Proteomic analysis of differentially expressed proteins in the serum of patients with acute renal allograft rejection using iTRAQ labelling technology

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Abstract. Transplantation is currently the best treatment for patients with end-stage renal disease. However, acute rejection (AR) is the major source of failure in renal transplantation. The current best practice for the diagnosis of AR involves renal biopsy, but it is invasive, time-consuming, costly and inconvenient. Sensitive and less invasive detection of AR episodes in renal transplant patients is essential to preserve allograft function. The present study applied isobaric tags for relative and absolute quantitation (iTRAQ) mass spectrometry to analyze serum protein expression in patients with AR and healthy controls. Overall, 1,399 proteins were identified. Using a cut-off of Q<0.05 and a fold change of >1.2 for the variation in expression, 109 proteins were identified to be differentially expressed between the AR and control groups, 72 of which were upregulated and 37 were downregulated. Several proteins, including properdin, keratin 1, lipoprotein(a) and vitamin D-binding protein, may have roles in the pathogenesis of AR. The present study focused on iTRAQ-based proteomic profiling of serum samples in AR. Insight from the present study may help advance the understanding of the molecular mechanisms of AR and identify potential novel biomarkers of AR for further characterization.

Introduction

At present, transplantation is the best treatment for end-stage renal disease. Survival and quality of life among transplant patients are significantly better than in dialysis patients (1). Despite the routine use of immunosuppressive therapies in the care of post-transplant patients, acute rejection (AR) of the renal allograft still occurs. Sensitive and early detection of AR episodes in patients with renal transplant is essential to preserve allograft function. The majority of patients with AR are asymptomatic and the detection of AR critically relies on regular monitoring for increases in serum creatinine, an insensitive laboratory biomarker of renal injury, as a sign of renal hypofunction (2,3). The current best practice for diagnosis of AR is renal biopsy, but this is invasive, time-consuming, costly and inconvenient. Therefore, noninvasive and sensitive methods would be valuable for the early detection of AR.

Biomarkers may be used for noninvasive prediction or diagnosis of AR in patients with kidney transplantation (4). Suitable candidate biomarker(s) should be based on a simple and cost-effective assay, requiring the noninvasive collection of a test sample, yet it should be specific and sensitive, as the results may prolong graft survival and improve patient health.
Proteomics have been widely employed in numerous fields of medical research (5-7). It is an interdisciplinary area for the discovery of candidate biomarkers that may be applied for noninvasive diagnoses.

Isobaric tags for relative and absolute quantitation (iTRAQ) is a multiplexed protein quantitative mass spectrometry (MS) technology based on isobaric reagents (8). It may be used to measure eight samples in one experiment (9). The iTRAQ reagent consists of a reporter group, a balance group and a peptide reactive group (PRG) (8). The reporter group ions that are generated appear as peaks in the low-mass region (113, 114, 115, 116, 117, 118, 119 and 121 Da) (10,11). As this region is free of other common fragment ions, signals found in this region are only due to contributions from the reporter ions from the corresponding labeled sample digests (12). Therefore, the relationships can be quantified by comparing the peak area of one reporter group peak with another. The ratio of one peak area to another represents the relative amount of a given peptide in each of the corresponding sample digests (12). The balance group ensures that an iTRAQ reagent-labeled peptide, whether labeled with iTRAQ reagent 114, 115, 116 or 117, displays at the same mass (12). The PRG covalently links an iTRAQ reagent isobaric tag with each lysine side chain and N-terminal group of a peptide. Multiple peptides in a sample digest are labeled (12). The advantage of iTRAQ is that it allows for concurrent quantization of complex samples but requires only a small amount of sample (13). Previous studies have indicated that quantitative proteomics using iTRAQ technology has the potential for diagnosis and treatment with AR after kidney transplantation (14,15). Therefore, the present study performed a quantitative proteomic analysis with an iTRAQ-based liquid chromatography electrospray ionization tandem MS (LC-ESI-MS/MS) approach to detect proteins differentially expressed in the serum of patients with AR and healthy individuals with no kidney transplant. Insight from the present study may help advance the understanding of the pathogenic mechanisms of AR and identify potential novel approaches for early diagnosis of AR.

Materials and methods

Serum sample collection. A total of 12 subjects, including patients with AR (1 male and 2 females; age, 51-61 years) and 9 age and gender matched healthy controls (6 males and 3 females; age, 40-55 years) were enrolled in the present study. All healthy controls were enrolled from the Guilin No. 924 Hospital (Guilin, China), with no prior history of chronic disease. Their characteristics are listed in Table I. The primary disease of the patients with AR was chronic glomerulonephritis. All patients with AR had received hemodialysis for >2 years prior to kidney transplantation and had no infectious diseases (such as hepatitis or tuberculosis) or autoimmune diseases. All kidney grafts were from donation after cardiac death of the donors and the patients had no history of organ transplantation. All patients with AR received tacrolimus, methylprednisolone and mycophenolate mofetil, to maintain triple immunosuppressive therapy. None of the subjects had a history of smoking or drinking. Serum specimens were collected from the Department of Nephrology at Guilin No. 924 Hospital (Guilin, China) between March and September in 2015. The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Guilin No. 924 Hospital (Guilin, China). Written informed consent was obtained from all participants. The serum samples (5 ml) were collected in serum separator Vacutainer tubes and were separated by centrifugation at 250 x g at 20°C for 10 min. Serum was divided into 0.5-ml aliquots and stored at -80°C until further analysis.

Protein preparation and tryptic digestion. Serum samples were disrupted in lysis buffer (7 M urea, 2 M thiourea, 0.2% SDS, 20 mM Tris) with enzyme inhibitors (1X protease inhibitor Cocktail, 1 mM EDTA). After centrifuging the mixtures at 25,000 x g at 4°C for 20 min, the supernatants were mixed with 5 volumes of cold acetone and stored at -20°C for 2 h to overnight. The mixtures were centrifuged at 25,000 x g for 20 min at 4°C, and the protein pellets were dissolved with lysis buffer, to which 10 mM dithiothreitol was added, prior to incubation at 56°C for 1 h in order to reduce the disulfide bonds of peptides. Then 55 mM iodoacetamide was added to the solution prior to storage in the dark for 45 min, followed by addition of 5 volumes of chilled acetone and storage at -20°C for 2 h. The solution was centrifuged at 25,000 x g for 20 min at 4°C and the pellet was dissolved with lysis buffer to obtain the protein solution. The protein concentration of the supernatant was determined with the Bradford assay method.

For each sample, 100 µg protein was digested with Trypsin Gold at a ratio of protein/trypsin of 20:1 at 37°C for 4 h. Fresh Trypsin Gold was added with the ratio of protein/trypsin of 20:1 again and the mixture was incubated at 37°C for an additional 8 h.

iTRAQ labelling and peptide fractionation. The peptides were vacuum centrifuged to dryness after trypsin digestion. The product was redisolved with 0.5 M triethylammonium bicarbonate and the iTRAQ labelling of peptide samples was performed using iTRAQ Reagent 8-plex kit (AB SCIEX) in accordance with the manufacturer's protocol. The peptides of the healthy controls were labelled with iTRAQ-113 isotopic tags and those from patients with AR with iTRAQ-121 isotopic tags and incubated for 2 h at 20°C. The iTRAQ-labelled peptides were fractionated using reversed-phase (RP) chromatography. For RP chromatography using a Shimadzu LC-20AB HPLC Pump system (Shimadzu Corp.), 100 µg digested peptides were reconstituted with 2 ml buffer A [5% acetonitrile (ACN), 95% H₂O, adjusted to pH 9.8 with ammonia] and loaded onto a 4.6x250 mm Gemini C₈ column containing 5-µm particles (Phenomenex Inc.). The peptides were eluted at 20°C at a flow rate of 1 ml/min with a gradient of 5% buffer B (5% H₂O, 95% ACN, adjusted to 9.8 with ammonia) for 10 min, followed by a 5-35% buffer B gradient for 40 min and a 35-95% buffer B gradient for 1 min. The system was maintained in 95% buffer B for 3 min, which was switched to 5% within 1 min prior to equilibrating with 5% buffer B for 10 min. Elution was monitored by measuring the absorbance at 214 nm and a different vial was used every min. The eluted peptides in 20 fractions were pooled and vacuum-dried.

LC-ESI-MS/MS analysis. Each fraction was resuspended in buffer A [5% ACN, 0.1% formic acid (FA)] and centrifuged
Table I. Basic characteristics of patients and healthy controls.

| Characteristic                      | Patients with acute rejection (n=3) | Healthy controls (n=9) |
|------------------------------------|------------------------------------|------------------------|
| Gender (male/female)               | 1/2                                 | 7/2                    |
| Age (years)                        | 53.0±7.2                           | 47.3±5.3               |
| Serum creatinine (µmol/l) (42-130 µmol/l) | 222.7±82.5                       | 89.4±10.3              |
| Blood urea nitrogen (mmol/l) (2.5-8.2 mmol/l) | 20.3±4.9                     | 4.6±0.8                |
| Uric acid (µmol/l) (male, 208-440 mmol/l; female, 155-360 mmol/l) | 410.7±81.3                      | 346.0±84.7             |

The normal ranges.

at 20,000 x g for 10 min at 4°C, and the final concentration of peptides was ~0.5 g/l. The supernatant was loaded on a LC-20AD nanoHPLC (Shimadzu Corp.) by an autosampler onto a 2-cm C18 trap column. The peptides were purified using an 18-cm analytical C18 column (inner diameter 75 µm, packed in-house). The samples were loaded and elution was performed in the following order: 8 µl/min for 4 min, 41 min gradient running at 300 nl/min from 5 to 35% B (95% ACN, 0.1% FA), 5 min linear gradient to 80% buffer B (maintained for 5 min), followed by a return to 5% within 1 min.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX) fitted with a Nanospray III source (AB SCIEX) and a pulled quartz tip as the emitter (New Objectives), and controlled with Analyst 1.6 software (AB SCIEX). Data were acquired under the following MS conditions: Ion spray voltage, 2.5 kV; curtain gas, 30 psi; nebulizer gas, 15 psi; and interface heater temperature, 150°C. The resolution was ~30,000. For information-dependent acquisition, survey scans were acquired at 250 msec and 30 production scans were collected if exceeding a threshold of 120 counts/sec and with a 2+ to 5+ charge state. The total cycle time was set to 3.3 sec. The Q2 transmission window was 100 Da for 100%. A total of four time bins for each scan was performed at a pulse frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode channel detection. An iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. The dynamic exclusion set for 1/2 of peak width (15 sec).

Proteomics data analysis. After separating the peptides, identification and quantification of detected proteins was performed. The MS/MS spectra were searched using Mascot software (version 2.3.02; Matrix Science, Ltd.). For protein identification, the search parameters were as follows: Fragment mass tolerance of 0.1 Da, peptide mass tolerance of 0.05 Da, MS/MS Ion as the type of search, trypsin as the enzyme, mass values of monoisotopic value, variable modifications of iTRAQ8plex (Y) and oxidation (M), fixed modifications of iTRAQ8plex (K), iTRAQ8plex (N-term) and carbamidomethyl (C) and database of human201512 (132,191 sequences). Protein identifications were considered reliable if they involved at least one unique peptide. An automated software called IQuant (16) was used for quantitatively analyzing the labeled peptides with isobaric tags. It integrates Mascot Percolator (17) and advanced statistical algorithms to process the MS/MS signals generated from the peptides labeled by isobaric tags. The main IQuant quantitation parameters were as follows: Quant_peptide of all unique peptides, Quant_number of at least one unique spectrum, Variance stabilization normalization, Protein_Ratio of weighted average and permutation tests as Statistical Analysis. High-confidence peptide identification was obtained by setting a false discovery rate of <1% at the peptide level. At least one unique peptide per protein group was required for identification of proteins and two quantified peptides for quantification of proteins. Functional enrichment analysis was performed using the Clusters of Orthologous Groups of proteins (COGs) (https://www.ncbi.nlm.nih.gov/COG) and the Gene Ontology (GO) (http://www.geneontology.org/) database. Pathway analysis was also performed by Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping (http://www.genome.jp/kegg/). For biological pathway analysis and GO term enrichment, P<0.05 was considered to indicate a statistically significant difference.

Results

Protein identification and quantification. Using Mascot Percolator (17), 1,399 proteins were identified and quantified using the cutoff of Q≤0.01 (Table SI). A total of 604 identified proteins contained >1 peptide, including 259 proteins with ≥5 peptides, 63 proteins with 4 peptides, 95 proteins with 3 peptides and 187 proteins with 2 peptides. Using a strict cut-off of a 1.2-fold change in expression and Q<0.05, 109 proteins [including 72 upregulated proteins (Tables II and SI) and 37 downregulated proteins (Tables III and SIH)] were found differentially expressed in AR specimens compared with those in the control samples. As presented in Fig. 1, a volcano plot of the log2 fold-change (x-axis) vs. -log10 Q-value (y-axis) was used to depict the differentially expressed proteins (DEPs).

Gene ontology analysis. These DEPs were analyzed by GO annotation and categorized into ‘molecular function’, ‘cellular component’ and ‘biological process’. ‘Molecular function’ describes activities, such as catalysis or binding, that occur at the molecular level. In this category, the proteins were indicated to be involved in 16 terms, including binding activity (48.95%), catalytic activity (27.27%), enzyme regulator activity...
Table II. Upregulated proteins in patients with acute rejection and control subjects.

| Protein     | Description                                      | Protein coverage (%) | Unique peptides | Q-value |
|-------------|--------------------------------------------------|----------------------|-----------------|---------|
| P35908      | Keratin, type II cytoskeletal 2 epidermal         | 22.8                 | 9               | 0.004   |
| P02741      | C-reactive protein                               | 43.3                 | 9               | 0.001   |
| Q14520      | Hyaluronan-binding protein 2                     | 19.6                 | 10              | 0.001   |
| P27918      | Properdin                                        | 25.4                 | 8               | 0.001   |
| Q7L523      | Ras-related GTP-binding protein A                | 1.9                  | 1               | 0.001   |
| Q53G71      | Calreticulin variant (fragment)                  | 35.2                 | 10              | 0.001   |
| P10645      | Chromogranin-A                                   | 9.4                  | 1               | 0.001   |
| Q1HP67      | Lipoprotein, Lp(A)                               | 6.2                  | 10              | 0.001   |
| B4DUV1      | Fibulin-1                                        | 40.1                 | 7               | 0.001   |
| K7ER74      | Protein APOC4-APOC2                              | 55.6                 | 5               | 0.001   |
| P12259      | Coagulation factor V                             | 25.9                 | 46              | 0.001   |
| P08311      | Cathepsin G                                      | 26.7                 | 6               | 0.001   |
| P04004      | Vitronectin                                      | 57.5                 | 16              | 0.001   |
| F5H6X6      | Neutral alpha-glucosidase AB                     | 14.0                 | 9               | 0.036   |
| P13645      | Keratin, type I cytoskeletal 10                  | 35.6                 | 12              | 0.001   |
| P02790      | Hemopexin                                        | 41.6                 | 15              | 0.001   |
| P69905      | Alpha-2 globin chain                             | 67.6                 | 7               | 0.001   |
| Q12794      | Hyaluronidase                                    | 26.4                 | 7               | 0.001   |
| P20742      | Pregnancy zone protein                           | 29.8                 | 25              | 0.001   |
| A0A0C4DH43  | Uncharacterized protein (Fragment)               | 36.1                 | 1               | 0.007   |
| H6VRG3      | Keratin 1                                        | 43.9                 | 1               | 0.001   |
| P03950      | Ribonuclease A A1                                | 44.9                 | 6               | 0.001   |
| P00450      | CP protein                                       | 49.7                 | 32              | 0.001   |
| D6RF35      | Vitamin D-binding protein                        | 65.5                 | 2               | 0.004   |
| P02675      | Epididymis secretory sperm binding protein Li 78p| 67.2                 | 22              | 0.001   |
| B4E380      | Histone H3                                       | 11.5                 | 2               | 0.009   |
| P14618      | Pyruvate kinase                                  | 46.9                 | 4               | 0.001   |
| B2RDW0      | cDNA, FLJ96792, highly similar to Homo sapiens    | 53.0                 | 5               | 0.038   |
|             | calmodulin 2 (phosphorylase kinase, delta), mRNA |                      |                 |         |
| P00734      | Prothrombin                                      | 93.2                 | 36              | 0.001   |
| Q5NV62      | V3-4 protein (fragment)                          | 18.2                 | 1               | 0.001   |
| P01008      | Serpin peptidase inhibitor, clade C (antithrombin), member 1, isoform CRA_a | 89.4 | 23 | 0.001 |
| P02656      | Apolipoprotein C-III                             | 99.9                 | 9               | 0.001   |
| P00451      | Coagulation factor VIII                           | 10.3                 | 13              | 0.001   |
| Q96IY4      | Carboxypeptidase B2                               | 25.8                 | 11              | 0.001   |
| P61626      | C-type lysozyme                                  | 52.0                 | 3               | 0.001   |
| Q8WWV5      | Putative uncharacterized protein (fragment)       | 65.8                 | 5               | 0.001   |
| P62805      | Histone H4                                       | 69.9                 | 7               | 0.001   |
| Q15113      | Procollagen C-endopeptidase enhancer 1           | 41.4                 | 12              | 0.001   |
| P34096      | Full-length cDNA clone CS0DF032YM23              | 55.1                 | 6               | 0.001   |
|             | of fetal brain of Homo sapiens (human)           |                      |                 |         |
| B0YIW1      | Apolipoprotein A-V variant 3                     | 37.7                 | 11              | 0.001   |
| B2R773      | cDNA, FLJ93312, highly similar to Homo sapiens   | 19.3                 | 3               | 0.001   |
|             | adipose most abundant gene transcript 1, mRNA    |                      |                 |         |
| P68871      | Hemoglobin, beta                                 | 84.4                 | 2               | 0.004   |
| G8JLA2      | Myosin light polypeptide 6                       | 33.6                 | 5               | 0.002   |
| P35527      | Keratin, type I cytoskeletal 9                   | 31.0                 | 14              | 0.001   |
| O00391      | Sulhydril oxidase 1                              | 46.7                 | 28              | 0.001   |
| B2R9V7      | Superoxide dismutase [Cu-Zn]                     | 17.1                 | 4               | 0.001   |
(5.85%) and structural molecule activity (4.03%; Fig. 2A). In the category ‘cellular component’, the proteins were enriched in 16 terms and the majority of them were located in the cell part (17.43%), cell (17.43%), organelle (12.96%) and organelle part (9.60%; Fig. 2B). In the category ‘biological process’, 23 terms were enriched, with 10.68% of the proteins participating in the cellular process, followed by single-organism process (9.82%), metabolic process (9.61%) and biological regulation (8.10%; Fig. 2C).

**COG analysis.** COGs were delineated by comparing protein sequences encoded in complete genomes, representing major phylogenetic lineages. COGs comprise a framework for functional and evolutionary genome analysis. In the analysis of COGs, all identified proteins were classified into 23 functional categories (Table IV), including posttranslational modification, protein turnover, chaperones (149 proteins, 20.67%), general function prediction only (119 proteins, 16.50%), energy production and conversion (56 proteins, 7.77%) and signal transduction mechanisms (47 proteins, 6.52%).

**Pathway enrichment analysis.** The KEGG database was used to identify the pathways in which the DEPs were involved. The results indicated that of these proteins were accumulated in 290 different pathways. The majority of the proteins were involved in ‘Complement and coagulation cascades’ (30 proteins, 18.18%), ‘Staphylococcus aureus infection’ (23 proteins, 13.94%), ‘Systemic lupus erythematosus’ (22 proteins, 13.33%) and ‘Amoebiasis’ (21 proteins, 12.73%). The top 30 DEPs in the KEGG pathway enrichment are presented in Table V. Protein interactions have an important role in certain biological functions, including immune response, blood coagulation, inflammatory response, ion homeostasis, cholesterol metabolism, actin binding, cell
motility, energy metabolism, RNA post-transcriptional modification, amino acid metabolism, small molecule biochemistry, cellular growth and proliferation (18-21). A pathway enrichment analysis of DEPs was implemented based on the KEGG database in AR patients and healthy controls (Fig. 3). All abbreviations are shown in Table SIV.

Table III. Downregulated proteins in patients with acute rejection and control subjects.

| Protein       | Description                                                                 | Protein coverage (%) | Unique peptides | Q-value |
|---------------|-------------------------------------------------------------------------------|----------------------|-----------------|---------|
| B4DRV4        | cDNA FLJ55667, highly similar to secreted protein acidic and rich in cysteine | 44.8                | 7               | 0.001   |
| H7C3N9        | Leucine-rich repeat flightless-interacting protein 2 (fragment)              | 8.2                 | 1               | 0.036   |
| Q71M29        | Putative uncharacterized protein FP3420                                     | 4.3                 | 1               | 0.027   |
| P05109        | Protein S100-A8                                                               | 11.8                | 1               | 0.032   |
| P22792        | Carboxypeptidase N subunit 2                                                 | 27.2                | 11              | 0.001   |
| D3J4V3        | C-X-C motif chemokine (fragment)                                             | 35.3                | 2               | 0.027   |
| B2R812        | cDNA, FLJ93914, highly similar to *Homo sapiens* histidine-rich glycoprotein, mRNA | 36.0                | 2               | 0.033   |
| B4DPP8        | cDNA FLJ53075, highly similar to kininogen-1                                  | 44.1                | 16              | 0.001   |
| P02765        | Alpha-2-HS-glycoprotein                                                      | 39.5                | 8               | 0.001   |
| Q6MZL2        | Putative uncharacterized protein DKFZp686M0562 (fragment)                    | 28.0                | 4               | 0.001   |
| P00739        | Haptoglobin-related protein                                                   | 72.1                | 9               | 0.016   |
| P09871        | Complement C1s subcomponent                                                   | 37.9                | 18              | 0.001   |
| P06396        | Gelsolin                                                                     | 35.2                | 1               | 0.001   |
| Q9UNU2        | Complement protein C4B frameshift mutant (fragment)                          | 61.6                | 1               | 0.038   |
| P27169        | Serum paraoxonase/arylesterase 1                                             | 52.1                | 11              | 0.001   |
| A4D1F6        | Leucine-rich repeat and death domain-containing protein 1                    | 0.7                 | 1               | 0.017   |
| P02760        | Protein AMBP                                                                  | 33.0                | 8               | 0.001   |
| F5GXQ5        | Glycine N-acyltransferase-like protein 3 (fragment)                          | 3.2                 | 1               | 0.001   |
| O95445        | Apolipoprotein M                                                              | 60.1                | 6               | 0.001   |
| O43866        | CD5 antigen-like                                                             | 43.5                | 13              | 0.001   |
| V9H1C1        | Gelsolin exon 4 (fragment)                                                    | 46.4                | 1               | 0.017   |
| F1CA47        | Monocyte differentiation antigen CD14                                        | 29.6                | 9               | 0.020   |
| P01024        | Epididymis secretory sperm binding protein Li 62p                            | 87.9                | 98              | 0.001   |
| Q5T985        | Inter-alpha-trypsin inhibitor heavy chain H2                                 | 44.4                | 31              | 0.001   |
| B7Z539        | cDNA FLJ56954, highly similar to Inter-alpha-trypsin inhibitor heavy chain H1 | 49.9                | 2               | 0.011   |
| B2R815        | cDNA, FLJ93695, highly similar to *Homo sapiens* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4, mRNA | 37.9                | 14              | 0.001   |
| A0A0A0MSP7    | FERM and PDZ domain-containing protein 3 (fragment)                          | 0.3                 | 1               | 0.010   |
| P80108        | Phosphatidylinositol-glycan-specific phospholipase D                          | 38.1                | 24              | 0.001   |
| P02652        | Apolipoprotein A-II                                                           | 99.9                | 8               | 0.001   |
| P51884        | Lumican                                                                      | 32.2                | 9               | 0.001   |
| B4DU16        | cDNA FLJ54550, highly similar to *Homo sapiens* fibronectin 1, transcript variant 6, mRNA | 78.7                | 1               | 0.004   |
| Q96PD5        | N-acetylmuramoyl-L-alanine amidase                                             | 35.1                | 10              | 0.001   |
| A0A087WX12    | IgGFc-binding protein                                                         | 2.6                 | 9               | 0.010   |
| P01031        | Complement C5                                                                | 50.1                | 69              | 0.001   |
| P15169        | Carboxypeptidase N catalytic chain                                            | 24.0                | 8               | 0.010   |
| A0A024R462    | Fibronectin 1, isoform CRA_n                                                  | 69.7                | 50              | 0.001   |
| A8K1K1        | cDNA FLJ76342, highly similar to *Homo sapiens* carnosine dipeptidase 1 (metallopeptidase M20 family), mRNA | 42.8                | 17              | 0.001   |

cDNA, complementary DNA; AMBP, alpha-1-microglobulin.
Discussion

The major impediment to success in kidney graft is acute graft rejection, which leads to loss of the organ occur mainly in the first year after transplantation (22). The lack of applicable biomarkers to predict rejection is the biggest challenge in AR. Renal biopsy offers an accurate detection method for AR but the invasiveness of this procedure and other adverse effects may limit its use in certain patients (23-25). Therefore, the reliable and timely identification of potential early biomarkers for rejection is essential. A number of studies have been performed over the past decade (26-29); the majority of them depend on the combination of stable isotopes for obtaining mass spectrometric ordering and relative quantification (30,31). Of these, iTRAQ technology, which is based on the labelling of peptides in up to 8 proteomes at the MS/MS level for relative and absolute quantization, is the most widely used for numerous types of diseases (18-20,32-34). Wu et al (14) used iTRAQ technology to detect DEPs in the plasma of patients with acute renal allograft rejection. The results demonstrated that NF-κB, and signal transducer and activator of transcription 1 and 3, are potential markers for AR and may lead to novel strategies for diagnosis and treatment. Freue et al (15) quantitated the relative plasma concentrations of proteins in patients with acute renal allograft rejection by using iTRAQ labeling and quantitative proteomic technology. The study indicated that the profiling of the plasma proteome provided a promising method to monitor the immunological course in patients with
Figure 2. Bioinformatics analysis of differentially expressed proteins. Gene Ontology terms in the categories (A) Molecular function, (B) Cellular component and (C) Biological process.
AR following renal allograft. The present study performed a proteomics analysis using iTRAQ technology and several proteins were identified as potential candidate biomarkers for the accurate diagnosis of AR. In the present study, 109 proteins with a fold change ≥1.2 were identified, 72 of which were upregulated and 37 were downregulated. GO analysis in the category ‘biological process’ revealed that alterations in the expression of identified proteins in patients with AR were involved in diverse biological processes, including a single-organism process, cellular process, biological regulation and metabolic process, confirming that the pathogenesis of AR is associated with different molecular mechanisms. Some of these proteins, including properdin and keratin 1 (KRT1), may have the potential to be used as a serum biomarker in the diagnosis of AR.

Fig. 3. Statistics of pathway enrichment of DEPs in patients with acute rejection and control subjects. The vertical axis represents the name of the pathway and the horizontal axis represents the corresponding enrichment factors. The enrichment factor is the ratio of the number of DEPs annotated to this pathway term to the total number of proteins annotated to this pathway term. A higher enrichment factor indicates greater intensiveness, a lower P-value means greater intensiveness. The dot size represents the number of DEPs annotated to the pathway. PPAR, peroxisome proliferator-activated receptor; DEP, differentially expressed protein.

| Code | Functional category                                      | Number of proteins |
|------|----------------------------------------------------------|--------------------|
| A    | RNA processing and modification                          | 1                  |
| B    | Chromatin structure and dynamics                         | 9                  |
| C    | Energy production and conversion                         | 56                 |
| D    | Cell cycle control, cell division, chromosome partitioning| 19                 |
| E    | Amino acid transport and metabolism                      | 42                 |
| F    | Nucleotide transport and metabolism                      | 20                 |
| G    | Carbohydrate transport and metabolism                    | 37                 |
| H    | Coenzyme transport and metabolism                        | 12                 |
| I    | Lipid transport and metabolism                           | 23                 |
| J    | Translation, ribosomal structure and biogenesis          | 26                 |
| K    | Transcription                                            | 21                 |
| L    | Replication, recombination and repair                     | 22                 |
| M    | Cell wall/membrane/envelope biogenesis                   | 13                 |
| N    | Cell motility                                            | 4                  |
| O    | Posttranslational modification, protein turnover, chaperones| 149                |
| P    | Inorganic ion transport and metabolism                   | 7                  |
| Q    | Secondary metabolites biosynthesis, transport and catabolism| 15                 |
| R    | General function prediction only                         | 119                |
| S    | Function unknown                                         | 24                 |
| T    | Signal transduction mechanisms                          | 47                 |
| U    | Intracellular trafficking, secretion, and vesicular transport| 9                  |
| Y    | Nuclear structure                                        | 1                  |
| Z    | Cytoskeleton                                             | 45                 |

Table IV. Protein number for each Cluster of Orthologous Groups of proteins function category.
Another significantly upregulated protein in AR identified in the present study was KRT1, which belongs to the keratin family. KRT1 is polymorphic (40) and has been reported to be expressed in endothelial cells (41). KRT1 is involved in the lectin complement pathway caused by oxidative stress in endothelial cells (42). Transplant recipients may have antibodies to endothelial cells (43-47). Guo et al (48) indicated that KRT1 antibodies were probably autoantibodies and the presence of KRT1 antibodies is significantly associated with deterioration of kidney allograft function. Analysis of the LC-ESI-MS/MS data indicated that KRT1 was differentially expressed between AR patients and healthy controls. This protein may be a valuable diagnostic marker for monitoring patients' conditions but its possible role in allograft rejection requires further investigation.

In addition, certain novel candidate protein markers identified in the present study, including lipoprotein (a) [Lp(a)] and vitamin D-binding protein (VDBP), were significantly upregulated between AR patients and healthy controls. Previous studies have revealed that Lp(a) promotes atherosclerotic diseases, including stroke and coronary heart disease (49,50). Shimoyamada et al (51) reported that smooth muscle cell proliferation could be induced by Lp(a) as a mechanism of atherosclerosis in the rejected kidney. The deposition of Lp(a) in lesions in vascular rejection of transplanted kidneys is similar to that in atherosclerotic lesions. VDBP is a multifunctional protein (52,53) that occurs in serum, cerebrospinal fluid and ascitic fluid, and is characterized as being capable of binding various forms of vitamin D (54). 1,25-dihydroxycholecalciferol [1,25-(OH)2D3; also known as calcitriol] is one of the active forms of vitamin D in the kidney. Previous studies have revealed that 1,25-(OH)2D3 has an essential role in immunoregulation and allograft rejection (55,56), and VDBP may be
one of the serum biomarker candidates of acute renal allograft rejection. These candidate proteins may provide a scientific foundation for the pathogenic mechanisms and potential therapeutic approach for AR that warrant further research.

The proteins identified as differentially expressed in patients with AR may be involved in the process of AR and have an important role in the development of the condition. In this study, the healthy controls were healthy individuals with no kidney transplant. Patients with kidney transplant but no AR were not included in the control group because these patients had other health conditions that were unsuitable for the control group. In future studies, it would be ideal to collect adequate specimens from patients with kidney transplant but no AR and with no other health conditions. One limitation of the present study was the small population due to AR being rare in the clinic and the number of patients with AR that met the selection requirements being small. The low sample numbers limit the ability to classify stages of acute renal allograft rejection. Due to the small sample size, it was not possible to determine the correlations of the levels of DEPs with multifarious risk factors or with a specific immune response in detail. If the number of samples was to be increased, different stages of acute renal allograft rejection would be able to be observed, different factors such as gender or age would be considered, and a more objective evaluation could be made for the iTRAQ labelling technique in acute renal allograft rejection. In addition, further validation studies are required to elucidate the mechanisms of the DEPs involved in the biological processes of acute renal allograft rejection, facilitating the development of novel biomarkers for rejection. Therefore, increasing the number of samples will be necessary in further research to obtain more objective and reliable results.

In conclusion, iTRAQ combined with LC-ESI-MS/MS has proven to be a potential and efficient quantitative proteomic technique. The iTRAQ labelling technique was applied to explore the pathogenic mechanisms of AR. The results proved that different protein profile alternations and different pathways were involved in AR. Certain representative candidates, including properdin, KRT1, Lp(a) and VDBP, may serve as potential and novel biomarkers for the early detection of AR.

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Availability of data and materials

The mass spectrometry proteomics data were deposited with the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/archive) with the dataset identifier PXD015336.

Authors’ contributions

The present study was conceived by YD and QY; the experiments were planned by YZ, MO, HL and LL; sample collection and experiments were performed by YZ, HL, HC, JC, WX, RZ, QG, DT, XS and JD; data analysis and interpretation were performed by YZ, MO, LL and WS; the manuscript was drafted by YZ, MO and HL. All aspects of the work were supervised by YD and QY. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed according to the Declaration of Helsinki and was approved by the Medical Ethical Committee of Guilin No. 924 Hospital (Guilin, China). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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