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# iTRAQ-based quantitative proteomic analysis reveals potential early diagnostic markers in serum of acute cellular rejection after liver transplantation

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ABSTRACT

Liver transplantation (LT) is the most effective treatment method for advanced stage liver disease but acute cellular rejection (ACR) seriously affects the prognosis of LT. To discover novel diagnostic biomarkers of ACR after LT, Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-based mass spectrometry was performed to characterize alterations of serum proteins among patients validated to be pathologically ACR or pathologically no-ACR after LT and healthy controls. As a result, 10 differentially expressed proteins were found out between the ACR group and the No-ACR group; 88 differentially expressed proteins were found out between the ACR group and the Healthy Control group; 39 differentially expressed proteins were found out between No-ACR group and Healthy Control group. After analysis and ELISA validation, the results showed that CFHR1, CFHR5 and CFH could be candidate protein biomarkers for the early diagnosis of ACR after LT.

## 1. Introduction

Since firstly used by Starzl at 1963, with half a century of development, liver transplantation (LT) has become the most effective treatment method for advanced stage liver disease [1,2]. Acute cellular rejection (ACR) is an important factor which affects the prognosis of LT [3]. Due to the application of potent immunosuppressive drugs, clinical occurrence of ACR was significantly reduced, and clinical symptoms and signs of ACR are lack of typicality. Moreover, some other pathological changes of the liver after LT may make the early diagnosis of ACR become more difficultly [4–6].

Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-based mass spectrometry, as an important proteomic technology, has been widely used to find differentially expressed proteins of many diseases [7–10]. But it's less used in the fields of diagnosis of allograft rejection in organ transplantation and finding rejection-related biomarkers. In this study, looking forward to finding specific protein markers associated with ACR, iTRAQ-based mass spectrometry and bioinformatic technologies were used in separating, screening and identifying differentially expressed serum proteins among patients after LT (classified in ACR and No-ACR groups) and healthy persons.

## 2. Materials and methods

### 2.1. Collection of serum samples

The subjects in this study were from Tianjin First Central Hospital from January 2013 to December 2014, totally including 6 ACR patients, 16 no-ACR patients and 6 healthy persons. All the ACR patients were confirmed by the tissue pathological analyses, which is the golden standard of the ACR. The no-ACR patients showed normal clinical symptoms and liver function test after LT. Due to the iTRAQ limiting condition, three ACR patients were selected in the ACR group and three no-ACR patients were selected in No-ACR group, who were all male and in the similar ages. The healthy persons were from the volunteers who also had the same gender and similar ages. The whole blood samples of ACR group were acquired during the rejection period, and which in No-ACR group were acquired at the 3rd and 4th weeks after LT. After collection, whole blood samples were placed at 4 °C for 60 min, and centrifuged at 2000 rpm for 30 min to collect serum. The serum samples were preserved at −80 °C for further analysis.

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**Table 1**
Clinical characteristics of ACR and No-ACR patients involved in iTRAQ experiment.

| Group | Gender | Age | Primary disease | ALT IU/L (5–35) | AST IU/L (0 − 31) | ALP IU/L (40–150) | Gln IU/L (5–36) |
|---|---|---|---|---|---|---|---|
| ACR | Male | 49 | Liver failure after LT | 148.9 | 84.6 | 118.9 | 196.6 |
| | Male | 48 | Hepatitis C cirrhosis | 55 | 78 | 73 | 210.3 |
| | Male | 59 | Hepatitis C cirrhosis | 37.9 | 117.1 | 253 | 417.8 |
| No-ACR | Male | 56 | Hepatitis B cirrhosis | 18 | 10.8 | 106.3 | 56.7 |
| | Male | 45 | Hepatitis B cirrhosis | 24.9 | 39.8 | 124.4 | 87.4 |
| | Male | 53 | Hepatitis C cirrhosis | 10.6 | 13.2 | 112.2 | 46.9 |

### 2.2. Preparation and labeling of serum proteins

To reduce individual differences, pipetted 200ul serum of six samples of Healthy Control (HC) group, mixed them, and then separate into two samples. Then high-abundance proteins in 3 ACR samples and 3 no-ACR samples and 2 mix Health Control samples were removed with the ProteoMiner protein enrichment kit (Bio-Rad). And the serum proteins concentration was measured by bradford method. Then the serum proteins were digested into peptides by trypsin at 37 °C for 12 h, labelled them by Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) reagent (3 serum samples of ACR group labelled 113, 114 and 115 and 3 serum samples of No-ACR group labelled 116, 117 and 118, 2 mix Health Control samples labelled 119 and 121).

### 2.3. LC-MS/MS analysis

Labelled peptides were fractionated by strong cation exchange liquid chromatograph (SCX) using Luna® 5 μm SCX 100 Å column (Phenomenex, USA). The flow rate was 1 ml/min, and the peptides were eluted by a step linear elution program: 0–20 min equilibration in 100% buffer A(10 mM $KH_2PO_4$, pH 3.0, 25% *v*/v ACN), 20–21 min fast elution with 0–5% buffer B (10 mM $KH_2PO_4$, pH 3.0, 2 M KCl and 25% v/v ACN), 21–41 min linear elution from with 5–30% buffer B, 41–61 min washing elution with 30–50% buffer B and 61–67 min elution with 100% buffer B. Then the eluates then loaded onto Q-Exactive MS (Thermo Fisher Scientific, USA) with full MS scans ranging from 350 to 2000 *m/z* at a resolution of 70,000.

### 2.4. iTRAQ data processing

The raw MS/MS data were converted into MGF format by Proteome Discoverer 1.3 (Thermo Scientific), and the exported MGF files were further compared with the Uniprot_human database using Mascot 2.3.0 (Matrix Science). The peptide searching parameters included a precursor mass tolerance of 15 ppm, a fragment ion mass tolerance of 20 mmu, a tolerance of one missed cleavage of trypsin, carbamidomethyl group of cysteine as the fixed modification, and Oxidation (M), Gln → Pyro-Glu (N-term Q), iTRAQ 8 plex (K), iTRAQ 8 plex (Y) and iTRAQ 8 plex (N-term), as variable modifications.

### 2.5. Analysis of the iTRAQ data

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was carried out to find out the important pathways of differentially expressed serum proteins and based on the latest KEGG database. Gene Ontology (GO) analysis was used find the unique biological significance of the differentially expressed serum proteins.

### 2.6. Elisa

To validate the differentially expressed proteins, we quantified serum content of CFH using commercially available ELISA kits (Human complement factor H, CFH ELISA Kit, CUSABIO, China) according to the manufacturer's instructions. The absorbances were examined at 450 nm using a microplate reader (Multiskan Spectrum Microplate Reader, Thermo Scientific, USA).

### 2.7. Statistical analysis

Statistical analyses were performed using SPSS18.0. and rank sum test were used to evaluate significant differences between different groups. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. iTRAQ-based profiling of the differentially expressed serum proteins from patients with ACR after LT

In this study, we profiled differentially expressed serum proteins in serum samples from 6 patients after LT (Table 1) and healthy controls (Fig. 1).

Overall, totally 488 proteins were identified by against the UniProt_human database. In this study, 1.2-fold change was chosen as variance threshold; ratio > 1.2 was up-regulated and ratio < 0.83 was down-regulated. As a result, ten differentially expressed proteins were found out between the ACR group and the No-ACR group, including 3 up-regulated proteins and 7 down-regulated proteins (Table 2). The heat map cluster analysis of these ten proteins was showed in Fig. 2. There were 88 differentially expressed proteins between the ACR group and the HC group, including 36 up-regulated proteins and 52 down-regulated proteins (Supplementary Table 1); there were 39 differentially expressed proteins between No-ACR group and HC group, including 17 up-regulated proteins and 22 down-regulated proteins (Supplementary Table 2).

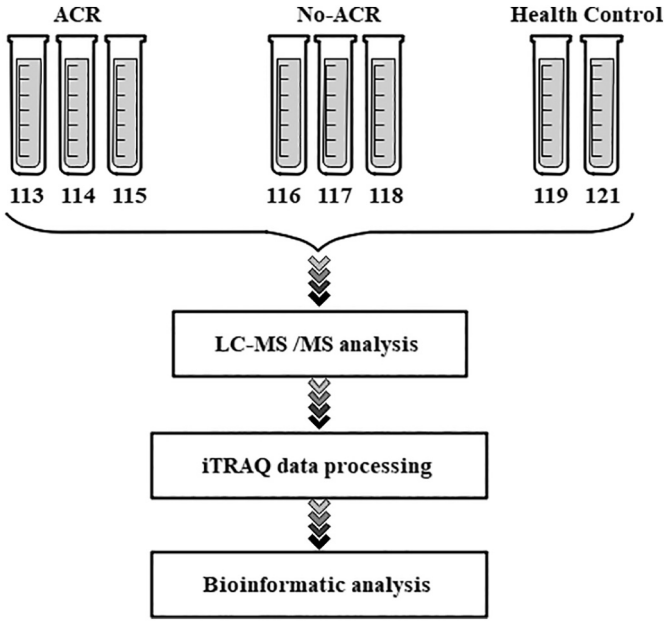


**Fig. 1.** Study flow chart.

**Table 2**
Differentially expressed proteins in serum between ACR and No-ACR.

| Response trend | Protein name | Accession number | Ratio | Unique Peptides | MW [kDa] | calc. pI |
|---|---|---|---|---|---|---|
| Up-regulated | Polymeric immunoglobulin receptor | P01833 | 2.486 | 19 | 83.2 | 5.74 |
| | Glutathione peroxidase 3 | P22352 | 1.371 | 9 | 25.5 | 8.13 |
| | Integral membrane protein 2B | Q9Y287 | 1.326 | 1 | 30.3 | 5.14 |
| Domn-regulated | Retinoic acid receptor responder protein 2 | Q99969 | 0.713 | 1 | 18.6 | 9.09 |
| | Complement factor H-related protein 1 | Q03591 | 0.666 | 3 | 37.6 | 7.39 |
| | Insulin-like growth factor-binding protein 1 | P08833 | 0.653 | 5 | 27.9 | 5.19 |
| | Ficolin-1 | O00602 | 0.466 | 1 | 35.1 | 6.86 |
| | Complement factor H-related protein 5 | Q9BXR6 | 0.426 | 22 | 64.4 | 7.06 |
| | Eukaryotic translation initiation factor 5A-1 | P63241 | 0.355 | 2 | 16.8 | 5.24 |
| | Apolipoprotein(a) | P08519 | 0.341 | 6 | 501 | 5.88 |

### 3.2. Bioinformatic analysis of our differentially expressed serum proteins

To further investigate the function of differentially expressed serum proteins, STRING, a database and web resource of known and predicted protein-protein interactions, was used to screen functional proteins and provide a protein–protein interaction networks. And the further KEGG pathway analysis showed that many differentially expressed serum proteins were found to be mostly enriched in complement and coagulation cascades pathway (Table 3 and Figs. 3, 4) both in ACR vs HC and No-ACR vs HC. Then the Venn diagram showed that there were 9 differentially expressed serum proteins overlapped in ACR vs No-ACR and ACR vs HC (Fig. 5). We utilized these 9 proteins for the GO analysis and found that the most enriched biological process was complement activation (Table 4). The 3 proteins involved in complement activation were CFHR1, CFHR5 and FCN1. However, FCN1 was one of the 2 proteins differentially expressed overlapped in No-ACR vs HC and ACR vs HC and its differential expression may due to the LT surgery. Therefore, CFHR1 and CFHR5 may be potential serum protein markers of ACR.

### 3.3. ELISA validation results and statistical analysis

CFHR1 and CFHR5 are members of the complement factor H family [7]. CFHR protein binds to C3b and acts around or in concert with the main complement alternative pathway regulator complement factor H (CFH) [8–10]. Therefore, we further hypothesized that the CFH may also dysregulated in patients occurred ACR after LT. Then we validated it by ELISA, and we also expanded the serum samples to compare the serum CFH content between ACR patients and No-ACR patients in different periods after LT (3 weeks and 4 weeks). The result of ELISA showed that compared with No-ACR, serum levels of CFH in patients occurred ACR after LT were significantly downregulated (Fig. 6). The result indicated CFH may be also a candidate potential diagnostic protein marker of ACR after LT.

## 4. Discussion

Organ transplantation had a great development during the past few decades, but there are still many problems. ACR not only is an important cause of early graft dysfunction, but also affect long-term graft survival [11–13]. These years, researchers begun to focus on non-specific immunity, especially the relationship between complement system and organ transplantation, for early diagnosis of rejection to improve the prognosis and function of transplanted organs [14,15].

Complement system is an important part of the immune system [16–19]. Inflammation and tissue damage could activate the complement system, not only via the classical pathway by antigen-antibody reaction, but also via the alternative pathway by the fragments of tissue damage. The activation products either kill target cells directly through the membrane attack complex or result in increased vascular permeability. Inflammatory cell accumulation caused or aggravated inflammation through activating fragments C2a, C3a, C4a, C5a as inflammatory mediators [20]. In addition, iC3b, C3d, C5a have immune regulatory effects and they could promote the proliferation of T, B lymphocytes or the production of antibodies and cytokines [21]. ACR after LT is an inflammatory process itself, thus in this inflammatory process, complement activation is undoubtedly detrimental [22–25]. Some studies have shown that the expression of complement regulatory proteins on liver parenchyma cell membrane is defective, which could not effectively suppress the activation of complement system on the surface of sinusoidal endothelial cells, hepatocytes and bile duct cells [26,27]. Moreover, multiple factors, such as rich blood supply, discontinuous holes on the sinusoidal endothelial and no basement membrane between sinusoidal endothelial cells and liver cells,make plasma components could freely enter the Disse space. Therefore, hepatocytes, sinusoidal endothelial cells and bile duct cells may be more susceptible to complement-mediated damages.

In this study, we profiled 2 differentially expressed proteins, CFHR1 and CFHR5 may be potential serum protein markers of ACR. CFHR1

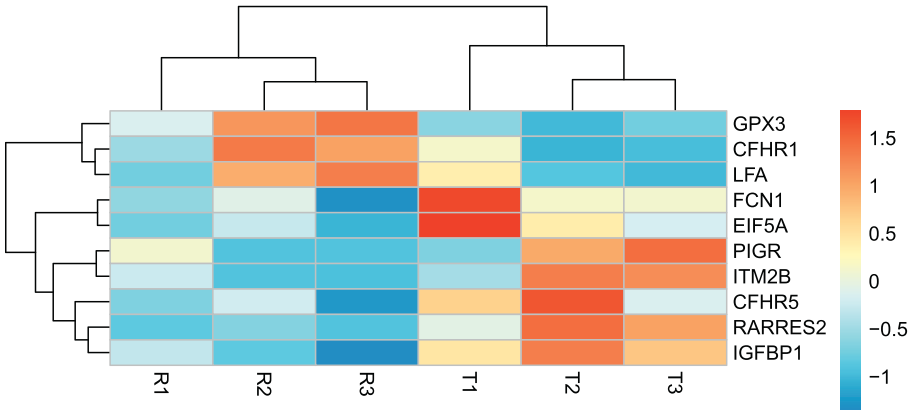


**Fig. 2.** The heat map cluster analysis of the ten proteins, which were found out between the ACR group and the No-ACR group.

**Table 3**
KEGG Pathways analysis.

| Group | Pathway ID | Pathway description | Count in gene set | False discovery rate |
|---|---|---|---|---|
| ACR vs HC | 04610 | Complement and coagulation cascades | 12 | 2.74e-14 |
| | 04977 | Vitamin digestion and absorption | 3 | 0.0202 |
| | 04512 | ECM-receptor interaction | 4 | 0.0455 |
| No-ACR vs HC | 04610 | Complement and coagulation cascades | 7 | 5.85e-09 |

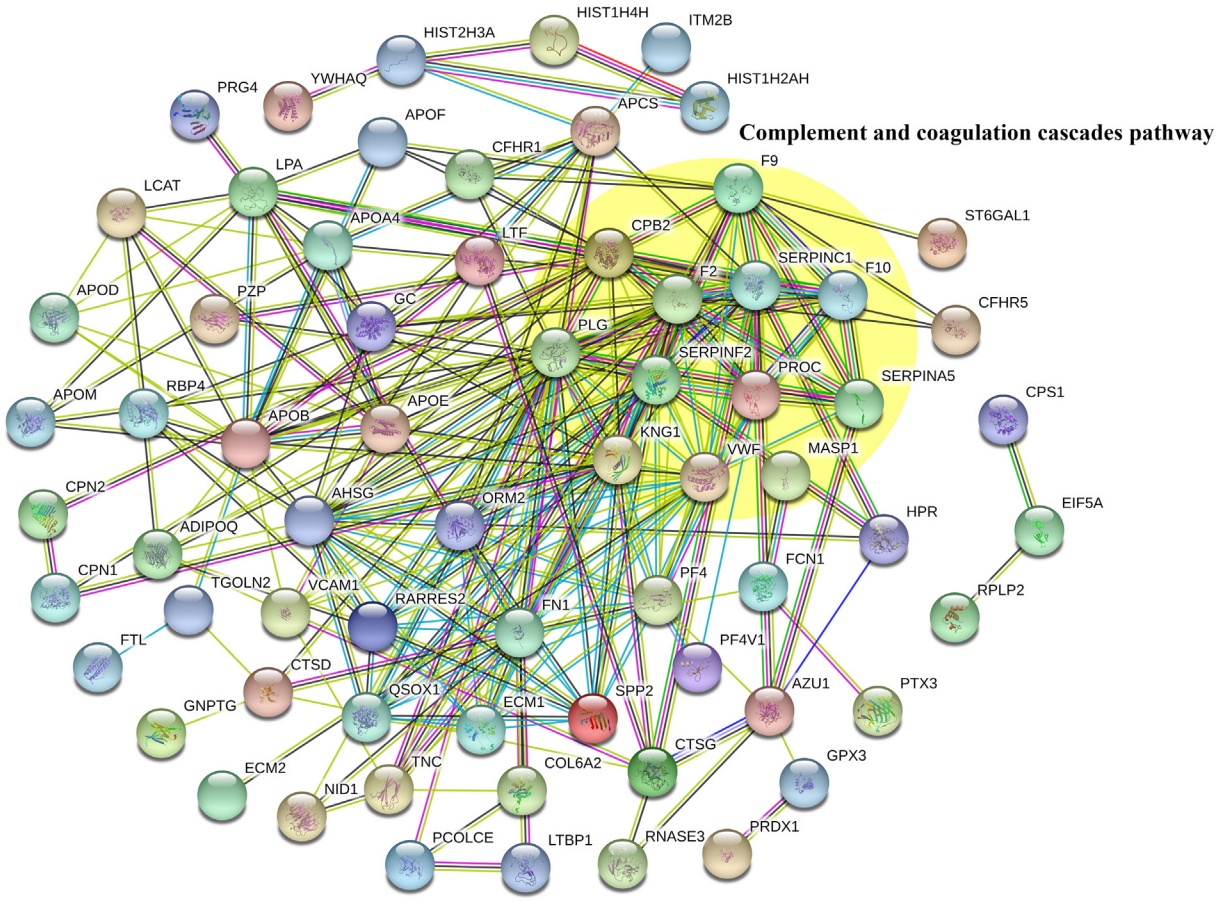


**Fig. 3.** The KEGG pathway analysis result of the differentially expressed serum proteins between ACR vs HC group.

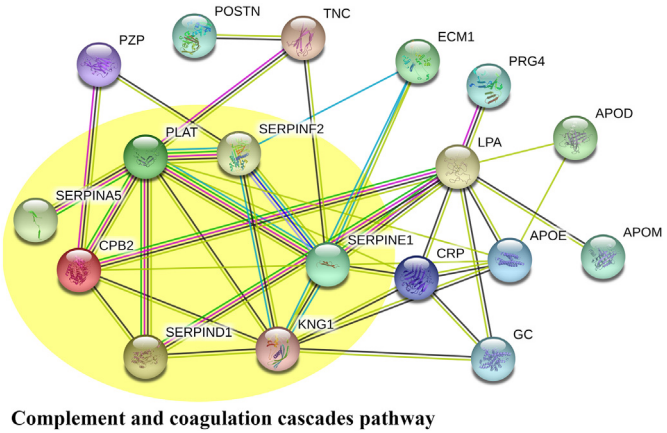


**Fig. 4.** The KEGG pathway analysis result of the differentially expressed serum proteins between No-ACR vs HC group.

and CFHR5 acts around or in concert with the main complement alternative pathway regulator CFH [7–10], so we validated the serum CFH content between ACR patients and No-ACR patients in different periods after LT and found the serum levels of CFH in patients occurred ACR after LT were also downregulated.

CFH plays a regulatory role on C3 convertase-C $\overline{3bBb}$ in the alternative pathway [28,29]. On one hand, CFH combines with C3b competitively with factor B or Bb, blocking the formation of C $\overline{3bBb}$ or making C $\overline{3bBb}$ dissociation. On the other hand, as a cofactor of factor I, CFH could make C3b easier to be hydrolyzed and inactivated by factor I to inhibit the activity of C $\overline{3bBb}$. If the serum level of CFH is reduced, its C3-convertase inhibiting function will be weaken or disappeared. Then the alternative pathway would be activated and the amplification mechanism be started, resulting in the rising of cytolysis effect, cytotoxic effect and ultimately leading to the occurrence of ACR after LT [25,30–33]. It was reported that CFH deficiency would cause atypical hemolytic uremic syndrome and LT could cure this disease to some extent [34]. In other words, LT itself would not reduce the CFH content in serum. Therefore, we believe that the downregulation of CFH is related to the occurrence of ACR after LT and CFH may be also a candidate of ACR diagnostic specificity protein biomarkers after LT.

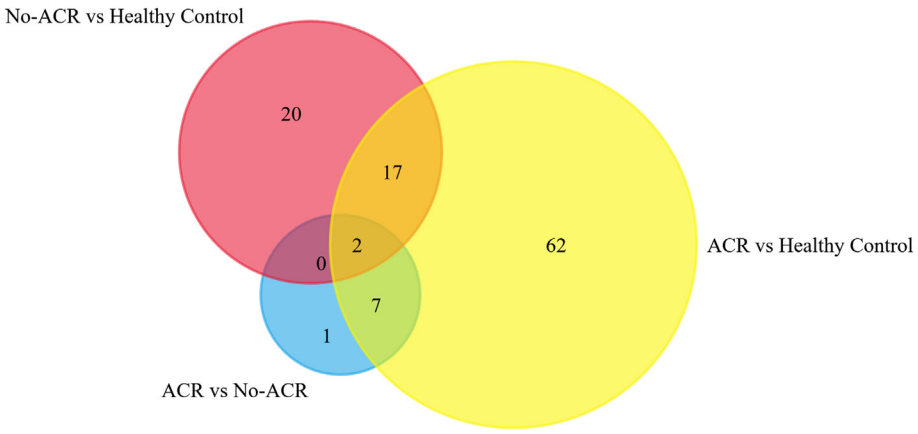


**Fig. 5.** The overlap of differentially expressed proteins between the all groups.

**Table 4**
GO analysis of 9 differentially expressed serum proteins both in ACR vs No-ACR and ACR vs HC.

| Function | Pathway ID | Pathway description | Count in gene set | False discovery rate |
|---|---|---|---|---|
| Biological Process | GO:0006956 | complement activation | 3 | 0.00993 |
| Cellular Component | GO:0005576 | extracellular region | 9 | 0.000863 |

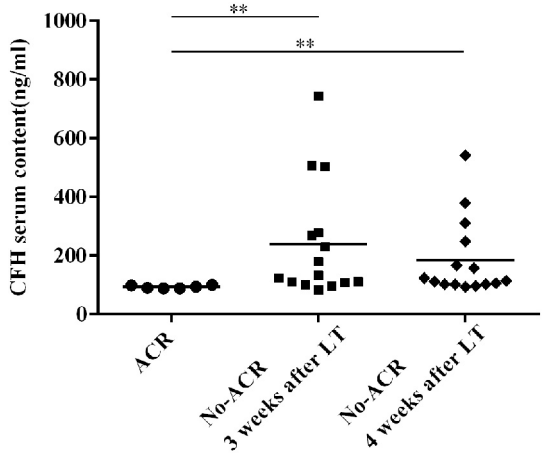


**Fig. 6.** The ELISA results of the CFH protein expression levels in ACR patients and No-ACR patients of different periods after LT (3 weeks and 4 weeks).

Currently, the gold standard of the clinical diagnosis of ACR is pathological examination after biopsy [35–38]. Due to the application of potent immunosuppressive drugs, the clinical incidence of ACR was significantly reduced, and the clinical symptoms and signs of ACR is a lack of typicality. Overlapped and compound pathology existing between other liver diseases after LT and ACR makes early diagnosis of ACR increasingly difficult [39–42]. In addition, due to the puncture site, pathological diagnosis is limited, which affects the detection rate of ACR. As the occurrence of ACR could cause serious damages to the patients, specific serum protein markers for early diagnosis of ACR were urgently to be found. Finding out specific serum protein markers could not only increase the positive detection rate of ACR, but also take the advantage of early diagnosis. We will study further on the differentially expressed proteins profiled in this study to provide more specific serum protein marker and theoretical basis for early prediction of ACR after LT.

## 5. Conclusion

The aim of this research was to find differentially expressed serum proteins associated with ACR after LT. After iTRAQ-based mass spectrometry, bioinformatics analysis and ELISA validation, we found CFHR1, CFHR5 and CFH were downregulated in patients occurred ACR after LT, and could be candidate protein biomarkers for the early diagnosis of ACR after LT.

## Conflict of interest

The authors declare that no conflict of interest exists.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2018.11.005.

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