

# Quantitative LC-MS/MS Uncovers The Regulatory Role of Autophagy in Immune Thrombocytopenia

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# Abstract

**Background:** Immune thrombocytopenia (ITP) is an autoimmune haemorrhagic disease whose pathogenesis is associated with bone marrow megakaryocyte maturation disorder and microenvironment destruction of haematopoietic stem cells.

**Method:** In this study, we report the qualitative and quantitative profile of the proteome in ITP. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted to detect the protein profiles in clinical bone marrow mononuclear cell (BMMC) samples from ITP patients and healthy volunteers (controls). Gene Ontology (GO) and Kyoto Encyclopedia Genes and Genome (KEGG) pathway analyses were performed for the annotation of differentially expressed proteins. The protein-protein interaction (PPI) network was constructed with the BLAST online database. Target proteins associated with autophagy were quantitatively identified by parallel reaction monitoring (PRM) analysis.

**Results:** Our approaches showed that of the differentially expressed autophagy-related proteins, namely, HSPA8, PARK7, YWHAH, ITGB3 and CSF1R, were the most changed. The expression of the CSF1R protein in ITP patients was higher than that in controls, while the expression of other autophagy-related proteins was lower in ITP patients than in controls.

**Conclusion:** Bioinformatics analysis indicated that the abnormal autophagy pathway is a potential pathological mechanism of ITP. These results can provide a new direction for exploring the molecular mechanism of ITP.

## 1. Introduction

Immune thrombocytopenia (ITP) is a multifactorial bleeding disease characterized by a breakdown of immune tolerance and results in a decreased platelet count. Blocking the maturation of megakaryocytes[1, 2] and variations in the bone marrow microenvironment[3] are important contributors to platelet destruction and/or suppression of platelet production in ITP[4]. Autophagy plays an important role in maintaining the microenvironment and stemness of haematopoietic stem cells[5] and in regulating megakaryopoiesis and platelet function[6].

Characteristic pathologic changes in megakaryocytes suggest an important role of bone marrow in ITP. Wang et al. reported that autophagy was inhibited by bafilomycin A1 or induced by rapamycin in bone marrow cells and observed a significant decrease in high ploidy megakaryocytes and a reduction in CD41 and CD61 (two markers of megakaryocytic cells) co-expressing cells, proplatelet or platelet formation[7]. Our previous study using the human Dami cell line confirmed that autophagy is involved in megakaryocyte endomitosis and platelet development in vitro[8]. As emerging roles of abnormal autophagy in megakaryopoiesis, thrombopoiesis and platelet function have been revealed in ITP patients, insights into signalling pathways may guide future research in this field.

As a powerful technique for biomarker discovery, proteomic strategies have been introduced in many haematological system malignant diseases, such as acute myelogenous leukaemia (AML)[9] and myelodysplastic syndrome (MDS)[10], revealing the great potential of bone marrow in biomarker discovery and clinical tests. In this study, we established an integrated workflow by the combination of proteome quantification and PRM validation of bone marrow from ITP patients and healthy controls. With the help of advanced bioinformatics, we hope to provide a new opportunity for understanding the pathogenesis and a novel therapeutic approach against ITP.

## **2. Materials And Methods**

### **2.1. Patient and control samples**

The ethical protocol for the collection of human bone marrow aspirate with informed consent was approved by the Shandong Provincial Hospital Affiliated to Shandong University and Shandong Provincial Hospital Affiliated to Shandong First Medical University. Twenty newly diagnosed primary ITP patients in the active phase (12 females and 8 males, age range of 18–70 years, median of 42 years) and 20 healthy controls (12 females and 8 males, age range of 18–55 years, median of 46 years) were enrolled in this study between May and November 2018. The diagnosis for ITP was made according to recently published criteria, including patient history, complete blood count, physical examination and peripheral blood smear examination[11]. The patients' platelet counts ranged between 1 and  $30 \times 10^9/l$ , with a median platelet count of  $11 \times 10^9/l$  (Table 1)[12]. Patients and controls were divided into four groups according to their age and sex to reduce differences and increase the accuracy of the results. None had been treated with glucocorticosteroids, immunoglobulins or immunosuppressants prior to sampling. Bone marrow aspirate was collected into heparin -containing vacutainer tubes. According to the manufacturer's instructions, bone marrow mononuclear cells (BMMCs) were isolated from heparinized bone marrow aspirate samples by gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden). BMMCs from ITP patients and control subjects were stored at  $-80\text{ }^\circ\text{C}$ .

### **2.2. Crude protein extraction and trypsin digestion**

The sample was removed by centrifugation at  $12000\text{ g }4^\circ\text{C}$  for 10 min, the cell debris was discarded. Transferred the supernatant to a new centrifuge tube. After precipitating protein with 20% cold TCA and washing protein with cold acetone, Abundant Protein Depletion Kit (Pierce Top 12, Thermo) used to remove the high abundance Protein. The protein was redissolved in buffer (8 M urea, 100 mM TEAB, pH 8.0) and the protein concentration was determined with BCA kit. For digestion, 5 mM dithiothreitol (Sigma) was used to reduce protein solution at  $56^\circ\text{C}$  for 30 min, and 11 mM iodoacetamide (Sigma) was alkylated in the dark at room temperature for 15 min. The protein sample was then diluted by adding 100 mM TEAB to decrease the urea concentration to less than 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and at a 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion to improve the digestion effect[11, 12].

## 2.3. High-performance liquid chromatography (HPLC) Fractionation and LC MS/MS analysis

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 (5 µm particles, 4.6 mm ID, 250 mm length). Peptides were separated into 60 fractions with a gradient of 8–32% acetonitrile (pH 9.0) in 60 min. Then, they were combined into 4 fractions and dried by vacuum centrifuging. The peptides were redissolved in solvent A (0.1% formic acid in 2% acetonitrile) and loaded onto a reversed-phase analytical pre-column (Acclaim PepMap 100, Thermo Scientific). The gradient was comprised of an increase from 6–25% solvent B (0.1% formic acid in 90% acetonitrile) over 40 min, 25–35% in 12 min, 35–80% in 4 min and climbing to 80% in 4 min then holding at 80% for the last 4 min, all at a constant flow rate of 500 nL/min on an EASY-nLC 1000 UPLC system. The peptides were subjected to NSI (nano-spray-ionization) source in Q Exactive™ Plus (Thermo) coupled online to the UPLC and were detected in the Orbitrap. A data-dependent procedure (DDA) that alternated between one MS scan followed by 20 tandem mass spectrometry (MS/MS) scans. Automatic gain control (AGC) was used to prevent overfilling of the Orbitrap. 5E4 ions were accumulated for generation of MS/MS spectra, the maximum injection time was set at 30 ms, and the signal threshold was set at 15000 ions/s.

## 2.4. Bioinformatics analysis/functional enrichment analysis

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8).

InterProScan soft was used to identify proteins domain functional based on protein sequence alignment method. Then proteins were classified by GO annotation based on three categories: biological process, cellular component and molecular function. KEGG online service tools KAAS used to annotate protein's KEGG database description. Then mapping the annotation result on the KEGG pathway database using KEGG mapper. Enrichment of GO analysis and KEGG analysis used a two-tailed Fisher's exact test to test the enrichment of the differentially expressed protein against all identified proteins. The corrected p-value < 0.05 was considered significant. Collated all categories after enrichment along with their P values, and then filtered for those categories which were at least enriched in one of the clusters with P value < 0.05. Cluster membership were visualized by a heat map using the "heatmap.2" function. All differentially expressed protein were searched against the STRING database version 10.1 for protein-protein interactions and visualized in R package "networkD3".

## 2.5. Parallel reaction monitoring (PRM) analysis

Target proteins associated with autophagy were quantitatively identified using mass spectrometry-based targeted proteome quantification. The proteins selected for PRM were based on the results of quantitative analysis of the bone marrow serum proteome. The steps of crude protein extraction and trypsin digestion were similar to those described above. The electrospray voltage applied was 2.0 kV. The peptide length was set as 7–25. Transition settings: precursor charges were set as 2, 3, ion charges were set as 1, ion

types were set as b, y. The product ions were set as from ion 3 to last ion, the ion match tolerance was set as 0.02 Da. The PRM data were analyzed using Skyline (v.3.6) software.

## 2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. Two groups were compared by two-tailed Student's t-tests in PRM data analysis. A p-value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Proteome quantification overview

In this study, we performed a quantitative analysis of human BMMCs by establishing an integrated quantification of the proteome. We calculated the data accuracy and validated the sample preparation fitting the requirement; we identified 829 proteins in human BMMCs, among which 613 proteins could be quantified. In total, 69 proteins were downregulated with a fold change < 1/1.5, while 26 proteins were upregulated with a fold change > 1.5 ( $p \leq 0.05$ ). To identified differentially expressed proteins, volcano plot analysis was conducted to visualize the differences between the ITP patient and control groups (Fig. 1).

### 3.2. Abnormal regulation of autophagy-related proteins (HSPA8, PARK7, YWHAH, ITGB3, and CSF1R)

To identify the functional classifications and pathways of changed proteins in ITP, we performed Kyoto Encyclopedia Genes and Genome (KEGG) pathway enrichment analysis. Our data showed that the upregulated differentially expressed proteins in ITP are most prominently enriched in the complement and coagulation cascades and that the downregulated differentially expressed proteins in ITP are enriched in carbon metabolism (Fig. 2A), regulation of actin cytoskeleton, vasopressin-regulated water reabsorption and tight junction (Fig. 2B). Using the networkD3 R package, we identified some highly connected subnetworks among autophagy proteins, including heat shock protein family A (Hsp70) member 8 (HSPA8), Parkinson disease (autosomal recessive, early onset) 7 (PARK7), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide, isoform CRA\_b (YWHAH), integrin beta-3 (ITGB3) and colony-stimulating factor 1 receptor (CSF1R). The expression of the CSF1R protein in ITP patients was higher than that in controls, while the expression of other autophagy-related proteins was lower in ITP patients than in controls.

The downregulated protein HSPA8 participates in signalling pathways associated with the longevity regulating pathway in multiple species and endocytosis (Supplementary Table S1). YWHAH was downregulated and was enriched in the cell cycle, vasopressin-regulated water reabsorption and Hippo signalling pathway, and ITGB3 was downregulated and enriched in focal adhesion, platelet activation and haematopoietic cell lineage (Supplementary Table S1). The autophagy-related CSF1R protein was

upregulated and closely related to haematopoietic cell lineage and cytokine-cytokine receptor interaction (Supplementary Table S1).

Protein functions are largely dependent on specific domain structures in the sequence. To assess the domain structures, a bubble chart was obtained by Fisher's exact test [ $\log_2(\text{p value})$ ] for protein domain enrichment analysis. In agreement with our findings, we revealed that protein domains involved in the lipocalin/cytosolic fatty-acid binding domain and calycin were enriched with the upregulated proteins (Fig. 2C) in ITP, while the thioredoxin-like fold was enriched most in the downregulated proteins (Fig. 2D). CSF1R, an autophagy-related protein, was mainly related to the immunoglobulin-like domain, serine-threonine/tyrosine-protein kinase catalytic domain and tyrosine-protein kinase catalytic domain (Supplementary Table S2). Other downregulated autophagy proteins were enriched in some important protein domains, such as the 14-3-3 domain of YWHAH and the C-terminal domain of HSPA8 (Supplementary Table S2).

### 3.3. Clustering analysis

To determine the correlation between the function of differentially expressed proteins in the ITP patient group, all the differentially expressed proteins were divided into four quantiles (Q1-Q4) according to expressed ratios as described above. Then, enrichment-based clustering analyses (Gene Ontology (GO), KEGG pathway and protein domain analyses) were performed (Fig. 3).

The biological process category was analysed as shown in Fig. 3A. The downregulated proteins were highly enriched in platelet activation, cell – cell adhesion, and the integrin – mediated signalling pathway (Q1), which may be associated with the autophagy process. The upregulated proteins were highly enriched in the negative regulation of adaptive immune response in Q3 and in the acute inflammatory response, positive regulation of adaptive immune response and negative regulation of cytokine production in Q4, which may be attributed to the mechanism of the unbalanced autoimmune response in ITP. In the cellular component category, the upregulated proteins were mainly located in the organism cell part (Q3) and extracellular space and fibrinogen complex (Q4), while the downregulated proteins were mainly located in the cytoplasmic region (Fig. 3B). Molecular function analysis is presented in Fig. 3C. Downregulated proteins with small molecule binding, signalling adaptor activity and transcription coactivation activity were enriched in Q1. The chaperone binding function of upregulated proteins was enriched in Q4.

KEGG pathway analysis of the quantitatively changed proteins in ITP showed several vital pathways (Fig. 4D). The tight junction pathway was enriched in Q1. The upregulated proteins are associated with the platelet activation pathway in Q4. CSF1R, YWHAH and ITGB3 were enriched in the PI3K/Akt/mTOR signalling pathway (Supplementary Table S3). In addition, CSF1R and HSPA8 also participated in the mitogen-activated protein kinase (MAPK) signalling pathway, which plays a crucial role in different antiproliferative events, including apoptosis and autophagy[17]. Therefore, autophagy-related proteins (HSPA8, CSF1R, YWHAH and ITGB3) are involved in the pathogenesis of ITP by affecting autophagy-related proteins and pathways.

Domain enrichment analysis of differentially expressed proteins (Fig. 3E) showed that the downregulated proteins clustered with the immunoglobulin E – set, thioredoxin domain and chemokine interleukin – 8-like domain, while the upregulated proteins clustered with the fibrinogen, alpha/beta/gamma chain and calycin – like lipocalin/cytosolic fatty – acid binding domain.

### **3.4. PPI network**

We visualized a protein-protein interaction (PPI) network of all quantified proteins on the basis of the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (V.10.5) database. A complete network of differentially expressed proteins was created and included 50 interactions. We found that autophagy-related proteins were clearly upregulated (CSF1R) in the ITP patient group, while a number of proteins (HSPA8, PARK7, YWHAH, and ITGB3) related to autophagy were downregulated. Our data set offers insights into the probability of interactions of autophagy proteins in ITP. A representative example is shown in Fig. 4A.

### **3.5. PRM analysis**

PRM quantification was carried out for 20 selected target proteins in all samples. Limited by the characteristics of some proteins and the abundance of their expression, we quantified 14 of these selected target proteins. The peak area is used for PRM quantitation analysis. The PRM quantitative results of autophagy-related proteins are presented in Fig. 4B, which further verified that the expression of HSPA8, PARK7, YWHAH, and ITGB3 was also downregulated and that the expression of CSF1R was upregulated in the ITP patient group compared with those in the control group, revealing the same conclusion.

## **4. Discussion**

Proteomics has the potential to provide answers in the pathogenesis of cancer and autoimmune disease by comprehensively analysing protein expression levels and activation statuses. In our study, we employed a quantitative proteomics strategy to compare the autophagy-related differentially expressed proteins of BMDCs in ITP patient and control groups and to explore the potential mechanism of ITP pathogenesis. The data showed that 26 upregulated proteins in ITP were enriched in the acute-phase response and regulation of the adaptive immune response, while 69 downregulated proteins were enriched in processes associated with binding, such as actin binding and cytoskeletal protein binding, which manifested as changes in the autophagy fractions. Then, we detected 5 abnormally expressed autophagy-related proteins that may be associated with the pathogenesis of ITP, among which 4 proteins (HSPA8, PARK7, YWHAH, and ITGB3) were downregulated and 1 protein (CSF1R) was significantly upregulated in ITP patients compared with those in controls. Clustering analysis showed that most of the autophagy-related differentially expressed proteins (YWHAH, ITGB3 and CSF1R) in this research were closely related to the PI3K/Akt/mTOR signalling pathway. The signalling pathway targeting mammalian target of rapamycin (mTOR) mediates many physiological functions, such as cell proliferation, differentiation, migration and apoptosis, and constitutes an important signalling pathway that regulates

autophagy[18, 19]. Studies have shown that the pathway controlling mTOR expression negatively regulates autophagy in cells stimulated by factors such as starvation and hypoxia[20].

YWHAH is a large family of phosphoregulatory proteins that exist primarily as homo- and heterodimers[21]. YWHAH proteins are involved in different signalling pathways that modulate cellular and whole-body energy and nutrient homeostasis, such as insulin signalling and mTOR- and AMP-dependent kinase signalling pathways (AMPK pathway), and regulate autophagy[22]. There is considerable cross-talk between the AMPK pathway and other key energy regulatory pathways, such as insulin signalling and mTOR signalling complex 1 (mTORC1)[22]. AMPK is reported to inhibit mTORC1 by activating TSC1/2 (tuberous sclerosis protein 1 and 2) and by inhibiting regulatory-associated protein of TOR (RAPTOR) by phosphorylation-induced binding of YWHAH[23], both of which will stimulate autophagy. Recently, a direct stimulatory path from AMPK to autophagy was described through the phosphorylation of ULK1[24], and complex formation between ULK1, mTORC1 and AMPK has been found to coincide with the phosphorylation of RAPTOR and binding of YWHAH[25]. We speculated that abnormal autophagy was associated in ITP patients with low expression of the YWHAH protein by the inhibition of RAPTOR, ultimately decreasing the function and quantity of megakaryocytes and platelets and leading to the onset of ITP.

KEGG enrichment analysis of differentially expressed proteins showed that the downregulated, autophagy-related protein ITGB3 was also enriched in platelet activation and haematopoietic cell lineage. ITGB3 is an important molecule involved in cell survival, proliferation and cancer metastasis[26]. ITGB3 has been reported to be upstream of the PI3K/AKT/mTOR signalling pathway in various cell types, and the pathway is activated when ITGB3 is overexpressed[27]. Studies have shown that ITGB3 upregulation inhibits the autophagic process in cardiomyocytes by activating AKT, suggesting that the expression status of ITGB3 may affect cell autophagy[28]. Our results showed that the expression of ITGB3 in the ITP patient group was lower than that in the control group, suggesting that the overexpression of autophagy may be caused by the downregulation of AKT activation by ITGB3.

Another important autophagy-related protein in this research is CSF1R. Autophagy mediated by CSF-1/CSF1R plays a crucial role during the differentiation of human monocytes into macrophages[29, 30], which induce typical autophagic structures, such as phagophores and autophagosomes, and results in the accumulation of LC3-II[29]. Tian et al. showed that the level of LC3-II in cells was lower in cells overexpressing CSF1R-Mut than in benign controls or CSF1R-WT cells when exposed to CSF-1 stimulation, indicating that the autophagy process might be disturbed by abnormal CSF-1/CSF1R signalling[31]. At the molecular level, E5[N-(3-((4(benzofuran-2-yl) pyrimidin-2-yl) oxy)-4-methylphenyl)-4-((4-methylpiperazin-1-yl) methyl) benzamide] was able to downregulate the mTOR pathway and to activate the MAPK/ERK pathway[17, 32], thus inducing the conversion of LC3-I to LC3-II, increasing the expression of Atg5 and restoring autophagy. In our results, the expression level of CSF1R was decreased in the control group compared with that in the ITP patient group, which may demonstrate that the abnormal autophagy mediated by CSF1/CSF1R signalling is involved in the pathogenesis of ITP.

HSPA8 was enriched in the longevity regulating pathway - multiple species in KEGG analysis. HSPA8 is a molecular chaperone involved in a wide variety of cellular processes and is located in the nucleus, the cytosol, extracellular exosomes, and the cell membrane[33]. HSPA8 is a detector of substrates that will be processed by chaperone-mediated autophagy (CMA)[34]. HSPA8 expression is altered in a number of immune disorders. For example, flow cytometry studies showed that the expression of HSPA8 is increased in B cells and T cells in the spleen of MRL/MpTn-gld/gld lupus-prone mice[35, 36]. HSPA8 is also involved in the molecular regulation of haematopoiesis[37]. PARK7 is a multifunctional protein involved in various cellular activities. One of its principle functions is antioxidative defence and maintains mitochondrial homeostasis[38]. The dysfunction of PARK7 leads to mitochondrial defects. Furthermore, CMA protects cells from mitochondrial toxin MPPC-induced changes in mitochondrial morphology and function and increases cell viability[39]. Under PARK7-deficient conditions in ITP, these protective effects may be lost.

In recent years, experimental and clinical evidence has concluded that autophagy plays an important role in maintaining the stemness and microenvironment of haematopoietic stem cells[5]. Perturbations of normal autophagy processes in ITP patients may be caused by the deletion of autophagy-related genes such as ATG7 and abnormal signalling due to the overexpression of mTOR. These changes are thought to affect markers of haematopoietic stem cells, such as CD41 and CD61, and the differentiation of megakaryocytes, ultimately decreasing the function and quantity of platelets and leading to the onset of ITP[3]. Ouseph et al. demonstrated that the autophagy process is essential for the normal functioning of platelet activation and aggregation[40]. In another study, they demonstrated that starvation induced substantial autophagy (above basal level), which was characterized by decreased platelet aggregation, reduced calcium mobilization and granule secretion, and decreased adhesion to immobilized fibrinogen, and eventually increased bleeding time[41].

In conclusion, autophagy-related differentially expressed proteins were found in ITP BMMC samples. GO, KEGG, protein domain enrichment and clustering analyses were performed to determine the correlation between the function and differential expression of proteins. PRM analysis further confirmed that the expression of autophagy-related proteins was significantly different in ITP patients. Furthermore, we indicate that the five autophagy-related differentially expressed proteins were closely related to mTOR signalling or similar pathways to regulate autophagy activity, which may provide useful information for ITP diagnosis and targeted treatment in the future.

## **Declarations**

### **Ethical approval and consent to participate**

Informed consent was obtained from each participating patient and/or legal guardian. Ethical approval for the study was obtained from the Medical Ethical Committee of Shandong Provincial Hospital Affiliated to Shandong University and Shandong Provincial Hospital Affiliated to Shandong First Medical University.

## Consent for publication

Not applicable.

## Availability of data and materials

The data of this manuscript have been presented in the main paper.

## Competing interests

The authors declare no competing interests.

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## Author's contributions

SRJ contributed to experimental design and data analysis. YD contributed to data analysis. YDM, LSY and ZJJ contributed to the statistical analysis. SNN obtained funding, experimental design and writing of the manuscript. All authors read and approved the final manuscript.

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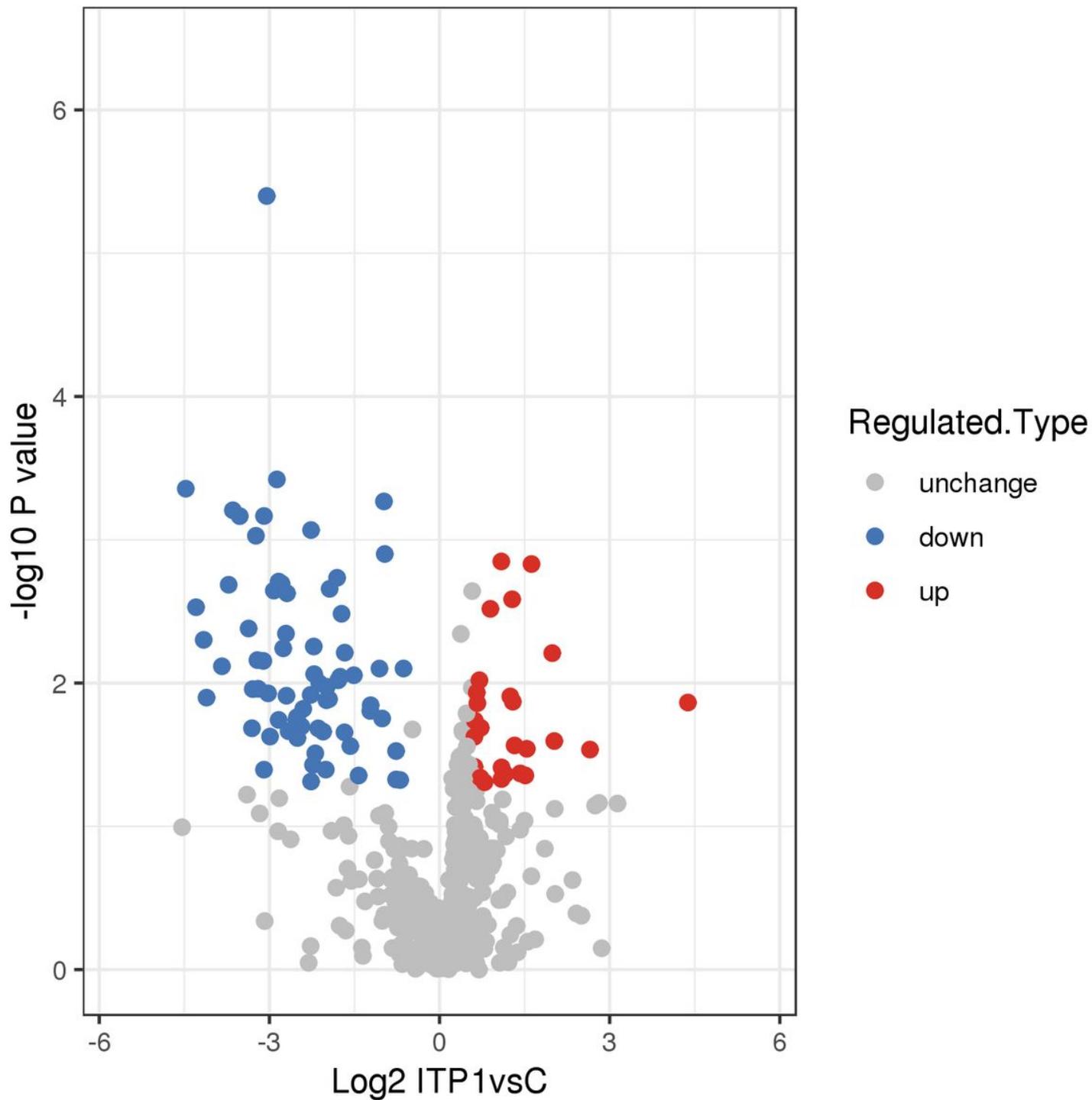
## Tables

**Table 1 Clinical characteristics of ITP patients**

Group	Group1	Group2	Group3	Group4	Median(min-max)
Age(year)/Sex	19/F	18/F	18/F	43/F	42 (18-70)
	38/F	27/M	33/M	69/F	
	43/F	41/F	48/F	48/F	
	55/M	45/M	43/F	25/M	
	70/M	65/F	60/M	39/M	
Platelet counts ( $\times 10^9/l$ )	9	30	11	1	11 (1-30)
	7	18	11	8	
	12	17	12	3	
	3	8	4	12	
	14	29	4	14	
Bleeding symptoms	EC, PT	EC	PT	PT, GH	
	GUH, PT	NONE	GH	PT, GUH	
	GH, EP	GUH, PT	GH	PT, GH	
	GH	GH, EC	EP, GH	GH $\boxtimes$ EP	
	NONE	EP $\boxtimes$ GH	EC, GH	GH	

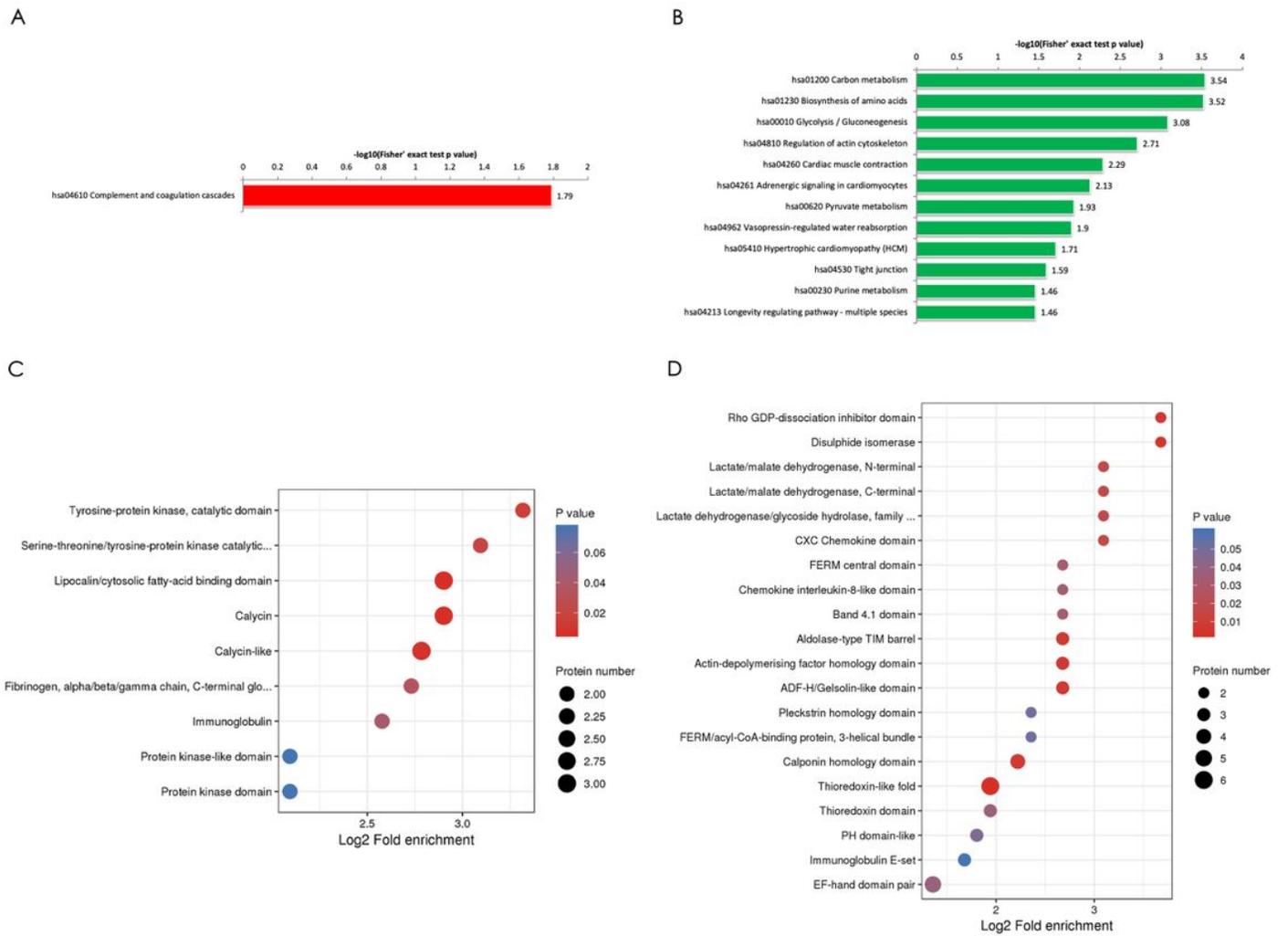
EC = ecchymoses, PT = Petechiae, GUH = genitourinary hemorrhage, GH = gingival hemorrhage, EP = epistaxis

## Figures



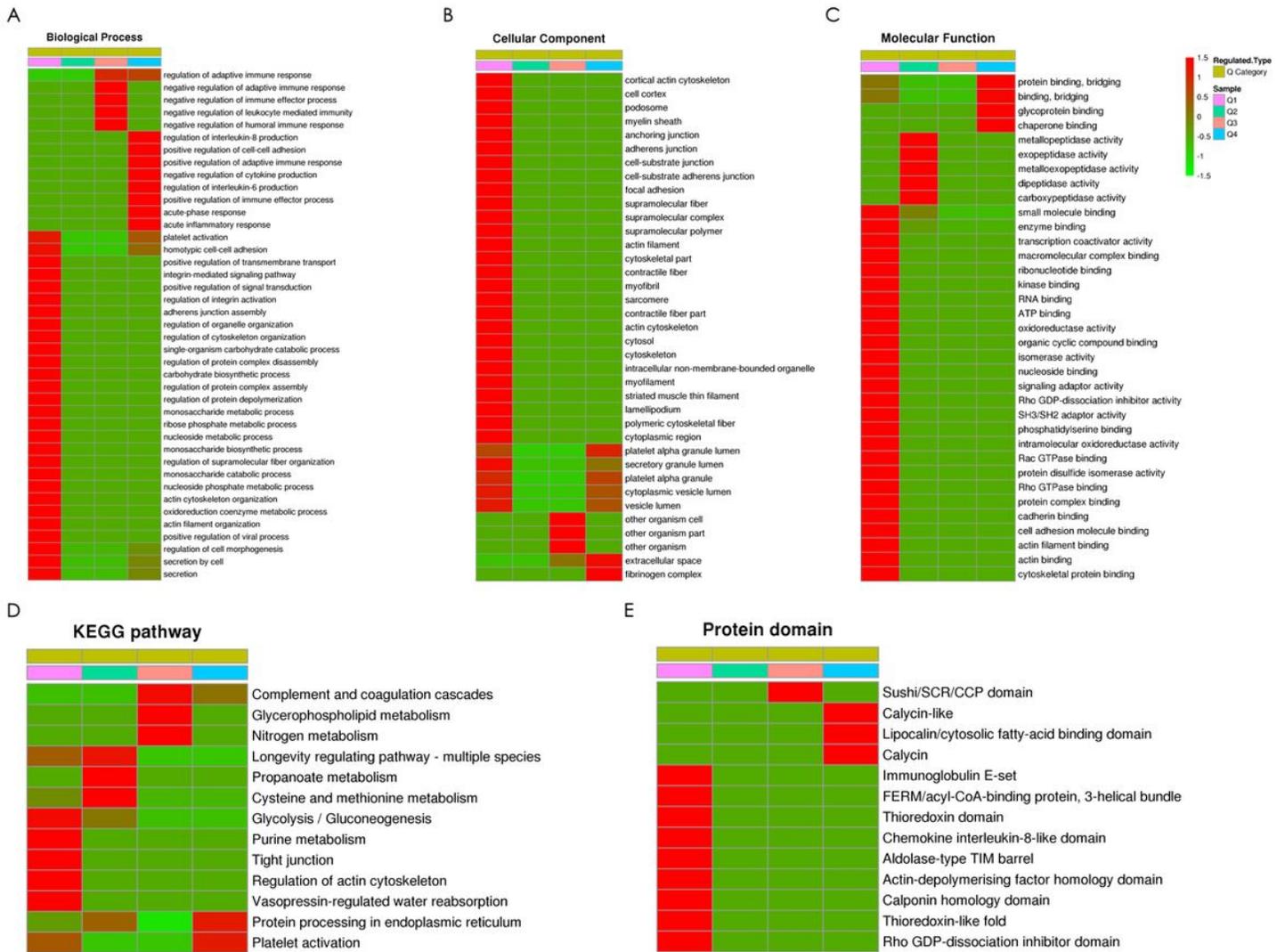
**Figure 1**

Volcano plots of all proteins identified in LC-MS/MS analysis; red dots in these plots represent the upregulated proteins with statistical significance, and blue dots in these plots represent the downregulated proteins (fold change  $\geq 1.5$ ,  $p \leq 0.05$ ).



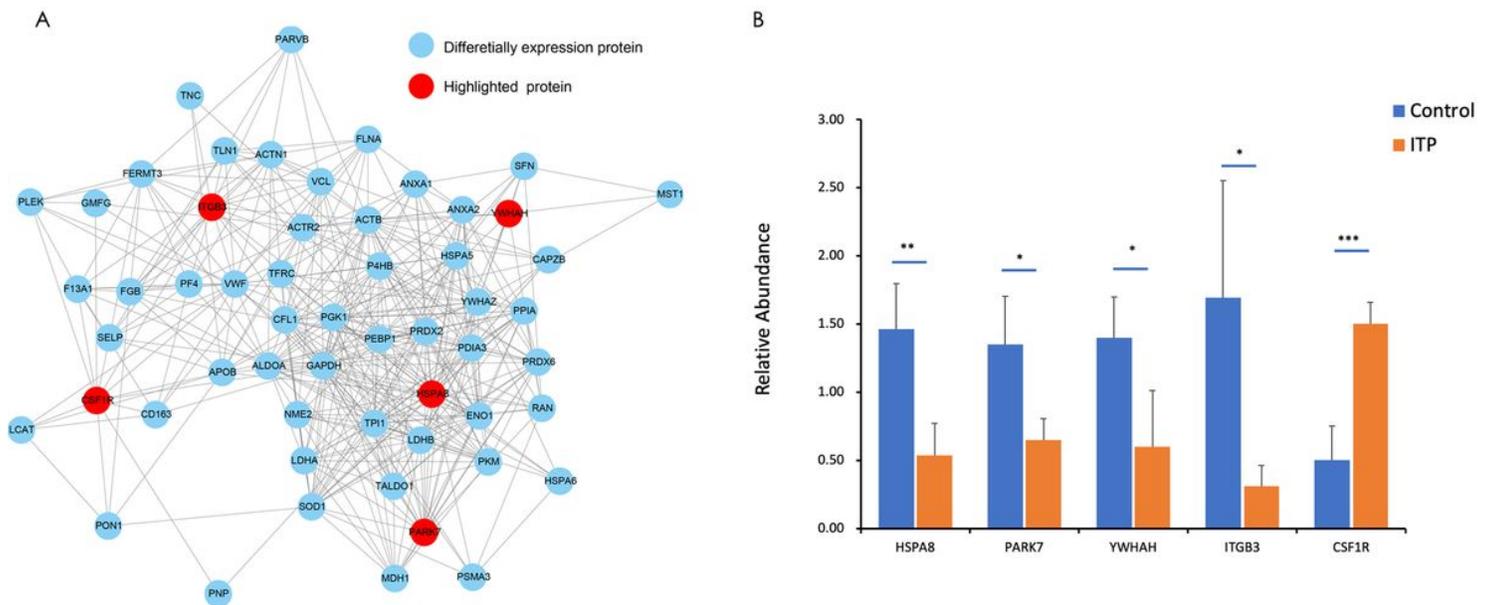
**Figure 2**

Functional enrichment of differentially expressed proteins. (A). KEGG enrichment analysis of the upregulated differentially expressed proteins by Fisher's exact test p value ( $-\log_{10}$ ). (B). KEGG enrichment analysis of the downregulated differentially expressed proteins by Fisher's exact test p value ( $-\log_{10}$ ). (C). Protein domain enrichment analysis of upregulated proteins. A bubble chart was used to display domain enrichment analysis of differentially expressed proteins. (D). Protein domain enrichment analysis of downregulated proteins. The circle size indicates the number of differentially expressed proteins, and the circle colour indicates the enrichment significance of the p value.



**Figure 3**

Heatmaps were presented by clustering based on (A) GO biological process, (B) GO cellular component, (C) GO molecular function, (D) KEGG pathway, and (E) protein domain enrichment. The differentially expressed proteins were divided into four quantitative categories according to the ITP patient group (P) to control group (C) ratio: Q1 ( $0 < P/C \text{ ratio} < 1/2$ ,  $p \text{ value} < 0.05$ ) and Q2 ( $1/2 < P/C \text{ ratio} < 1/1.5$ ,  $p \text{ value} < 0.05$ ) represent downregulated proteins, and Q3 ( $1.5 < P/C \text{ ratio} < 2$ ,  $p \text{ value} < 0.05$ ) and Q4 ( $P/C > 2$ ,  $p \text{ value} < 0.05$ ) represent upregulated proteins.



**Figure 4**

PPI network and PRM analysis of autophagy-related proteins. (A). A complete PPI network was performed. Five autophagy-related proteins (HSPA8, PARK7, YWHAH, ITGB3 and CSF1R) were highlighted from the differentially expressed proteins. (B). Quantitative analysis results of autophagy-related proteins (HSPA8, PARK7, YWHAH, ITGB3 and CSF1R) were verified based on PRM. With the exception of CSF1R, the expression of these autophagy-related proteins was significantly decreased in the ITP group compared with that in the control group. The data are represented as the Mean  $\pm$  SD; two-tailed Student's t-test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .