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Original Article

Differential protein expression in patients with urosepsis

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ABSTRACT

Purpose: Urosepsis in adults comprises approximately 25% of all sepsis cases, and is due to complicated urinary tract infections in most cases. However, its mechanism is not fully clarified. Urosepsis is a very complicated disease with no effective strategy for early diagnosis and treatment. This study aimed to identify possible target-related proteins involved in urosepsis using proteomics and establish possible networks using bioinformatics.

Methods: Fifty patients admitted to the Urology Unit of Lanzhou General PLA (Lanzhou, China), from October 2012 to October 2015, were enrolled in this study. The patients were further divided into shock and matched-pair non-shock groups. 2-DE technique, mass spectrometry and database search were used to detect differentially expressed proteins in serum from the two groups.

Results: Six proteins were found at higher levels in the shock group compared with non-shock individuals, including serum amyloid A-1 protein (SAA1), apolipoprotein L1 (APOL1), ceruloplasmin (CP), haptoglobin (HP), antithrombin-III (SERPINC1) and prothrombin (F2), while three proteins showed lower levels, including serotransferrin (TF), transthyretin (TTR) and alpha-2-macroglobulin (A2M).

Conclusion: Nine proteins were differentially expressed between uroseptic patients (non-shock groups) and severe uroseptic patients (shock groups), compared with non-shock groups, serum SAA1, APOL1,CP, HP, SERPINC1and F2 at higher levels, while TF, TTR and A2M at lower levels in shock groups.these proteins were mainly involved in platelet activation, signaling and aggregation, acute phase protein pathway, lipid homeostasis, and iron ion transport, deserve further research as potential candidates for early diagnosis and treatment. (The conclusion seems too simple and vague, please re-write it. You may focus at what proteins have been expressed and introduce more detail about its significance.)

Introduction

Urosepsis is defined as sepsis caused by a urogenital tract infection. Urogenital tract infections are considered to be responsible for about 30% sepsis cases, with a mortality of 20%–40%. As the population ages, the incidence of urosepsis is likely to rise.[1,2] Although blood culture has been considered the gold standard for sepsis diagnosis, it is too slow and limited by false negatives.[3] This has prompted studies to identify methods for sepsis diagnosis in the early-stages, where treatment outcomes are more favorable. Indeed, the prognosis of urosepsis must be assessed as early as possible. Recently, proteomic technologies have been used to detect new biomarkers. A large number of differentially expressed proteins have been reported as potential biomarkers for the diagnosis and prognosis of several diseases. In this study, two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) was applied to assess protein profiles in uroseptic patients with different prognoses, to identify potential prognostic biomarkers.

Methods

Patients

Fifty patients with urinary tract infection, admitted to the Urology Unit of Lanzhou General PLA (Lanzhou, China) from

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October 2012 to October 2015 and diagnosed according to the 2001 and 2008 International Sepsis Definition Conference,[4,5] were enrolled in this study. According to their conditions, these patients were divided into shock and matched-pair non-shock groups. The study was approved by the Lanzhou General PLA Hospital Ethics Committee; informed consent was obtained from all patients or their relatives. Exclusion criteria were: (1) liver dysfunction, (2) abnormal blood coagulation, (3) age<18 years, (4) patients with a history of cancer, and (5) autoimmune diseases.

Blood collection and serum preparation

Blood samples were drawn within 24 h of diagnosis from septic patients, and centrifuged at 15,000 g in the absence of prostacyclin, followed by addition of 20 μL/ml protease inhibitor cocktail to the resulting serum samples. Storage was carried out at −80℃.

Two-dimensional gel electrophoresis

All protein samples from non-shock and shock groups were assessed by 2-DE; a minimum of three samples were run per group to ensure reproducibility. In the first dimension of the 2-DE, protein samples (80 μg) were applied to immobilized pH gradient (IPG) strips (17 cm, pH 3–10, NL; Fig. 1), which were rehydrated overnight at room temperature according to the manufacturer's instructions with DeStreak Rehydration Solution containing 0.4% ampholytes with pH 3–10. The IPG strips were initially run at 50 V for 12 h, and subsequently focused at 250 V for 30 min, 1000 V for 1 h, 10000 V for 5 h, and maintained at 10000 V until a total of 60,000 V/h was achieved. Following is on electric focusing (IEF), the IPG strips were equilibrated with 1.5 M Tris-HCl (pH 8.8), 6 M urea, 87% glycerol, 2% sodium dodecyl sulfate (SDS) and 0.2% bromophenol blue. After initially treatment with 15 ml 1% DTT for 10 min with constant shaking, alkylation with 15 ml 2.5% indole-3-acetic acid was performed for 15 min. Following IEF, the equilibrated strips were subjected to second dimensional separation (SDS-PAGE) using 1.0 mm-thick 10% polyacrylamide gels on a Bio-Rad PROTEAN II xi Cell system. Staining was carried out with silver nitrate (CWBIO, Beijing, China).

Image acquisition and data analysis

The 2D gels were scanned on a Densitometer GS-800 (Bio-Rad). Spot detection, quantification and analysis were performed with the PDQuest8.0.1 software (Bio-Rad), followed by manual matching.

Protein identification by mass spectrometry

MALDI-TOF MS/MS analysis of protein spots was conducted on an Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA, USA). Differentially expressed protein spots were excised from the 2-DE gels and washed with water before digestion. Digestion and peptide extraction were performed according to previous methods.[6] Mass spectra of peptide mixtures were obtained using an Applied Biosystems 4700 Proteomics Analyzer operated in delayed reflector mode with an accelerated voltage of 20 kV. Spectra were calibrated using trypsin auto-digested ion peaks ($m/z = 842.5$ and 2211.1) as internal standards. Peptide mass finger printing (PMF) data were used to search for candidate proteins using the MASCOT (http://www.matrixscience.com) software. A hit was considered to be positive when MASCOT revealed a global score exceeding 60 ($p < 0.05$).

Interaction network of the identified proteins

We used a web-based bioinformatics tool to analyze the interacting proteins: STRING software version 10.0[7] (http://string-db.org). The results included a detailed network featuring several core proteins.

Statistical analysis

SPSS 16.0 (SPSS, Chicago, IL, USA) was used for data analysis. Measured data were expressed as mean ± standard deviation (SD), and compared by *t*-test. Qualitative variables were expressed as percentage, and compared by the Chi-square test. $p < 0.05$ was considered statistically significant.

Results

Identification of differentially expressed 2-DE spots by mass spectrometry

Table 1 summarizes the clinical data of all the patients involved in this study. Protein samples of the non-shock and shock groups were run on 2-DE gels, and each sample was run at least three times to ensure reproducibility. For the non-shock group, an average of 645 ± 45 spots was obtained (Fig. 1A). For those of the shock group, 704 ± 84 spots were obtained (Fig. 1B), indicating no difference in spot count between the two groups ($p > 0.05$). Up- and down-regulation in the shock group was defined with a fold change cutoff value of 2.0 in comparison with the non-shock group. Eight

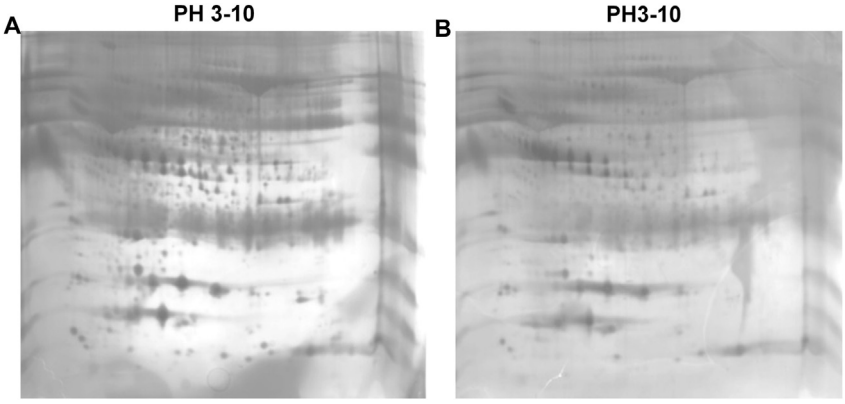

Fig. 1. DE gel from (A) non-shock and (B) shock groups.

Table 1
Clinical characteristics of patients involved in this study.

Characteristics	Non-shock	Shock	*p* value
	n = 25	*n* = 25	
Age (years)	49.64 ± 18.02	49.16 ± 15.62	0.903
Gender (Male/Female)	11/14	12/13	0.500
WBC counts (× 10^9/L)	13.12 ± 2.72	14.32 ± 3.29	0.176
CRP (mg/dl)	14.32 ± 4.44	14.74 ± 4.34	0.778
Seruim PCT (ng/ml)	5.76 ± 2.31	7.88 ± 2.19	0.001
Etiological factors (*n*, %)			
ESWL	7 (28)	8 (32)	0.758
PCNL	8 (32)	11 (44)	0.382
BPH	4 (16)	1 (4)	0.157
Prostate biopsy	1 (4)	2 (8)	0.552
Urinary bladder reflux	2 (8)	1 (4)	0.552
Bactermia	3 (12)	2 (8)	0.637
Pathogens detected (*n*, %)			
E.coli	7 (28)	9 (36)	0.544
Proteus.spp	3 (12)	2 (8)	0.637
Klebsiella	4 (16)	3 (12)	0.684
Fungi	2 (8)	3 (12)	0.637

protein spots with differential levels were statistically significant at $p < 0.01$, including spots 10, 19, 2, 24, 31, 26, 9 and 30 (Fig. 2).

Identification of differentially expressed proteins by MALDI-TOFMS

Spots representing the differentially expressed proteins were excised from stained gels, *in situ* digested with trypsin, and analyzed by MALDI-TOFMS. The identified proteins using MASCOT scores, MS/MS matched sequences, apparent and theoretical MWs, pI values, sequence coverage and regulation are listed in Table 2. There were 9 differentially expressed proteins. Among them, 6 proteins (SAA1, APOL1, CP, HP, SERPINC1 and F2) were upregulated, while another 3 proteins (IF, TTR, and A2M) were down regulated in the shock group compared with the non-shock group.

MALDI-TOF mass spectrum and database query results of the representative spot 19 are shown in Figs. 3, 4, 5. A total of 22 monoisotopic peaks were input into the Mascot search engine to search the Swiss-Prot database, and query results showed that protein spot 19 was APOL1.

Protein–protein interaction analysis

Protein–protein interaction (PPI) analysis was performed through STRING. A total of 9 differentially expressed proteins were imputed into STRING, and a complex network was obtained. The obtained protein–protein interactions are shown in Fig. 6. In biological process analysis, these proteins were mainly involved in platelet activation, signaling and aggregation, acute phase proteins highly expressed in response to inflammation and tissue injury, lipid homeostasis, cartilage development, iron ion transport, and select metabolic processes.

Discussion

Urosepsis mainly results from obstructed uropathy of the upper urinary tract, with ureterolithiasis being the most common cause. The complex pathogenesis of sepsis is initiated when pathogen or injury-associated molecular patterns recognized by pattern recognition receptors of the host innate immune system generate pro-inflammatory cytokines.[6,7] In this study, the 2-DE-based proteomics approach was undertaken to identify altered proteins in shock group compared with the non-shock group. Nine differentially expressed proteins were successfully identified by MALDI-TOF-MS, with six upregulated (SAA1, APOL1, CP, HP, SERPINC1 and F2) and three proteins downregulated (TF, TTR and A2M).

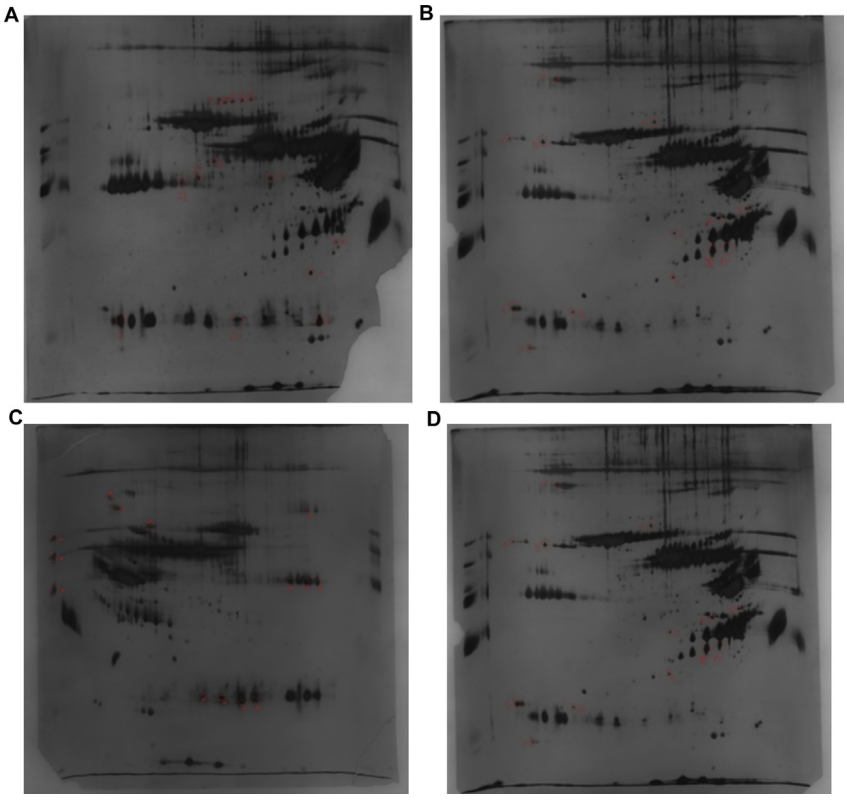

Fig. 2. Results of 2-DE analysis. A, C: Representative 2-DE images of non-shockgroup samples; B, D: Representative 2-DE images of shock group samples.

Table 2
Differentially expressed proteins identified by MALDI-TOF-MS.

Spot No.	Target protein	Aliases	Nominal mass (Mr) KD	Calculated pI	Protein score	Sequence coverage (%)	Expression
10	Serotransferrin	TF	79.294	6.81	88	7	Decreased
19	Serum amyloid A-1 protein	SAA1	13.581	6.28	341	63	Increased
2	Transthyretin	TTR	15.877	5.52	68	8	Decreased
19	Apolipoprotein L1	APOL1	44.004	5.60	119	15	Increased
24	Ceruloplasmin	CP	122.983	5.44	53	4	Increased
31	Haptoglobin	HP	45.861	6.13	69	9	Increased
26	Antithrombin-III	SERPINC1	53.025	6.32	280	15	Increased
9	Prothrombin	F2	71.475	5.64	70	7	Increased
30	Alpha-2-macroglobulin	A2M	163.188	6.03	26	0	Decreased

Analysis of proteins involved biological pathways such as platelet activation, signaling and aggregation, acute phase protein pathway, folate metabolism and the selenium pathway, as well as the implications of these changes are presented for further understanding of urosepsis.

Platelet activation, signaling and aggregation

TF (serotransferrin) related pathways are platelet activation and signaling and aggregation, as well as transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds.[8] The inflammatory process is associated with alterations in iron metabolism. Transferrin, an acute-phase *N*-glycosylated glycoprotein, plays an important role in iron transport.[9] Decreased expression of TF is associated with sepsis, and TF is consistently low during sepsis.[10] ChiarlaC- hypothesized that change in transferring sialylation could reflect the intensity of the inflammatory response, and is insufficient if under-expressed and detrimental when over-expressed.[11–13] In this study, TF showed lower levels in the shock group; the most likely explanation is transferring degradation by neuraminidase. Further studies, including measurement of blood neuraminidase concentrations and activity, are needed to understand the exact role of sialic acid decrease in septic patients.[9]

SERPINC1 (antithrombin-III) inhibits thrombin and other serine proteases of the coagulation system, and regulates the blood coagulation cascade. The related pathways encompass platelet activation, signaling and aggregation, as well as the clotting cascade. Postoperative hemostatic disorders manifest as both hypo- and hyper-coagulation, with thrombinemia in most cases. Patients with urosepsis show latent hyper-coagulation phase of DIC. There is insufficient evidence to support SERPINC1substitution in any category of critically ill participants, including those with sepsis and DIC. SERPINC1 does not impact mortality, but increases the risk of bleeding.[14,15] SERPINA1 is a highly effective inhibitor of neutrophil elastase, plasmin, thrombin, trypsin, chymotrypsin, and plasminogen activator.[16–18] In addition, it is an acute-phase protein and positively associated with necrosis and inflammation.[19] As shown above, compared with the non-shock group, SERPINA1showed higher levels in the shock group.

A2M (alpha-2-macroglobulin) is a protease inhibitor and cytokine transporter. It inhibits many proteases, including trypsin, thrombin and collagenase. Kelly BJ reported that A2M concentrations were significantly lower in surgical intensive care unit (SICU) patients; meanwhile, PCT levels were significantly higher in subjects with bacterial sepsis. Interestingly, combination of A2M and PCT can discriminate bacterial sepsis from other SIRS among SICU patients with suspected sepsis.[20] A2M is also a protein with modulatory properties in inflammation. A previous study indicated that A2M has been microencapsulated could be an effective strategy to harness the complex biology of A2M, enhancing outcomes of fundamental processes of the innate immune response; this paves the way to potential future strategies in controlling sepsis.[21,22] The current findings corroborate previous reports demonstrating that A2M plays an important role in urosepsis regulation.

F2 (prothrombin) coagulation factor II is proteolytically cleaved to form thrombin in the first step of the coagulation cascade, which ultimately results in blood loss. F2 also plays a role in maintaining vascular integrity during development and postnatal life. It was suggested that prothrombin gene-variant associated with sepsis, respiratory distress syndrome, and perinatal asphyxia, as well as other thrombophilic disorders, could be a risk factor for the development of neonatal thrombus.[23,24]

There are many complex pathophysiological changes of the coagulation system in sepsis. Survivors and non-survivors show significant differences in anti-thrombin as revealed by coagulation tests.[25,26]

Acute phase proteins

SSA1 (serum amyloid A-1 protein) is a major acute phase protein that is highly expressed in response to inflammation and tissue injury. This protein also plays an important role in HDL metabolism and cholesterol homeostasis. Apolipoprotein A is a major apoprotein (45%) constituting HDL in the early phase of sepsis, and slowly replaced by apolipoproteinA-1 during recovery. In severe sepsis, HDL is shifted to acute phase HDL, which is enriched in serum amyloid A and depleted of cholesterol and apolipoprotein A-1.[27] In addition, high apolipoprotein A-1 levels are found in patients with sepsis.[28] Liu MJ demonstrated that Zn deficiency enhanced the acute phase response and particularly the JAK-STAT3 pathway, resulting in increased serum amyloid- A production.[29]

HP (haptoglobin) binds free plasma hemoglobin, allowing degrading enzymes to reach hemoglobin, while preventing iron loss through the kidneys, which are therefore protected from hemoglobin induced damage. In critically ill patients with sepsis, elevated plasma levels of haptoglobin are associated with a decreased risk of in-hospital mortality, independently of confounders. Increased haptoglobin amounts may play a protective role in sepsis patients with elevated levels of circulating cell-free hemoglobin, beyond its previously described function of acute phase reactant.[30]

Lipid homeostasis, iron ion transport, and select metabolic processes

APOL1 (apolipoprotein L1) plays a role in lipid exchange and transport throughout the body, and it is involved in reverse cholesterol transport from peripheral cells to the liver. Several transcript variants encoding different isoforms have been described for this gene.[23] A negative correlation between ApoL 1 expression in neutrophils and *C*-reactive protein (CRP) levels was reported as well as a positive association of apoptotic neutrophil count with ApoL1 and 2 mRNA levels. The degree of neutrophil apoptosis in critically ill patients is therefore correlated with modified expression levels of ApoLs.[31]

MS/MS Fragmentation of VNEPSILEMSR
Found in APOL1_HUMAN in SwissProt, Apolipoprotein L1 OS=Homo sapiens GN=APOL1 PB=1 SV=5

Match to Query 55: 1289.563624 from(1290.570900,1+) intensity(0.0000) index(4)
Title: Label: B14, Spot_Id: 21141, Peak_List_Id: 224827, MSMS Job_Run_Id: 13456, Comment:

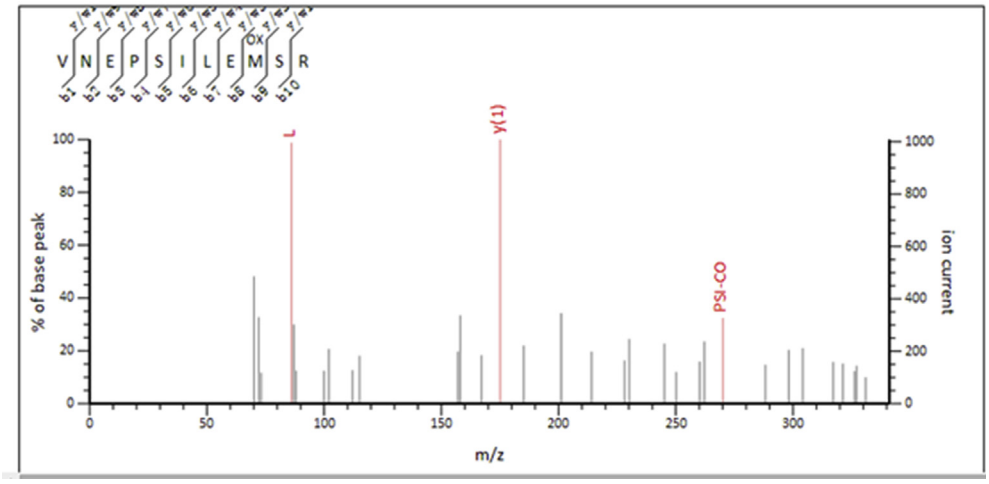

Fig. 3. MALDI-TOF MS mass spectrum of protein spot 19 identified as APOL1 according to the matched peaks.

Protein sequence coverage: 15%

Matched peptides shown in ***bold red***.

```
  1 MEGAALLRVS VLCIWMSALF LGVGVRAEEA GARVQQNVPS GTDTGDPQSK
 51 PLGDWAAGTM DPESSIFIED AIKYFKEKVS TQNLLLLLTD NEAWNGFVAA
101 AELPRNEADE LRKALDNLAR QMIMKDKNWH DKGQQYRNWF LKEFPRLKSE
151 LEDNIRRLRA LADGVQKVHK GTTIANVVSG SLSISSGILT LVGMGLAPFT
201 EGGSLVLLEP GMELGITAAL TGITSSTMDY GKKWWTQAQA HDLVIKSLDK
251 LKEVREFLGE NISNFLSLAG NTYQLTRGIG KDIRALRRAR ANLQSVPHAS
301 ASRPRVTEPI SAESGEQVER VNEPSILEMS RGVKLTDVAP VSFFLVLDVV
351 YLVYESKHLH EGAKSETAEE LKKVAQELEE KLNILNNNYK ILQADQEL
```

Unformatted sequence string: 398 residues (for pasting into other applications).

Fig. 4. Protein sequence of APOL1, and matched peptides are bold red.

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 31 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

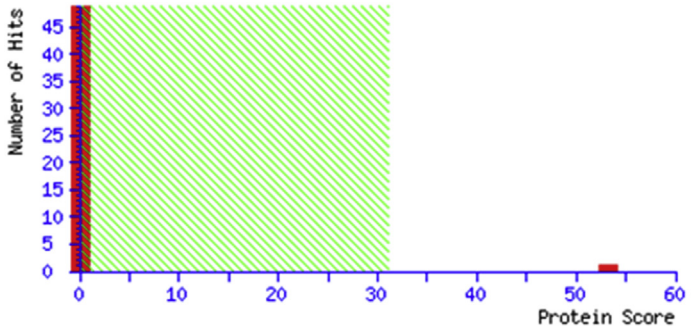

Fig. 5. Database query results and score of protein spot 19.

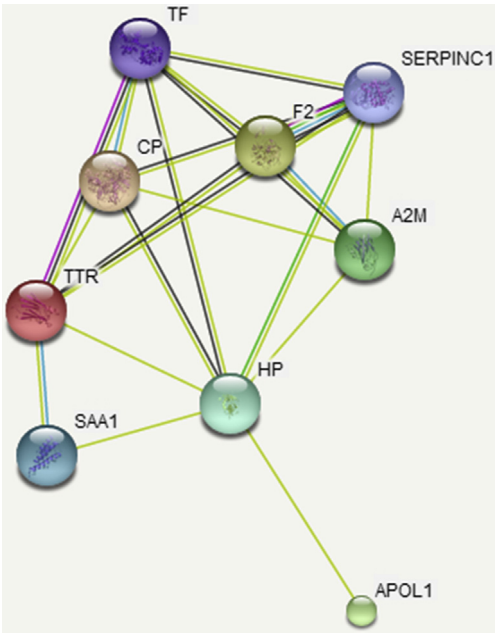

Fig. 6. Protein–protein interactions.

CP (ceruloplasmin) is a metalloprotein that binds most of the copper in plasma; it is involved in the peroxidation of Fe (II) transferrin to Fe (III) transferrin. Serum CP level estimate may help diagnose neonatal septicemia, but is not useful as a tool for early prognosis.[32] A study reported 5 proteins included in a protein-protein interaction network for sepsis differentiation: cadherin 1, haptoglobin, complement 3, alpha-1-antitrypsin, and ceruloplasmin.[33]

TTR (transthyretin) is a carrier protein that transports thyroid hormones in plasma and the cerebrospinal fluid, as well as retinol (vitamin A) in plasma. Raju M S reported a delayed increase in Hp, A1AT, ORM1, S100A9 and SAA levels, whereas TTR levels increased during the early stages of sepsis in non-survivors (NS). Therefore, a weaker acute phase response in early sepsis stages, combined with an inefficient inflammatory response, may contribute to mortality in NS.[30] Transthyretin is a marker predicting outcome in critically ill patients.[34]

Conclusion

This study firstly provided an overview of serum proteomics changes between patients with urosepsis (non-shock group) and severe urosepsis (shock group). Interestingly, 9 differentially expressed proteins were found in the serum of severe urosepsis cases compared with the urosepsis group, and mainly involved in platelet activation, signaling and aggregation, acute phase protein pathway, lipid homeostasis, and iron ion transport. This study revealed molecular markers of early diagnosis and prognosis of urosepsis, and the differentially expressed proteins might be new therapeutic targets for urosepsis treatment.

Fund

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cjtee.2018.07.003.

Conflict of interests

All authors declare that they have not any conflict of interests.

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