer whether the return to normal of the proteomic profile of tears from a traumatic vegetative state patient may suggest the patient's conscious state. In future studies, the proteomic profile of tears from persistent traumatic vegetative state patients can be compared with that of patients with MCS.

Conclusions

In summary, an altered proteomic profile was found in tears from traumatic vegetative state patients compared to healthy controls. A seven-protein panel was able to distinguish traumatic vegetative state patients from healthy controls with high sensitivity and specificity. The results of the tear proteomics study suggested that post-injury repair functions and related pathways are activated. Our study demonstrates the potential of using tears as a non-invasive tool for diagnosis/prognosis of the traumatic vegetative state.

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