

Urine Myoglobin is Associated with Different Severities and Depths for Burn Patients

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Research Article

Keywords: Burns, Urine Proteomics, Burn Depth, Severity, Label-freeMass Spectrometry

Posted Date: February 4th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1311947/v1>

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Abstract

Backgrounds It is important to make reasonable clinical decision based on precise burn depth for burn specialists. However, the identification of burn depth based upon clinical assessment was not accurate. It still needs more biomarkers to rapidly distinguish different depths for burn specialists. The potential value of urine myoglobin (UM) is still unknown in burn patients.

Patients and methods The urine of 30 burn patients and 10 Healthy volunteers (Discover set) were collected from January 2017 to December 2020. Urine proteomics of all samples was identified by Label-free mass spectrometry and functional analysis for differential expression proteins (DEPs) was also analyzed. The Receiver Operating Characteristic curve to evaluate the value of UM from another 67 burn patients (Validation set).

Results A total of 8253 peptides and 1465 proteins were identified in four groups. For severe burn patients, 402 DEPs (279 up-regulated and 123 down-regulated) were identified and functional analysis demonstrated that neutrophil-mediated immunity, response to stimulus, and complement and coagulation cascades pathway were the most enrichment function. UM had significant differences in burn patients with different severities and depths (Discover and Validation set; $P < 0.05$). UM as a novel biomarker could rapidly distinguish different depths for burn patients (Discover set, $AUC = 0.8203$, 95%CI: 0.6448~0.9957; Validation set, $AUC = 0.7393$, 95%CI: 0.618~0.8606).

Conclusion The urine DEPs were mainly identified in the severe group. UM might as a novel biomarker help burn specialists to rapidly determine different depths for burn patients.

Introduction

Approximately 800,000 people were burned each year in China, and about half of patients need to be hospitalized [1]. There were 3275 burn patients who eventually died in the USA in 2016 and also face serious challenges to decrease the rate of mortality [2]. The depths of burn were commonly divided into three degrees, including superficial (I degree), partial-thickness (II degrees), and full-thickness (III degrees). The depth of the burn has a tremendous impact on the course of treatment and prognosis. Patients with different depths have been received various treatments, such as observation, common treatment, and skin grafting [3]. When it comes to the diagnostics of burn depth, the main challenge is the ability to distinguish between superficial partial-thickness and deep partial-thickness burns, which impact the dermis to varying degrees [4, 5]. The significance of distinguishing partial-thickness burns and full-thickness burns was influenced the critical clinical decision that whether a patient should be allowed to heal on their own or if the wound should be surgically excised and replaced with a graft [6].

Burn depth analysis has traditionally been estimated via physical examination by the burn specialist. The surgeon would assess pain and tenderness, formation of blisters, the appearance of the dermis, color, and if the skin blanched when pressure was applied. However, this method has been shown to be only 60 to 75% accurate in correctly identifying the depth [7]. Many technologies have been emerged to distinguish the depth of burn, including radioactive phosphorus, vital dyes, and thermography [8-10]. The most recent wave of depth analysis techniques includes laser Doppler imaging and indocyanine green videoangiography [11]. However, based on clinical assessment and various technologies are still not accurately assessing burn depth. Therefore, it still needs more biomarkers to easily distinguish different depths for burn patients and make a nearly clinical decision.

Mass spectrometry-based proteomic analysis has been being a powerful screening tool for the early detection of renal function and cancer-related biomarkers. This analysis has been successfully utilized to identify protein biomarkers from body fluids [12-14]. Unlike blood samples, urine could be collected non-invasively and timely, moreover, urine could better reflect the changes in the human body. Besides, the protein compositions of urine samples are relatively simple, stable, and easier to analysis [15].

Based on these, Label-free mass spectrometry (MS) was used to analyze the changes in urine proteins between burn patients with different severity and healthy volunteers. bioinformatics analysis was also carried out for different expression of proteins (DEPs). Our study is to find specific urine proteins for different severities and depths and further evaluate the potential clinical value, which may lay a foundation for potential biomarkers for burn patients.

Patients And Methods

Patients

Thirty burn patients (10 mild, 10 moderate, and 10 severe), who were hospitalized for treatment within 24 hours after burned, were selected before they underwent treatment at Beijing Shijitan Hospital, Capital Medical University from April 2017 to December 2020. The study was approved by the Ethics Committee of Beijing Shijitan Hospital, and all burn patients and healthy volunteers were provided written consent and assent. All experiments conformed to the Declaration of Helsinki.

Exclusion criteria: patients whose age was less than 18 or more than 65 years old were excluded. Patients who have chronic diseases such as chronic kidney disease, diabetes, hyperlipidemia, rheumatism, hepatitis, history of cancers, and blood system diseases were also excluded. Besides, Patients with the usage history of medicine 2 weeks prior to the collection of the urine sample were also excluded. The detailed information for our study was shown in **Figure 1**.

According to the classification proposed by the burn conference, the mild group refers to I degree burn patients who had <9% total body surface area (TBSA) burn; moderate group refers to II degree burn patients who had 10~29% TBSA burn; severe group refers to 30%~49% TBSA burn (III degree) or 10%~19% TBSA burn (II degree), or the total burn area is less than 30%, but one of the following conditions exists: patients with severe general condition or shock; patients with multiple injuries or combined injury; patients with moderate or severe inhalation injury; especially severe burn refers to total area $\geq 50\%$ or I degree burn area $\geq 20\%$. The detailed clinical features were shown in **Table 1**.

To ensure the accuracy and comprehensiveness of reporting in this case-control biomarker study, ten healthy volunteers were selected as the healthy control group (HC) and the detailed clinical features were also shown in **Table 1**.

Evaluation of burn depth

Burn depth evaluation has traditionally been based on the histological assessment of dermal microvascular occlusion in burn biopsies in our hospital.

The analysis of UM

In the present study, each patient's midstream urine was obtained before treatment and the value of urine MB was tested by Beckman ACCESS2, USA. The test of urine MB was carried out under the guidance of the instructions.

Urine samples collection

Midstream urine samples were collected in the morning into dry and clean containers from all volunteers. Immediately after collection, urine samples were centrifuged at 4000r/min for 5 minutes to remove cell debris and casts. Then we divided the supernatants into aliquots and froze them at -80°C until use.

Protein extraction and digestion

To find more urine proteins, ten samples in each group were mixed into one sample and was repeatedly tested three times. The detailed information for test as followed: The frozen urine sample was reconstituted at 4°C , centrifuged at 2000g for 10min, and the protein concentration was determined using Bradford method, rest was frozen to -80°C .

Take $10\mu\text{g}$ of each sample for SDS-PAGE electrophoresis, stain with Coomassie blue for 30 minutes, and decolor until the background is clear. The gel was then washed by destained buffer and treated with ACN as above. When the enzyme solution is completely absorbed by colloidal particles, then made up to $100\mu\text{L}$ with 25 mM ABC, proteins were digested overnight at 37°C .

Next day, after centrifugation in low speed, the supernatant was collected, $200\mu\text{L}$ of ACN was added, put under vortex and mixed. The supernatant was extracted in $100\mu\text{L}$ of 0.1% formic acid (FA). Combined the supernatant and centrifuged at 12000 g for 5 min at room temperature and then lyophilized. The powder was dissolved and mixed in 0.1% of formic acid. The supernatant was slowly loaded to the C18 desalting column, washed with washing solution (0.1% FA, 3% ACN) 3 times, then eluted twice by some elution buffer (0.1% FA, 70% ACN). The eluents were collected and lyophilized.

Peptide identification, quantitative profiling and statistics by LC-MS/MS

Mobile phase A (100% water, 0.1% formic acid) and B solution (100% acetonitrile, 0.1% formic acid) were prepared. Peptides were separated in a analytical column, using a linear gradient elution. The separated peptides were analyzed by Orbitrap Fusion mass spectrometer (Thermo Fisher) and Full scan range from m/z 350 to 1550 with resolution of 120000 (at m/z 200), an automatic gain control (AGC) target value was 2×10^5 and a maximum ion injection time was 50ms.

The resulting spectra from each fraction were searched separately against human database by the search engines MaxQuant. The MS/MS spectra results were queried against the SwissProt human database within Uniprot (www.uniprot.org) using the Proteome Discoverer software suite (v2.1, Thermo Fisher Scientific). Searches were performed using a peptide tolerance of 20 ppm and a product ion tolerance of 0.05 Da. At the protein level, a 1% FDR was used as a filter, and each protein contained at least 1 unique peptide. After filtering the results as described above, the peptide abundances in the different reporter ion channels of the MS/MS scan were normalized. The protein abundance ratios were based on unique peptide results and proteins with a fold change ≥ 1.5 were considered significantly altered.

Functional evaluation and pathway analysis

Online resources such as Database for Annotation, Visualization, and Integrated Discovery (Metascape: <https://metascape.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG: <https://www.genome.jp>), and Geneontology: <http://geneontology.org>) were applied to investigate gene ontology and signaling pathways of candidate biomarkers. STRING protein-protein interactions network analysis (<https://string-db.org>) was used to determine the relationships and net-works between identified proteins.

Statistical analysis

Statistical analyses were performed R 4.0.1 version and visualized by GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA). Data were expressed as median (range) and were tested by ANOVA test and Student-Newman-Keuls (SNK) test. The results were considered to be statistically significant at $P < 0.05$.

Results

Demographic characteristics of burn patients and HC

There were no significant differences in age and gender in each group. In discover set, compared with HC, patients with mild or moderate only had lower level of TP and other features were not found. However, burn patients with severe had lower level of TP and ALB ($P < 0.05$, **Table 1**) than HC. In validation set, there were also significant difference in TP and ALB between severe and non-severe group ($P < 0.05$, **Table 1**) and other clinical features were not found.

Overview of urinary proteomics for burn patients

Our study compared the urine protein expression between burn patients with mild, moderate, severe degree and HC through the approach of Label-free mass spectrometry and tandem mass spectrometry as described in Methods. A total of 8253 peptides and 1465 proteins with a protein FDR of $< 1\%$ and with at least two unique peptides were identified in four groups of urine sample (**Figure 2A, B, and C**).

Heatmap and Venn diagram were performed based on the logarithm of peak intensity in Label-Free Quantification to visualize proteins with differential expression in burn patients. A total of 402 DEPs were identified in severe group ($\log_2 FC > 1.5$; $P < 0.05$; FDR $< 1\%$; **Figure 2F**), of which 279 proteins were up-regulated, and 123 proteins were down-regulated.

However, there was only one DEP between the mild and HC groups (**Figure 2D**), whereas 11 proteins (7 up-regulated and 2 down-regulated; **Figure 2E**) were found between the moderate and HC groups. In summary, our study found that mild and moderate groups were not identified obviously urine DEPs.

Bioinformatics analysis of proteomes for severe group

To further understand the function of DEPs, GO analysis showed that neutrophil-mediated immunity for biological process (BP), collagen-containing for cellular component, and enzyme inhibitor for molecular function were the most enrichment function (**Figure 3A**). Complement and coagulation cascades pathway was most enrichment function through KEGG pathway analysis ($P_{\text{adjust}} < 0.05$; **Figure 3B**).

Furthermore, the metascape analysis further proved that the top three levels of BP were negative regulation of the biological process, immune system process, and response to stimulus, which might further support that urine proteomics could reflect the dynamic change of immunity functions for burn patients (**Figure 3C**).

The STRING analysis of MB

As one of the top up-regulated urine protein, the results of STRING analysis demonstrated that the function of MB was most related to the creatine kinase activity and oxygen carrier activity ($P < 0.05$, **Figure 4**). Besides, *Hp*, *APOA1*, *TNNI2*, and *CYCS* were detected in the data of discover set and related information was shown in **Table 2**.

The difference of UM in patients with different severities and depths

In discover set, there was a significant difference in UM among mild, moderate, and severe groups ($P = 0.0058$, **Figure 5A**) and also in III degrees depth and non-III degrees depth groups ($P = 0.0437$, **Figure 5B**).

In validation set, the same differences in burns patients were also founded ($P < 0.05$, **Figure 5C and 5D**).

The value of UM to distinguish different depths for burn patients

To further evaluate the potential clinical value of UM, UM might could as a biomarker to rapidly distinguish patients with III degrees depth and non-III degrees depth through the ROC curve (Discover set, AUC = 0.8203, 95% CI: 0.6448 ~ 0.9957, **Figure 6A**; Validation set, AUC = 0.7393, 95% CI: 0.618 ~ 0.8606, **Figure 6B**).

Discussion

Blood and urine are common frequently liquids used to the discovery of biomarkers of human diseases as both can be collected frequently and non-invasively. It is a challenge to obtain peripheral venous blood and will increase the incidence of severe infection by the usage of central venous catheter for severe patients. Therefore, Urine as a body fluid has several advantages over blood: easily obtained in large volumes, less complex, and

lower dynamic range allowing low abundance but functionally important proteins [16, 17]. In the present study, only little significant DEPs were found in mild group, which could be interpreted that the lower TBSA could not or slightly activate the response to a stimulus or the immune response. For the moderate group, only 9 DEPs were identified and 5 proteins were also found in the severe group (data was not shown). However, there were 402 DEPs in severe group, moreover, GO and KEGG analysis for DEPs demonstrated that the response to stimulus and complement and coagulation cascades pathway were the most enrichment. Burns that affect $\geq 20\%$ of the TBSA trigger a major inflammatory response and lead to capillary leakage, which mainly caused hypoalbuminemia [18]. Acute kidney injury, Severe infections, and Hypoproteinemia are the most adverse complications for burn patients who were admitted to hospital. Furthermore, the prognosis of those patients seems not well [19-21]. The severe burn patients had lower albumin compared healthy volunteers and further support the results of our study. It is important to make reasonable clinical decision based on precise burn depth for burn specialists and found more reliable biomarker to distinguish the depth of burn.

MB as one of the up-regulated protein was founded in severe group and our study demonstrated that there were significant differences in different severities and depths. Interestingly, our study revealed that UM as a novel biomarker could distinguish different depths in burn patients, which might assist burn specialist to quickly assess the depth of burn. Our study showed that UM play an important role in predicting the depth of burn. We further consider the reasons why UM plays a key role in burn patients. Mb is in the cytoplasm of muscle and cardiac tissue, iron-containing porphyrin and hemoglobin homologous intracellular chromoprotein containing a 153 amino polypeptide chain composed of acid residues and a heme prosthetic group, moreover, Mb could promote the oxygen supply in tissues and has functions such as oxygen transporter, intracellular catalyst and oxygen storage [22]. As we all know, Trauma, infection, drugs, immune diseases, endocrine and metabolic disorders and other factors can cause damage to skeletal muscle and cardiac muscle, followed by dissolution, changes in cell membrane integrity, and the contents of muscle cells enter the extracellular fluid and blood circulation system [23, 24]. Resulting in the increase of serum Mb content, this process involves many pathophysiological mechanisms such as activation of phospholipase A2, mitochondrial dysfunction, continuous contraction of muscle cells, and oxidative stress caused by free radicals. UM was mainly used to predicting the acute kidney injury for burn patients [25]. However, Gosling P and colleagues found that UM was not increased in burn patients [26]. In our study, UM was greatly increased patients with three degrees depth. We further hypothesized that the onset of burn caused the broken of red blood cell. Mb was released into the blood and further excretion in the urine.

To the best of our knowledge, our study is the first report on the identification of urine proteins as a urinary biomarker for burn patients. DEPs were mainly founded burn in severe burn patients. Interestingly, our study revealed that UM could be served as a biomarker to rapidly distinguish different burn depths. However, our study also has some limitations: The sample was not large and collected from a single center. The UM still has certain diagnostic limitations and larger samples need to be validated in the future.

Conclusions

This is the first comprehensive study to demonstrate the landscape of urine proteomics for burn patients. UM could serve as a novel and non-invasive biomarker for distinguishing burn depth. No matter what, our study provides a new insight for burn patients and looking forward to seeing these results translated into clinical practice after validation based on a large sample.

Declarations

Acknowledgement

None

Authors' contributions

TL data analysis and draft the manuscript; QM Z, JT W, and TC L sample and data collection; M Z designed the study and revised the manuscript. All authors reviewed the manuscript.

Funding

This work was supported by the foundation of Beijing Key Clinical Specialty Program (2020ZDZK2).

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author upon a reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Characteristics	Discoverycohort				Validationcohort			
	HC (N=10)	Mild (N=10)	Moderate (N=10)	Severe (N=10)	P value	Mild and Moderate (N=33)	Severe (N=34)	P value
Age, range (median)	23~46 (33.5)	28~54 (36.0)	24~48 (35.0)	24~56 (34.0)	0.519	23~67 (46)	21~66 (40)	0.214
Gender, male, n (%)	5 (50%)	4 (40)	5 (50)	5 (50)	1.0	26 (78.78)	28 (82.35)	0.765
Total protein (g/L)	57.8~75.9 (66.15)	62.8~79.5 (73.4)	52.5~78.0(63.55)	32.4~53.1(49.55)	0.001	53.3~75.9 (62.7)	37.8~71.8(56.35)	0.001
ALB (g/L)	31.7~47.4 (38.05)	36.2~50.4 (44.1)	33.1~44.9 (41.3)	15.5~29 (26.0)	0.001	26.2~47.4(41.3)	18~44.5(30.35)	0.001
BUN	3.52~9.12 (5.09)	3.9~7.12 (5.775)	1.8~6.88 (5.22)	4.1~9.5 (6.4)	0.089	3.2~9.12(4.99)	2.7~16.5(5.7)	0.233
Serum creatinine	47~78 (59.7)	38~81 (54)	42.5~70 (50.6)	36.9~178.1(73.95)	0.496	41~97(61)	40.3~116.8(60.2)	0.792

Note:a: P value was compared between severe and HC group.TP: total protein; ALB:albumin.

Protein ID	Protein name	Gene	Log ₂ FC	P value	FDR
P00738	Haptoglobin	HP	3.124	0.000006	0.00036
P02647	Apolipoprotein A-I	APOA1	2.703	0.000218	0.00201
A0A087WXS0	Troponin I, fast skeletal muscle	TNNI2	3.128	0.000422	0.00291
C9JFR7	Cytochrome c	CYCS	2.972	0.001749	0.00661

Figures

Figure 1

The workflow of this study.

Figure 2

B: The ROC curve in Validation set (AUC=0.7393,95%CI: 0.618~0.8606).