

# 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.

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*Abbreviations:* AR, acute rejection; GO, Gene Ontology; COGs, Clusters of Orthologous Groups of proteins; iTRAQ, isobaric tags with relative and absolute quantitation; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; KRT1, keratin 1; Lp(a), lipoprotein (a); VDBP, vitamin D-binding protein

*Key words:* acute rejection, renal allograft, isobaric tags with relative and absolute quantitation, proteomics, serum

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 redissolved 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 isobaric tags and those from patients with AR with iTRAQ-121 isobaric 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% H<sub>2</sub>O, adjusted to pH 9.8 with ammonia] and loaded onto a 4.6x250 mm Gemini C<sub>18</sub> 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% H<sub>2</sub>O, 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 ( $\mu\text{mol/l}$ ) (42-130 $\mu\text{mol/l}$ <sup>a</sup> )	222.7±82.5	89.4±10.3
Blood urea nitrogen (mmol/l) (2.5-8.2 mmol/l <sup>a</sup> )	20.3±4.9	4.6±0.8
Uric acid ( $\mu\text{mol/l}$ ) (male, 208-440 mmol/l; female, 155-360 mmol/l <sup>a</sup> )	410.7±81.3	346.0±84.7

<sup>a</sup>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 C<sub>18</sub> trap column. The peptides were purified using an 18-cm analytical C<sub>18</sub> column (inner diameter 75  $\mu\text{m}$ , packed in-house). The samples were loaded and elution was performed in the following order: 8  $\mu\text{l}/\text{min}$  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 were 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, 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 with 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 SII) and 37 downregulated proteins (Tables III and SIII)] were found differentially expressed in AR specimens compared with those in the control samples. As presented in Fig. 1, a volcano plot of the log<sub>2</sub> fold-change (x-axis) vs. -log<sub>10</sub> 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 <i>Homo sapiens</i> calmodulin 2 (phosphorylase kinase, delta), mRNA	53.0	5	0.038
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
Q8WVW5	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 of fetal brain of <i>Homo sapiens</i> (human)	55.1	6	0.001
B0YIW1	Apolipoprotein A-V variant 3	37.7	11	0.001
B2R773	cDNA, FLJ93312, highly similar to <i>Homo sapiens</i> adipose most abundant gene transcript 1, mRNA	19.3	3	0.001
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	Sulfhydryl oxidase 1	46.7	28	0.001
B2R9V7	Superoxide dismutase [Cu-Zn]	17.1	4	0.001

Table II. Continued.

Protein	Description	Protein coverage (%)	Unique peptides	Q-value
F2RM37	Coagulation factor IX	32.1	12	0.001
P08670	Epididymis luminal protein 113	27.3	8	0.001
Q6N095	Putative uncharacterized protein DKFZp686K03196	37.7	1	0.002
P55056	Apolipoprotein C-IV	52.8	5	0.001
P02649	Apolipoprotein E	100	9	0.001
P13798	N-acylaminoacyl-peptide hydrolase, isoform CRA_b	10.2	7	0.003
P23142	Fibulin-1	31.7	7	0.001
B3KRF9	cDNA FLJ34156 fis, clone FCBBF3013266, highly similar to Tsukushi (leucine-rich repeat-containing protein 54)	31.2	7	0.037
Q8NG19	Multi-functional protein MFP	33.7	7	0.004
Q86UD1	Out at first protein homolog	31.1	6	0.001
C9JEU5	Fibrinogen gamma chain	31.5	2	0.019
P20160	Azurocidin	22.3	5	0.040
P55072	Epididymis luminal protein 220	33.7	6	0.001
B2RA39	cDNA, FLJ94686, highly similar to <i>Homo sapiens</i> complement factor H-related 5, mRNA	17.6	9	0.001
A0A075B785	LisH domain and HEAT repeat-containing protein KIAA1468	1.2	1	0.003
P02671	Fibrinogen alpha chain	30.7	15	0.003
P04406	GAPDH	57.0	14	0.001
P11586	C-1-tetrahydrofolate synthase, cytoplasmic	6.1	5	0.017
Q02818	Nucleobindin-1	25.4	9	0.006
P05164	Myeloperoxidase	64.0	31	0.001
B3KQ20	cDNA FLJ32635 fis, clone SYNOV2000178, highly similar to Proteoglycan-4	34.6	13	0.001
A0A024R7F1	Protein kinase C substrate 80K-H, isoform CRA_a	14.0	6	0.007
H9KV75	Alpha-actinin-1	26.5	9	0.001
B4DWH0	cDNA FLJ55670, highly similar to EGF-containing fibulin-like extracellular matrix protein 1	42.9	10	0.001
Q13103	Secreted phosphoprotein 24	32.2	6	0.001
Q9UBX5	Fibulin 5, isoform CRA_b	14.1	6	0.017

cDNA, complementary DNA; EGF, epidermal growth factor; APOC, apolipoprotein C; CP, ceruloplasmin.

(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

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
D3JV43	C-X-C motif chemokine (fragment)	35.3	2	0.027
B2R8I2	cDNA, FLJ93914, highly similar to <i>Homo sapiens</i> 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
F1C4A7	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
B2R8I5	cDNA, FLJ93695, highly similar to <i>Homo sapiens</i> 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 <i>Homo sapiens</i> fibronectin 1, transcript variant 6, mRNA	78.7	1	0.004
Q96PD5	N-acetylmuramoyl-L-alanine amidase	35.1	10	0.001
A0A087WXI2	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 <i>Homo sapiens</i> carnosine dipeptidase 1 (metallopeptidase M20 family), mRNA	42.8	17	0.001

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

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.

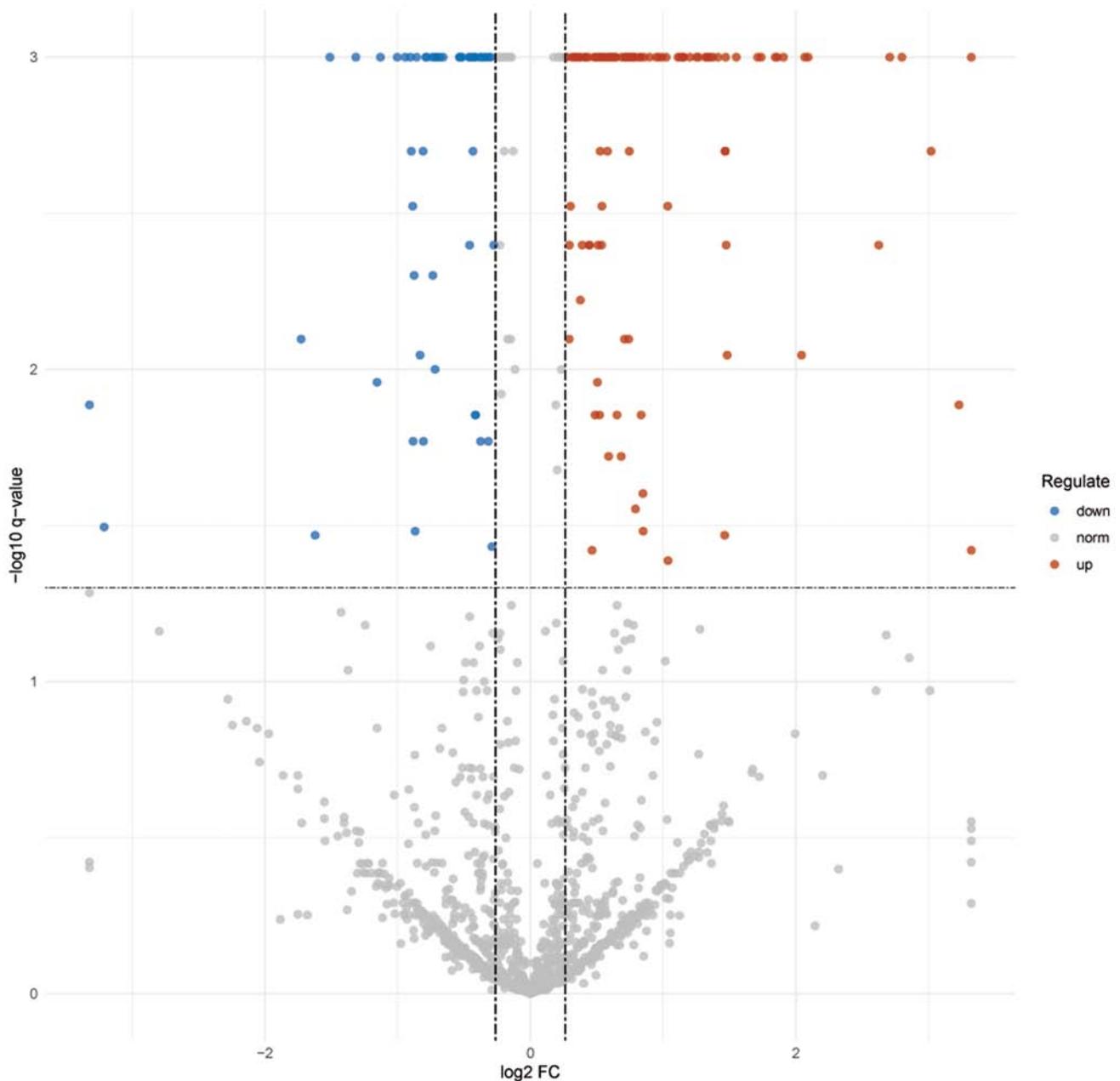


Figure 1. Volcano plot of DEPs. This plot depicts the  $\log_2$  FC (x-axis) vs.  $-\log_{10}$  Q value (y-axis, representing the probability that the protein is differentially expressed).  $Q \leq 0.05$  and  $FC \geq 1.2$  were set as the significant threshold for differential expression. Dots in red denote significantly upregulated proteins which passed the screening threshold. Dots in green denote significantly downregulated proteins which passed the screening threshold. Gray dots indicate non-significantly DEPs. FC, fold change; up, upregulated; down, downregulated; norm, not differentially expressed; DEP, differentially expressed protein.

## 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- $\kappa$ B, 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

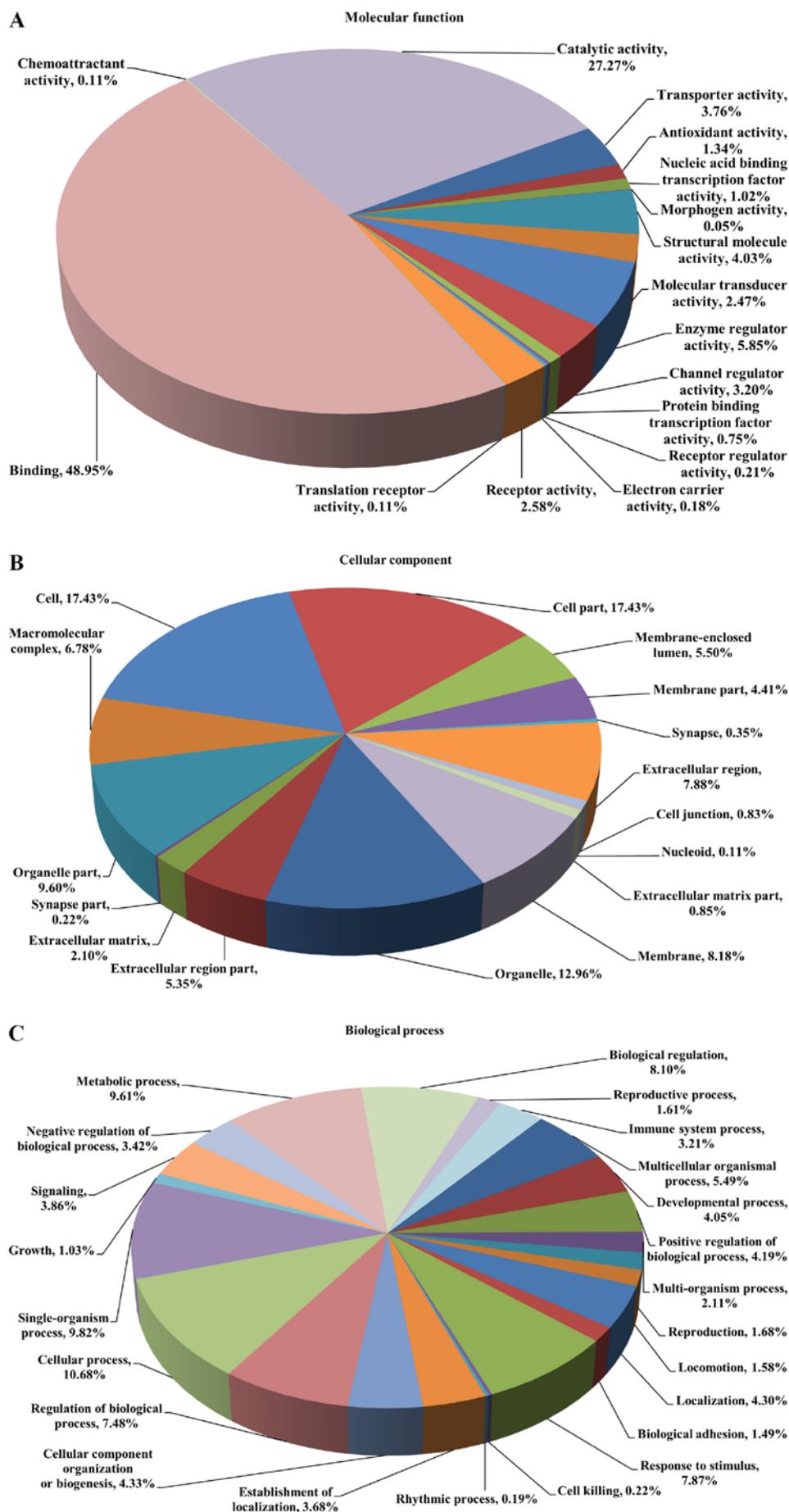


Figure 2. Bioinformatics analysis of differentially expressed proteins. Gene Ontology terms in the categories (A) Molecular function, (B) Cellular component and (C) Biological process.

Table IV. Protein number for each Cluster of Orthologous Groups of proteins function category.

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

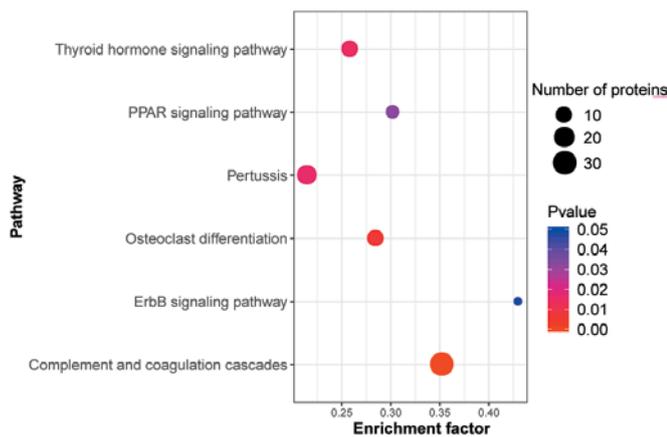


Figure 3. Statistics of pathway enrichment of DEPs in patients with acute rejection and control subjects. The vertical axis represents the name of the pathway and the horizontal axis represents the corresponding enrichment factors. The enrichment factor is the ratio of the number of DEPs annotated to 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.

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  $\geq 1.2$  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.

Complement proteins have an important role in the ischemia/reperfusion injury (IRI) (35), which prominently contributes to morbidity and mortality in acute renal allograft failure (36). Properdin is a  $\gamma$ -globulin protein made up of multiple identical monomeric subunits and is a stabilizer of surface-bound C3bBb. It facilitates the complement alternative pathway for C3-convertase formation (37). Properdin is the only known positive regulator of complement activation (38). Miwa *et al* (39) suggested that properdin has a major pathogenic role during early renal IRI and anti-properdin therapy may have a beneficial effect in human IRI. Consistent with the literature, properdin was also significantly upregulated in the serum of patients with AR in the present study. According to these results, it may be hypothesized that high levels of properdin may be significantly involved in the development of AR. However, its specific roles in AR have not been well studied and require to be further investigated.

Table V. Top 30 DEPs mapped to pathways.

Pathway	DEPs with pathway annotation	All proteins with pathway annotation	P-value	Pathway ID
Complement and coagulation cascades	30 (18.18)	86 (6.63)	2.940498e-08 <sup>a</sup>	ko04610
Osteoclast differentiation	11 (6.67)	39 (3.00)	0.006834358 <sup>a</sup>	ko04380
Thyroid hormone signaling pathway	11 (6.67)	43 (3.31)	0.01483172 <sup>a</sup>	ko04919
Pertussis	18 (10.91)	85 (6.55)	0.01614457 <sup>a</sup>	ko05133
PPAR signaling pathway	6 (3.64)	20 (1.54)	0.03239792 <sup>a</sup>	ko03320
ErbB signaling pathway	3 (1.82)	7 (0.54)	0.04790133 <sup>a</sup>	ko04012
Systemic lupus erythematosus	22 (13.33)	125 (9.63)	0.06066206	ko05322
Malaria	9 (5.45)	41 (3.16)	0.06572233	ko05144
Salivary secretion	3 (1.82)	8 (0.62)	0.0696401	ko04970
VEGF signaling pathway	3 (1.82)	8 (0.62)	0.0696401	ko04370
Regulation of actin cytoskeleton	18 (10.91)	100 (7.70)	0.07168303	ko04810
mTOR signaling pathway	2 (1.21)	4 (0.31)	0.08102746	ko04150
Herpes simplex infection	5 (3.03)	19 (1.46)	0.08226199	ko05168
Chagas disease (American trypanosomiasis)	4 (2.42)	14 (1.08)	0.090641	ko05142
Staphylococcus aureus infection	23 (13.94)	139 (10.71)	0.09910894	ko05150
Glucagon signaling pathway	4 (2.42)	15 (1.16)	0.1118566	ko04922
MicroRNAs in cancer	10 (6.06)	52 (4.01)	0.1129492	ko05206
Phototransduction - fly	2 (1.21)	5 (0.39)	0.1239715	ko04745
Protein export	1 (0.61)	1 (0.08)	0.1271186	ko03060
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	1 (0.61)	1 (0.08)	0.1271186	ko00563
Leukocyte transendothelial migration	15 (9.09)	87 (6.70)	0.1274251	ko04670
Proteoglycans in cancer	15 (9.09)	88 (6.78)	0.1370228	ko05205
Rap1 signaling pathway	14 (8.48)	82 (6.32)	0.1463614	ko04015
Amoebiasis	21 (12.73)	132 (10.17)	0.1525601	ko05146
Focal adhesion	16 (9.70)	97 (7.47)	0.1572612	ko04510
HIF-1 signaling pathway	5 (3.03)	23 (1.77)	0.1576914	ko04066
Hippo signaling pathway	7 (4.24)	36 (2.77)	0.1626743	ko04390
Renin secretion	2 (1.21)	6 (0.46)	0.1708845	ko04924
Type II diabetes mellitus	2 (1.21)	6 (0.46)	0.1708845	ko04930
Renin-angiotensin system	2 (1.21)	6 (0.46)	0.1708845	ko04614

<sup>a</sup>P<0.05. DEP, differentially expressed protein. Values are expressed as n (%).

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)<sub>2</sub>D<sub>3</sub>; also known as calcitriol] is one of the active forms of vitamin D in the kidney. Previous studies have revealed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> 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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