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Original Article

Proteomic characteristics of circulating microparticles in patients with newly-diagnosed type 2 diabetes

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Abstract: Objective: This study aimed to evaluate the proteomic characteristics of plasma microparticles (MPs) from patients with newly diagnosed type 2 diabetes (T2DM). Methods: The subjects comprised eight male T2DM patients recruited between December 2013 and March 2014, as well as eight age and sex-matched healthy controls enrolled during the same period. Plasma microparticles (MPs) were extracted from the blood of each subject, and subjected to proteomics analysis using label-free methods. Bioinformatic analyses were performed using specialized software. Results: 3,148 unique peptides and 496 proteins were identified, among these, 46 proteins were differentially expressed between the two groups. Among these 46 candidates, 20 proteins had higher expression in T2DM group compared with the control group, whereas 3 proteins displayed lower expression. There were 17 proteins only detected in T2DM group, and 6 proteins only detected in the control group. Gene ontology (GO) analysis revealed significant differences between the two groups in some functional nodes, including neutrophil accumulation, chemokine production, platelet activation, and blood coagulation. Pathway analysis showed that proteins involved in platelet activation, cell adhesion, focal adhesion, and extracellular matrix-receptor interaction were differentially expressed between the 2 groups. Network analysis indicated that ubiquitin was the protein with the highest degree of connectivity. Conclusions: Blood MPs from T2DM patients are enriched in proteins involved in platelet activation, cell adhesion, and inflammation. Therefore, MPs in T2DM patients might be associated with hypercoagulable state in diabetic patients and the development of diabetic complications.

Keywords: Microparticles, diabetes, proteomic characteristics

Introduction

MPs are vesicles released by cells when stimulated by physical (e.g. shear force) or chemical (e.g. agonists) factors, as well as when cells are apoptotic or exposed to inflammatory conditions [1]. MPs are 100-1000 nm in diameter, have membrane cytoskeletons, express phosphatidylserine (PS) on the surface, and lack nuclei [2]. Many studies have been conducted on MPs since Wolf *et al*. initially described platelet-derived MPs (PMPs) as platelet "dust" in 1967 [3]. Nearly all cell types, including erythrocytes, leukocytes, platelets, vascular endothelial cells, smooth muscle cells, retinal cells, and cancer cells, can release MPs [4, 5]. Surface molecules, enzymes, RNA and DNA are conveyed via MPs from origin cells to target cells [6, 7].

As mediators of information transfer, MPs have been proposed to have pro-inflammatory and pro-coagulant effects in many disease states, such as cancer [8], venous thromboembolism [9], arteriosclerosis, and diabetes mellitus [10]. MPs play a pro-inflammatory role mainly by expressing bioactive surface lipids and inflammatory cytokines. Mesri *et al*. reported that leukocyte-derived MPs can stimulate endothelial cells to secrete Interleukin 6 (IL-6) and Interleukin 8 (IL-8), and upregulate expression of the cell adhesion molecules: Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) *in vitro* [11]. In addition, *in vitro* experiments by Barry *et al*. showed that peanut-derived arachidonic acid on the surface of platelet-derived MPs can promote cyclooxygenase-2 (COX-2) expression in endothelial cells and monocytes [12]. MPs exert pro-coagulant effects by expressing PS, tissue factor (TF), as well as other surface molecules, such as thrombin receptors. Expression of PS and TF on the surface of MPs is the main

feature enabling a pro-coagulant effect. PS is negatively charged, which provides a catalytic surface for the function of coagulation proteins, clotting factors, and thrombin, which all contain cationic domains [13]. TF on the surface of MPs can function as blood-borne TF to initiate the clotting process. As a FVII/VIIa receptor, TF can interact with FVII/VIIa to form a TF: FVIIa complex with protease activity, leading to activation of FX and FIX and initiation of the clotting process [14]. On the surface of platelet-derived MPs, the FVIII receptor [15] and FVa receptor [16], as well as high/low affinity FIXa binding sites [17] are expressed, which can bind corresponding thrombin factors to promote the coagulation cascade. Extra-large vWF is expressed on the surface of endothelium-derived MPs and binds platelets and platelet-derived MPs to form a strong network structure that promotes blood coagulation [18].

The hypercoagulable state associated with diabetes is well recognized [19, 20], and 80% of T2DM patients have died from thrombotic diseases [21]. The incidence of myocardial infarction in diabetic patients is 1.5-2-fold higher compared with the incidence in non-diabetic patients [22]. MPs have been increasingly investigated in diabetes studies. The total number of MPs in the blood of diabetic patients is elevated and the number of MPs derived from different cell types and their pro-coagulant activity are different compared with values observed in non-diabetic control subjects. Sabatier *et al*. found that the total MP concentrations in patients with either T1DM or T2DM were higher than that of healthy controls. EMP and PMP concentrations were higher in T1DM patients compared with those in healthy controls [10]. Esposito *et al*. reported that even in the blood of diabetic patients without complications and optimal glucose control, as well as in patients with newly diagnosed diabetes, levels of pro-coagulant platelet-derived, monocyte-derived and endothelium-derived MPs were all increased compared with values observed in non-diabetic controls [23]. Diabetic patients with different complications have increased levels of MPs to various extents. Tsimerman *et al*. reported that total MP and PMP concentrations were increased in T2DM patients with diabetic foot compared with those in healthy controls, furthermore, the EMP concentration and TF/TFPI ratio were increased in T2DM patients with severe diabetic foot. In addition, HUVECs co-cultured with MPs from patients with severe diabetic foot displayed a five-fold increase in expression of TF mRNA and protein, and a three-fold increase when these cells were co-cultured with MPs from diabetes patients with retinopathy and heart disease [24]. These results indicate that MPs of diabetic patients, especially from patients with severe diabetic foot, have high pro-coagulant activity. Plasma MPs from patients with diabetes also influence cellular functions. Nomura *et al*. reported that in patients with T2DM, platelets and PMPs promote an interaction between endothelial cells and monocytes [25]. These results suggest that MPs are potentially implicated in the onset of diabetes-associated hypercoagulable state, inflammation, and secondary complications. However, most previous studies on diabetes and MPs only evaluated their plasma concentration, type, source, and pro-coagulant activity in patients with different complications. Few reports have studied the proteomics features of plasma MPs in patients with diabetes. Therefore, the purpose of this study was to identify and characterize MP-associated proteins in newly diagnosed diabetic patients.

Materials and methods

Biological materials and sample collection

Sixteen male subjects were enrolled in the study, including eight patients with newly diagnosed T2DM and eight age-matched healthy controls. Patients were recruited from inpatient and outpatient departments of Tongji Hospital between December 2013 and March 2014. Control subjects were recruited from healthy staff in Tongji hospital during the same period. Patients were diagnosed according to WHO criteria. Informed consent was provided by all study participants, and the protocol was approved by the ethics committee of Tongji Hospital. Inclusion criteria included normal ECG, seated blood pressure (BP) < 150/90 mmHg, no history or symptoms of cardiovascular disease, glutamic-pyruvate transaminase (GPT) < 60 U/L, serum creatinine (SCr) < 1.25 mg/dl, no pathogen infection, no acute diabetic complications including diabetic ketoacidosis or a hyperglycemic hyperosmolar state. Inclusion criteria for healthy controls included a body mass index (BMI) < 25 kg/m^2. The demo-

Table 1. Demographic characteristics of the study subjects

	DM	HC	*P* value
Age (years)	53.50±10.41	49.88±9.61	0.481
BMI (kg/m^2)	28.03±3.72	22.38±2.21	0.002
LDL-C (mmol/L)	2.90±0.53	3.00±0.37	0.648
Cr (μmol/L)	82.38±16.62	80.75±8.81	0.812
GPT (U/L)	33.88±14.34	24.00±15.06	0.201
FPG (mmol/L)	8.49±1.93	4.92±0.41	0.001
HbA1C (%)	8.75±1.90	5.51±0.19	0.002

graphic characteristics of the study subjects are summarized in **Table 1**. The data were analyzed using Spss19.0 software and the results are presented by mean ± standard deviation.

Plasma preparation

Platelet-poor plasma (PPP) was obtained from study subjects following a standardized protocol [26]. Briefly, venous blood samples (2 × 5 ml) were drawn from the antecubital vein and collected in citrate tubes (containing 3.2% sodium citrate) using a 21-gauge needle (BD Vacutainer Safety-Lok, USA). The first few milliliters were used for other biochemical tests. All blood samples were collected at room temperature (RT) and processed within two hours. After collection, blood cells were removed by centrifugation at 2,500 × *g* for 15 min at 20°C with no break applied. The supernatant was collected, leaving one centimeter of plasma above the buffer layer, with care taken not to disturb it. The plasma was centrifuged a second time at 2,500 × *g* for 15 min at 20°C. The resulting PPP was transferred into a fresh tube using a pipette, while leaving approximately 100 μl of PPP at the bottom of the tube. PPP was then divided into 250 μl and 1000 μl aliquots and stored at -80°C until analysis.

MP isolation

PPP (1 ml) was thawed in 37°C water and centrifuged at 20,000 × *g* at 4°C for 30 min. After centrifugation, 950 μl of supernatant was discarded. Remaining material was resuspended in 950 μl of PBS-citrate at pH 7.4. After resuspension of the MP pellet, the centrifugation step was repeated twice, with 950 μl of supernatant removed each time. Purified MPs (50 μl) were used for proteomic analysis. For proteomics, MPs from every two individuals in the same group were pooled to obtain sufficient material for analysis. Each pooled sample was supplemented with 55 μl of SDT buffer (4% SDS, 150 mM of Tris-HCl pH 8.8), and shaken for 30 min at 37°C. Samples were boiled for 5 min and centrifuged at 14,000 × *g* for 15 min. Supernatants were collected and protein concentrations were determined using a BCA Protein Assay Kit (Bio-Rad, USA). The data were analyzed using SPSS 19.0 and there was no statistics differences in protein concentrations between two groups (*P=0.168*).

Protein digestion

Protein digestion (75 μg from each pooled sample) was performed according to the FASP procedure described by Wisniewski *et al*. [27]. Briefly, 75 μg of protein was added to DTT at a final concentration of 100 mM, boiled for 5 min and then cooled to room temperature (RT). The DTT detergent and other low-molecular weight components were removed using 200 mL of UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0) by repeated ultra-filtration (Microcon units, 30 kD) facilitated by centrifugation at 14,000 × *g* for 15 min. Next, 100 μL of IAA (50 mM IAA in UA) was added to block reduced cysteine residues and samples were incubated for 30 min in darkness. The filter was washed with 100 μL of UA buffer twice and then three times with 100 μl of 25 mM NH_4HCO_3. Finally, the protein suspension was digested with 40 μl of trypsin buffer (3 μg of trypsin in 40 μl of 25 mM NH_4HCO_3) overnight at 37°C and 25 μl of 25 mM NH_4HCO_3 was added prior to centrifugation at 14,000 × *g* for 30 min. The resulting peptides were collected as a filtrate. Peptide content was estimated by UV light spectral density at 280 nm using an extinction coefficient of 1.1 of a 0.1% (g/l) solution calculated on the basis of the frequency of tryptophan and tyrosine residues in vertebrate proteins.

Nano-liquid chromatography (LC) - electrospray ionization (ESI) tandem MS (MS/MS)

MS experiments were performed using a Q-Exactive mass spectrometer coupled to an Easy-nLC 1000 (Thermo Finnigan, USA). Peptide (1 μg) was loaded on a trap column (Thermo EASY column SC001, 150 μm × 20 mm) desalted in-line and separated on an analytical column (Thermo EASY column SC200 150 μm × 100 mm). The flow rate was 400 nl/

Table 2A. Differentially expressed proteins in MPs of T2DM patients that were selected using a cutoff point of $p < 0.05$

Protein ID	Protein Name	Gene Name	Change	DM/C ratio	t-test *p* value
P16157	Ankyrin-1	ANK1	↑	12.9803709	0.00024191
Q8IWB9	Testis-expressed sequence 2 protein	TEX2	↑	3.033393156	0.000917079
Q0ZCI4	Immunglobulin heavy chain variable region	N/A	↑	2.955014629	0.001365084
P05109	Protein S100-A8	S100A8	↑	21.94417224	0.001474215
P21926	CD9 antigen	CD9	↑	5.00062732	0.003568371
P32119	Peroxiredoxin-2	PRDX2	↑	3.000585839	0.004047499
P06702	protein s100-A9	S100A9	↑	3.576446564	0.004106192
A2MYC8	V5-2 PROTEN	V5-2	↑	5.701744852	0.007383447
Q8WWU7	Intelectin-2	ITLN2	↑	19.42986746	0.015549027
P08514	Integrin alpha-IIb,CD41	ITGA2B	↑	3.25373108	0.015569025
Q86UX7	Fermitin family homolog 3	FERMT3	↑	3.275755085	0.016935154
P02730	Band 3 anion transport protein, CD233	SLC4A1	↑	2.368997286	0.017763551
Q6PIK1	IGLC2 protein	IGLC2	↑	3.287573019	0.018170724
P61224	Ras-related protein Rap-1b	RAP1B	↑	2.957510253	0.019828426
P16188	HLA class I histocompatibility antigen, A-30 alpha chain	HLA-A	↑	5.469089803	0.022349402
P69905	Hemoglobin alpha chain	HBA1	↑	1.562586471	0.025623744
P27918	Properdin, Complement factor P	CFP	↑	3.913525581	0.025959527
P10643	Complement component C7	C7	↑	1.667145763	0.042331979
P27105	Erythrocyte band 7 integral membrane protein brane protein	STOM	↑	2.718224198	0.043582008
P60174	Triosephosphate isomerase	TPI1	↑	5.766182194	0.049350022
P35858**	Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	↓	0.299654681	0.018213325
P02452**	Alpha-1 type I collagen	COL1A1	↓	0.282184209	0.023446723
Q5SRP5**	Apolipoprotein M	APOM	↓	0.507962709	0.04775203

Note: DM/C ratio: the ratio fold of a protein in the diabetic group relative to the control group; **indicates proteins which were decreased in the T2DM group.

min. The mobile phases consisted of buffer A (2% v/v acetonitrile and 0.1% v/v formic acid) and buffer B (84% v/v acetonitrile and 0.1% v/v formic acid). The gradient went from 0 to 45% B in 100 min, followed by 8 min from 45 to 100% B, and held for 12 min with 100% B. The column was re-equilibrated with solvent A. MS data were acquired using a data-dependent method dynamically selecting the most abundant top 20 precursor ions from the survey scan (300-1800 *m/z*) for HCD fragmentation. Survey scans were acquired at a resolution of 70,000 at *m/z* 200 and the resolution for HCD spectra was set to 17,500 at *m/z* 200. MS experiments were performed twice for each sample.

Sequence database searching and data analysis

MS data were searched against the UniProt Human FASTA database (136,615 total entries, downloaded 05/07/14). Peptides were identified and quantified using MaxQuant (version 1.3.0.5) with the following settings: main search ppm: 6; missed cleavage: 2; MS/MS tolerance ppm: 20; de-isotopic: TURE; enzyme: trypsin; database: uniprot_Human.fasta; Fixed modification: Carbamidomethyl (C); variable modification: oxidation (M), acetyl (protein N-term); decoy database pattern: reverse; iBAQ: TURE; match between runs: 2 min; peptide FDR: 0.01; protein FDR: 0.01. The original data were then analyzed using Perseus software (version 1.3.0.4). Student's *t*-tests were performed to compare the four disease group samples with the four control group samples. A *p*-value less than 0.05 was used to define statistically significant differential protein expression.

GO analysis, pathway analysis, and protein network analysis

Next, the GSEABase package of R project (http://www.r-project.org/) was used for GO analysis. Differential genes were mapped to the appropriate GO database to calculate the number of genes at each node. Differentially expressed genes were independently classified according to BP (biological process), CC (cellular component), and MF (molecular function). For identification of the molecular pathways in which the differential proteins are involved, we

Table 2B. The seventeen proteins identified only in MPs of the T2DM group

Protein IDs	Protein Name	Gene Name	Value in Patients	value in Controls
p03989	HLA class I histocompatibility antigen	HLA-B	1805275	UD
P0CG48	Ubiquitin	UBC	397340	UD
P08575	Receptor-type tyrosine-protein phosphatase C, CD45	PTPRC	143929.5	UD
Q96T46	Hemoglobin alpha 2	HBA2	567127.5	UD
O00602	Ficolin-1	FCN1	132564.75	UD
P04083	Annexin A1	ANXA1	186665	UD
G1ENK8	MHC class I antigen (Fragment)	HLA-B	271230	UD
Q15404	Ras suppressor protein 1	RSU1	301592.5	UD
P02792	Ferritin light chain	FTL	954150	UD
P01615	Ig kappa chain V-II region FR	N/A	2175125	UD
P01344	Insulin-like growth factor II	IGF2	569210	UD
A2NWW1	VH-3 family (VH26)D/J protein	VH-3 family (VH26)D/J	3973200	UD
P60660	Myosin light polypeptide 6	MYL6	749737.5	UD
P10599	Thioredoxin	TXN	2507350	UD
Q5CZ94	Putative uncharacterized protein DKFZp781M0386	DKFZp781M0386	5838300	UD
Q6N091	Putative uncharacterized protein DKFZp686C02220	DKFZp686C02220	1272485	UD
P30408	Transmembrane 4 L6 family member 1	TM4SF1	2602905	UD

Note: UD means “undetectable”.

Table 2C. The six proteins identified only in MPs of the HC group

Protein IDs	Protein Name	Gene Name	Value in Patients	value in Controls
P20742	Pregnancy zone protein	PZP	UD	79230
P01719	Ig lambda chain V-V region DEL	N/A	UD	753030
P08670	Vimentin	VIM	UD	1057650
A2N2F4	VK3 protein	VK3	UD	1159185
Q0ZCI6	Immunglobulin heavy chain variable region	N/A	UD	1503450
P68431	Histone H3.1	HIST1H3A	UD	3784175

Note: UD means “undetectable”.

mapped differentially expressed genes to the KEGG pathway database using GenMAPP software (version 2.1). Gene enrichment (enrichment *p*-value) of each pathway was analyzed, and the pathways with $p < 0.01$ were listed and considered significantly different. For protein network analysis, interactions between genes were analyzed using the KEGGSOAP package (http://www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html) and R project. Three different types of interaction were analyzed using MIPS and the PubMed database. Results for the three types of analysis were integrated to a gene inter-relationship network, which was displayed in the form of graphs using Medusa software.

Results

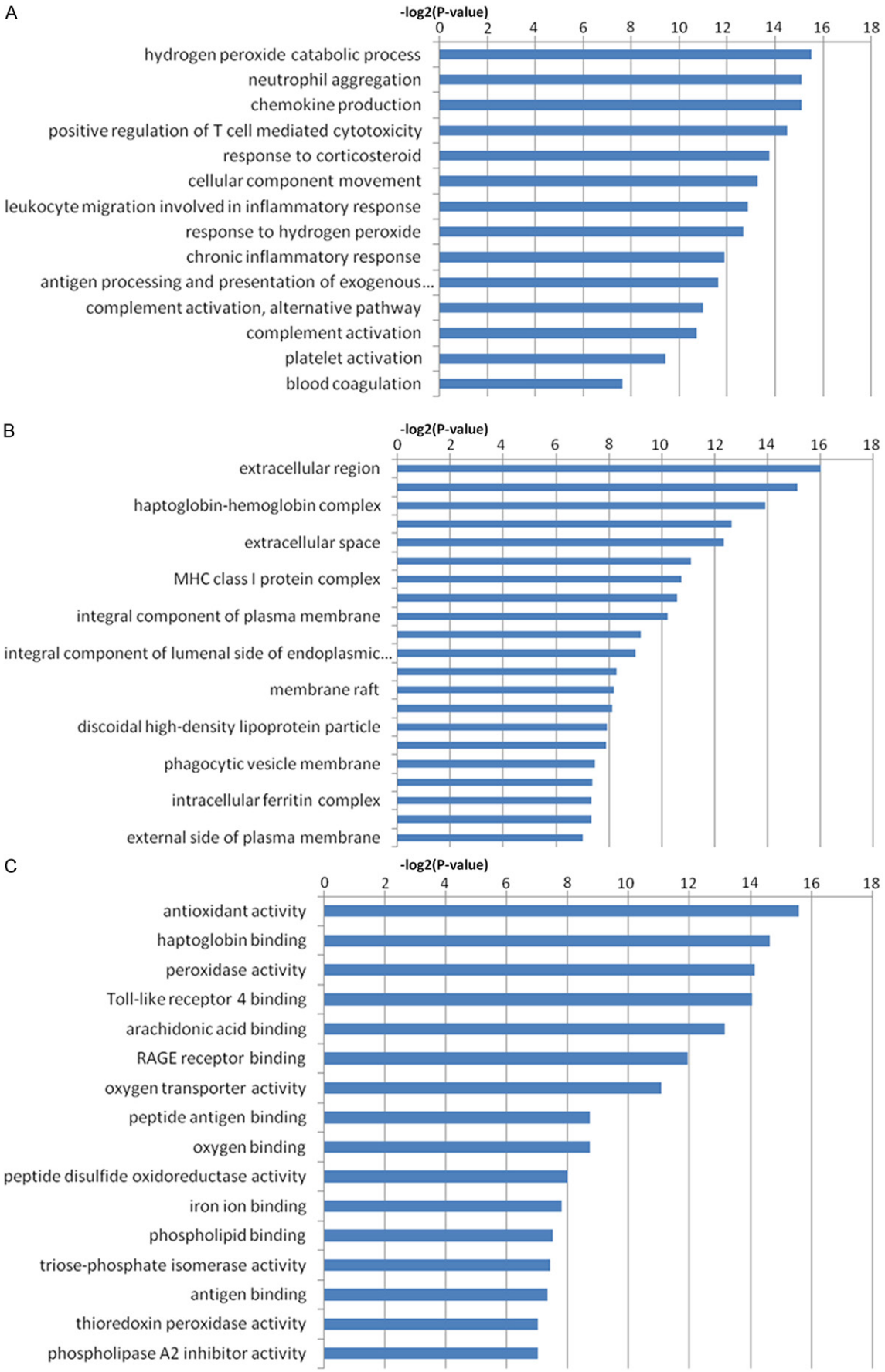
Screening for differentially expressed proteins

Protein identification was based on two peptides with 95% confidence. In our study, 3,148 unique peptides were found and 496 proteins were identified. There were 46 proteins which were differentially existed between diabetic patients and healthy controls: Using $p < 0.05$ as cutoff point (**Table 2A**), the contents of 20 proteins of MPs were increased whereas those of 3 proteins were decreased in patients vs those of controls; the other 17 proteins were identified only in MPs of patients (**Table 2B**) and the other 6 proteins were identified only in MPs of healthy controls (**Table 2C**). Differentially expressed proteins included the following: S100A8, S100A9, CD45, fermitin family homolog 3, histone H3.1 (HIST1H3A), Ras-related protein Rap-1b (RAP1B), CD9, integrin alpha-IIb (ITGA2B, CD41), and alpha-1 type I collagen (COL1A1).

Table 3. GO and KEGG pathway analyses for parts of differentially expressed proteins involved in platelet activation, cell adhesion

Protein name	Gene ontology			KEGG pathway
	CC (cellular component)	BP (biological process)	MF (molecular function)	
S100A8 S100A9	cytoskeleton; cytosol; nucleus; plasma membrane;	cell-cell signaling cytokine production; chemokine production inflammatory response; leukocyte migration involved in inflammatory response; neutrophil aggregation; neutrophil chemotaxis;	Antimicrobial; arachidonic acid binding; calcium ion binding; zinc ion binding; RAGE receptor binding; Toll-like receptor 4 binding;	
Integrin alpha-IIb (CD41)	blood microparticle; focal adhesion; plasma membrane;	blood coagulation; cell adhesion; cell-matrix adhesion; platelet activation; platelet aggregation; platelet degranulation; positive regulation of leukocyte migration;	extracellular matrix binding; identical protein binding; metal ion binding;	Platelet activation*; Focal adhesion*; Hematopoietic cell lineage*; ECM-receptor interaction*; Rap1 signaling pathway;
Fermitin family homolog 3	cell junction; membrane;	leukocyte cell-cell adhesion; platelet aggregation; regulation of cell-cell adhesion mediated by Integrin; substrate adhesion-dependent cell spreading;	integrin binding;	Platelet activation*;
Ras-related protein Rap-1b	cell-cell junction; cytosol; plasma membrane;	blood coagulation; platelet activation; Rap protein signal transduction;	GDP binding; GTPase activity; GTP binding;	Platelet activation*; Focal adhesion*; Rap1 signaling pathway;
Ras-related protein Rap-1b		regulation of insulin secretion	protein complex binding	Leukocyte transendothelial migration; Ras signaling pathway; MAPK signaling pathway;
Alpha-1 type I collagen	collagen type I trimer; endoplasmic reticulum lumen; Golgi apparatus;	blood coagulation; blood vessel development; cartilage development involved in endochondral bone morphogenesis; collagen biosynthetic process; collagen catabolic process; leukocyte migration; osteoblast differentiation; platelet activation; positive regulation of canonical Wnt signaling pathway;	extracellular matrix structural constituent; metal ion binding; platelet-derived growth factor binding;	Platelet activation*; Focal adhesion*; ECM-receptor interaction*; Protein digestion and absorption;
Receptor-type tyrosine-protein phosphatase C (CD45)	external side of plasma membrane; focal adhesion; plasma membrane;	cell surface receptor signaling pathway; negative regulation of cell adhesion involved in substrate-bound cell migration; negative regulation of cytokine-mediated signaling pathway; negative regulation of protein kinase activity; positive regulation of protein kinase activity; protein tyrosine phosphatase activity; release of sequestered calcium ion into cytosol;	protein kinase binding; protein tyrosine phosphatase activity; transmembrane receptor protein tyrosine phosphatase activity;	Cell adhesion molecules (CAMs)*; T cell receptor signaling pathway;
Histone H3.1	extracellular region; membrane nuclear; chromosome; nucleus	blood coagulation; chromatin organization; DNA replication-dependent nucleosome assembly; regulation of gene silencing;	DNA binding;	Systemic lupus erythematosus; Alcoholism; Transcriptional dysregulation in cancer;

Note: *$p < 0.05$ in KEGG pathway analysis; *T2DM: type 2 diabetes mellitus, HC: healthy control.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oxygen binding
peptide disulfide oxidoreductase activity
iron ion binding
phospholipid binding
triose-phosphate isomerase activity
antigen binding
thioredoxin peroxidase activity
phospholipase A2 inhibitor activity

Figure 1. Partial results of the GO analysis are shown. A: Biological process analysis shows that differential genes (proteins) are involved in hydrogen peroxide catabolic process, neutrophil aggregation, and chemokine production. B: According to the cellular components analysis, different proteins are mainly distributed in extracellular region, extracellular vesicular exosome, and plasma membrane. C: Molecular functions of differentially expressed proteins mainly include antioxidant activity, haptoglobin binding, toll-like receptor 4 binding, and phospholipid binding.

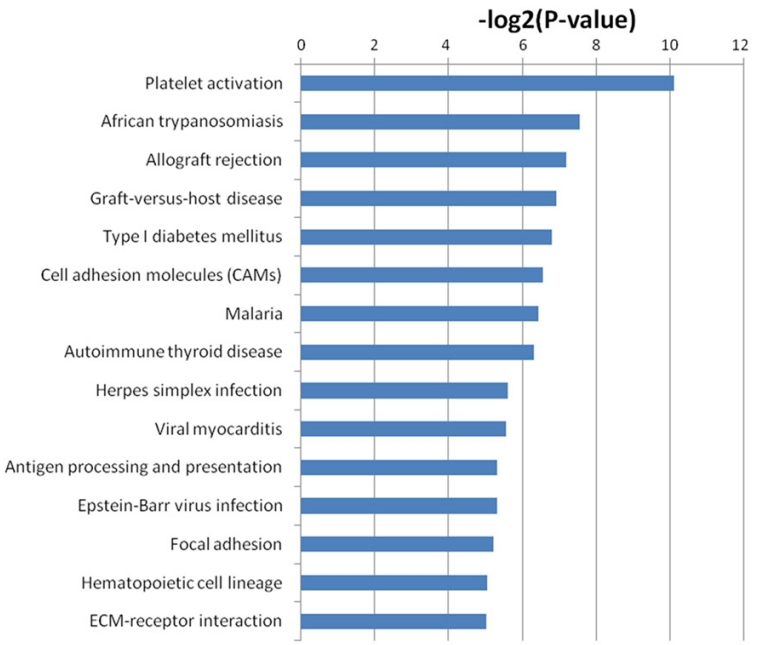

Figure 2. Pathway analysis shows that differentially expressed proteins enrich inplatelet activation, type 1 diabetes mellitus, cell adhesion molecules (CAMs), and focal adhesion.

GO analysis of differentially expressed proteins and pathway analysis

The results of the GO analysis are shown in **Table 3** and **Figure 1A-C**. Some biological processes, such as neutrophil aggregation, chemokine production, platelet activation, and blood coagulation were significantly dysregulated. Accordingly, S100A9, S100A8 (for neutrophil aggregation, chemokine production), RAP1B, CD9, and ITGA2B (for platelet activation) were upregulated in patients with T2DM. Details of the GO analysis and pathway analysis are shown in **Table 3**. As shown in **Table 3** and **Figure 2**, we also observed significant differences in pathways associated with platelet activation, cell adhesion molecules (CAMs), focal adhesion, and ECM-receptor interaction. These results demonstrated that in patients with T2DM, proteins carried by MPs are potentially involved in platelet activation, blood coagulation, and cell adhesion.

Protein network analysis

The differential protein network was established by integrating three types of interactions: enzyme-enzyme relation, indicating two enzymes that catalyze successive reaction steps; protein-protein interaction, such as binding and modification; gene expression interaction, indicating a relation between transcription factors and target genes. Results are shown in **Figure 3A**. **Figure 3B** showed that the *UBC* gene (ubiquitin C) displayed the highest degree of connectivity and interacted with 12 genes with a z-test *p*-value of 0.0017.

Discussion

This study systematically analyzed the differences in plasma MP proteins between patients with newly diagnosed diabetes and healthy controls. Using bioinformatic analyses, we identified biological processes regulated by plasma MPs in patients with diabetes. GO analysis of differentially expressed proteins showed that proteins related to certain biological processes, such as neutrophil accumulation, chemokine production, platelet activation, and blood clotting, were significantly dysregulated in patients.

Expressions of RAP1B, CD9, and CD41 in MPs from patients with diabetes were significantly higher compared with those of the healthy control group, while expression of COL1A1 was lower in the disease group. These four proteins are all related to blood coagulation and platelet activation. CD41, also named platelet glycoprotein IIb, is an important protein implicated in platelet activation and aggregation. PMPs and megakaryocyte-derived MPs were major MPs that express CD41, which has been used as

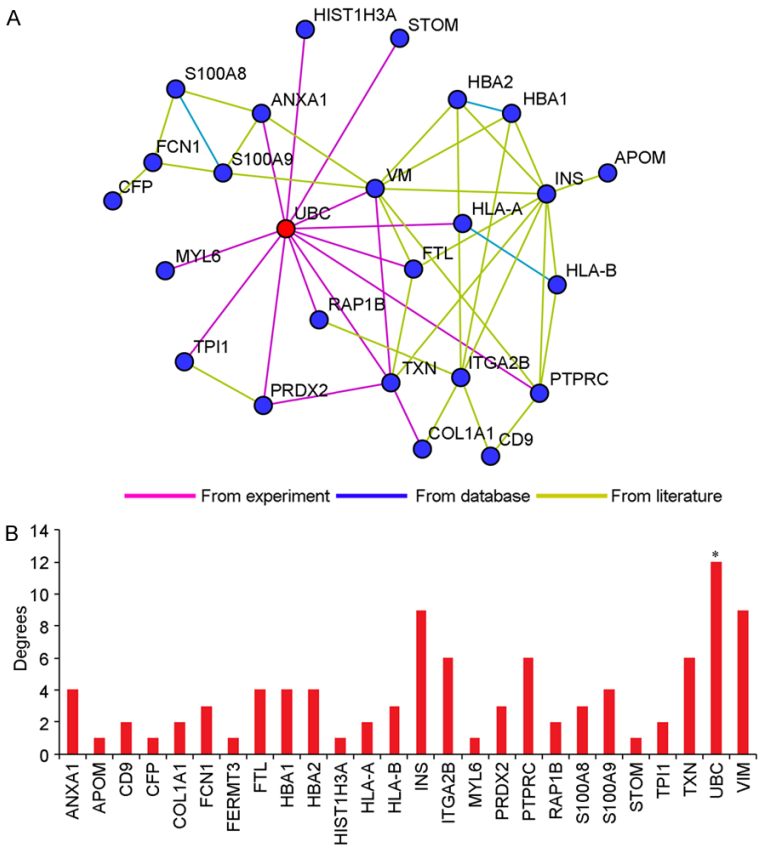

Figure 3. Protein network analysis. A: The differential protein network was established by integrating three types of interaction. B: *UBC* gene had the highest degree of connection and interacted with 12 genes with a z-test *p*-value of 0.0017.

one of the surface antigens to identify PMP in flow cytometric analysis [28]. Previous experiments have shown that PMP concentration in diabetic patients was elevated. Zhang *et al*. reported that PMP concentration was increased in patients with T2DM compared with that in healthy controls, independently of obesity [29]. Salem *et al*. reported that PMP concentrations was elevated in patients with T1DM and that PMP could be considered an early marker of microvascular complications and subclinical atherosclerosis [30]. These results suggested that PMPs could play a role in promoting a hypercoagulable state and arteriosclerosis in diabetic patients. However, the specific proteins involved in promoting these conditions have not been elucidated. CD41 in PMPs have been shown to be involved in platelet aggregation and thrombosis by combining with fibrinogen and vWF, which contributed to pro-coagulant activity of MPs [31]. The adhesion between platelet and endothelial cells, which was the base of leukocyte aggregation in initial inflammation, was enhanced when CD41 adhered to fibrinogen [32]. In our study, CD41 expression in MPs of diabetic patients was 3.5-fold higher compared with that of healthy controls. We speculated that CD41 could participate in arteriosclerosis and promotion of the diabetes-associated hypercoagulable state through these mechanisms.

The expressions of S100A8 and S100A9 in MPs from diabetic patients were 21.9- and 3.6- fold higher, respectively, compared with those in healthy controls. Previous studies have found that S100A8/A9 proteins are closely associated with inflammation, atherosclerosis, diabetic foot, and other complications observed in patients with diabetes. Krisp *et al*. reported that expression of S100A8/A9 increased more than ten-fold in chronic diabetic foot secretions [33]. Compared with individuals that were islet autoantibody (IAA) -positive but without diabetes, expressions of S100A8 and S100A9 were upregulated in patients with T1DM. Especially in diabetic patients with neurological complications, upregulation of S100A8 and S100A9 expressions was statistically significant compared with islet autoantibody-positive subjects [34]. Using an experimental diabetic rat model, researchers found that compared with controls, expressions of S100A8 and S100A9 were upregulated in carotid artery endothelial cells of Zucker obese rats. *In vitro* experiments showed that expression of S100A8/A9 was increased in rat aortic endothelial cells (RAOEC) and in human aortic endothelial cells (HAEC) cultured under high glucose conditions. Interestingly, shRNA-induced S100A8 silencing decreased endothelial cell injury caused by high glucose levels [35]. These findings suggest that S100A8/A9 play an important role in

the pathological processes associated with endothelial dysfunction and other complications in diabetic patients.

The exact mechanism by which activated immune cells and epithelial cells secrete extracellular S100A8/A9 proteins under disease or inflammatory conditions is currently unknown [36]. There were studies suggesting that the process is mediated through the non-Golgi secretory system [37, 38]. There have not been previous reports associating S100A8/A9 with MPs. In our experiments, S100 levels were increased in plasma MPs of diabetic patients. Therefore, we speculate that MPs are potentially involved in diabetes-associated pathological processes by expressing S100A8/A9, which were at least partly secreted by MPs.

Previous studies have confirmed that TF is expressed in MPs, including monocyte-derived MPs, leukocyte-derived MPs, and endothelial cell-derived particles [39, 40]. Expression of Tissue Factor Pathway Inhibitor (TFPI) in MPs was also reported [41, 42]. However, TF and TFPI were not included in the 496 proteins identified in our study. Previous proteomic MP studies about patients with deep venous thrombosis have also failed to detect TF [43]; we speculate that this was because the protein concentration was possibly below the detection threshold.

MPs could have anti-inflammatory, anticoagulant and fibrinolytic effects through expression of TGFb-1, endothelial cell protein C receptor (EPCR), as well as urokinase plasminogen activator (uPA) and its receptors (uPAR). However, there were no significant differences in these proteins and other concerning proteins between diabetic patients and healthy controls in our study, therefore, it seems that the main effects of MPs in diabetes are pro-inflammatory and coagulant. In the future clinical research, we might design some strategies to decrease MP concentration or the concentrations of some MP-enriched proteins, including CD41 and S100, to develop an individualized anticoagulation treatment, and the measurements for MP concentration or the concentrations of some MP-enriched proteins might be used to assess the risk of atherosclerosis and thrombotic events.

In summary, blood MPs from T2DM patients are enriched in proteins (such as S100A8, S100A9, CD41 *et al.*) involved in platelet activation, cell adhesion, and inflammation. microparticles may play a part in hypercoagulable state in patients with diabetes and be associated with the development of diabetic complications.

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Disclosure of conflict of interest

None.

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