World Journal of

Diabetes

World Journal of _Diabetes_

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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

 WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

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ORIGINAL ARTICLE

Case Control Study

Functional annotation and enrichment analysis of differentially expressed serum proteins in patients with type 2 diabetes after dapagliflozin

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Abstract

BACKGROUND

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differen-tially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

AIM

To identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM.

METHODS

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

RESULTS

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin (ITGA2B), and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

CONCLUSION

Dapagliflozin has hypoglycemic effects and regulates the serum expressions of MPO, ITGA2B, and PCX, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

Core Tip: This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with type 2 diabetes mellitus. Changes in blood indexes were examined in 20 patients treated with dapagliflozin for 3 mo. Quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. Dapagliflozin has hypoglycemic effects and regulates the serum expressions of myeloperoxidase, alpha II B integrin, and podocalyxin, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

INTRODUCTION

With the aging global population and the increase in the prevalence of obesity, it is expected that type 2 diabetes mellitus (T2DM) will affect more than 381.8 million people worldwide in 2035[1]. In the United States alone, T2DM is projected to affect nearly one in three people by 2050[2]. T2DM manifests through the development of fasting and postprandial hyperglycemia, which is the primary contributor to numerous life-threatening complications and co-morbidities[3,4]. These alarming projections suggest an urgent need for the development and implementation of novel preventative and treatment strategies to fight the rise in T2DM prevalence worldwide[4]. Unfortunately, despite the best care, only 50% of patients with T2DM can control their blood glucose levels[5-7].

In recent years, the participation of the kidney in glucose metabolism and homeostasis attracted much attention, and this participation has begun to be explored in clinical studies[8]. The mechanisms mainly include the renal tubular reabsorption of glucose, largely dependent on the expression of sodium-glucose co-transporter 2 (SGLT-2) localized at the proximal small tubules S1 and S2[9]. Dapagliflozin is a selective inhibitor of SGLT-2, reducing the reabsorption of SGLT-2 receptor glucose in renal tubular epithelial cells and allowing excess glucose to be excreted in the urine[10]. Thus, the insulin sensitivity of the liver and peripheral tissues can be improved, and the hepatic glucose output can return to the normal range[10,11]. Furthermore, many investigators proposed that SGLT-2 inhibitors have renal and cardiovascular protective roles in addition to their glucose-lowering effects[12-14]. Thereby, dapagliflozin is recommended for T2DM patients[15].

Several studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection[13-15]. Powell *et al*[16] suggested that SGLT2 inhibitor alone could reduce hemoglobin A1c (HbA1c) by 0.37%-1.16%. Several randomized, double-blind, controlled trials have confirmed that dapagliflozin can significantly reduce HbA1c (by up to 1.16%) and blood glucose and that the efficacy of dapagliflozin (10 mg) in reducing HbA1c is

comparable to that of metformin sustained-release tablets (2000 mg)[17,18]. Ji *et al*[19] proposed that SGLT2 inhibitors can reduce blood glucose and hyperglycemic toxicity by reducing the stress reaction in the endoplasmic reticulum and reducing the beta-cell apoptosis caused by glycolipid toxicity, thereby improving insulin secretion. They also proposed that the damaged function of the beta-cells will also be improved[19]. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially abundant proteins in serum of T2DM patients have not been intensively explored so far.

Proteomics techniques have attracted more and more attention since these techniques can be used to identify the expression of differential proteins in cells and tissues of patients with T2DM[20]. Label-free quantitative proteomics can replace or supplement the traditional two-way gel electrophoresis approach, and it has become an important mass spectrometry method in recent years because of its powerful protein identification ability[21]. The changes in protein abundances of different samples can be analyzed by comparing mass spectrometry frequency or mass spectrometry peak intensity[22]. Without expensive isotope labeling and with liquid chromatography-mass spectrometry analysis of peptides obtained from protein enzymatic digestion, the relative abundance of the corresponding proteins can be quantified according to the signal strength of the peptide segments[22]. In this study, using this technique, we explored the differentially abundant proteins in the serum samples of T2DM patients before and after dapagliflozin treatments and conducted functional annotation analysis of the differential proteins. In addition, the levels of some differential proteins in serum samples were validated, and the correlations between their levels and clinical indexes were analyzed.

MATERIALS AND METHODS

Patients

Forty-six patients with T2DM were enrolled at the Department of Endocrinology, Affiliated Hospital of Inner Mongolia Medical University, between January 1, 2017 and December 1, 2018. There were 26 participants in the dapagliflozin group, and 20 participants who controlled their blood glucose levels through diet and exercise alone were enrolled in the control group during the same period (these patients did not receive drugs as per their own choice but were still followed in case diet and exercise became insufficient to control their T2DM). All participants met the diagnostic criteria for T2DM according to the World Health Organization diagnostic criteria for type 2 diabetes in 2017[23] The course of T2DM was < 5 years. All participants were 25-55 years of age, and they did not have a blood relationship. All participants had complete physical examination data and other disease information. The exclusion criteria were: (1) Acute or chronic complications of T2DM; (2) Cardiovascular and cerebrovascular diseases such as hypertension or coronary heart disease; (3) Other antidiabetic, antihypertensive, or lipid-regulating drugs; (4) Ketoacidosis, genital fungal infection, or urinary tract infection; (5) Recent history of surgery and trauma; or (6) Infectious diseases, tumors, hematological diseases, severe impairment of heart, liver and kidney functions, autoimmune diseases and other endocrine and metabolic diseases.

This study was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. All participants signed the informed consent form before the start of the study.

Study groups

For the participants in the dapagliflozin group, their HbA1c levels ranged from 7.0% to 10.0%. If the participants were taking other hypoglycemic drugs, the participants were admitted to the group after a wash-out period of 1-2 wk. The participants were instructed to adhere strictly to diet and exercise during the wash-out period. After wash-out, the participants were treated with dapagliflozin alone after clinical evaluation. The participants without the need for wash-out were treated with dapagliflozin directly after clinical evaluation. In order to avoid complications such as urinary tract infection and ketoacidosis, the participants were advised to drink more water during the study period, which was also conducive to the excretion of urinary glucose. No other drugs, such as lipid-lowering drugs, were allowed during the study. After 3 mo of treatment, the participants with HbA1c < 7.0% were considered as reaching the HbA1c target level. Six patients in the dapagliflozin group dropped out due to self-discontinuation, failure to meet the HbA1c target, or refusal to be reviewed after 3 mo. The average age of the 20 eligible patients in this group was 39.8 ± 5.1 years.

For the participants in the control group, their HbA1c levels reached the target level (HbA1c < 7.0%). These participants did not take any hypoglycemic drugs or other drugs within 3 mo before sampling, and they controlled their blood glucose levels only through diet and exercise. There were no dropouts. Their average age was 39.8 ± 6.0 years.

All participants received diet and exercise guidance. The dietary guidance referred to the balanced dietary plan recommended by the Chinese Guidelines for the Prevention and Treatment of Type 2 Diabetes (2013 edition) and suggested 1/3 structure of the energy intake ratio of three meals or 2:2:1 distribution. According to the "Guidelines for Exercise in Type 2 Diabetes Mellitus", the exercise program was mainly composed of low-intensity aerobic exercise such as walking, swimming, cycling,

etc. After a meal, the participants were required to exercise for about 30 min and 3-5 times per week. During the study, no adverse reactions such as urinary tract infection, ketoacidosis, or other adverse reactions occurred.

Data collection

Fasting venous blood was drawn from the participants in the morning. Blood samples were subjected to centrifugation. The supernatant was stored at -80 °C until analysis. Blood samples were also sent to the laboratory professionals of Affiliated Hospital of Inner Mongolia Medical University for quantification of indicators, including retinol-binding protein 4 (RBP4), fasting blood glucose (FBG), fasting C-peptide (FCP), fasting plasma insulin level (FINS), HbA1c, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol(LDL-C), triglycerides(TG), total cholesterol (TC), apolipoprotein A1 (ApoA1), ApoB100, homocysteine (HCY), non-esterified fatty acids (NEFA), C-reactive protein. These indicators were determined using Roche Cobas 8000 and Aikelai H-8160 automatic biochemical analyzers in the Laboratory Department of Affiliated Hospital of Inner Mongolia Medical University. Quality control was carried out by the professionals in the Laboratory Department. Insulin resistance indexes, including updated homeostasis model assessment-insulin resistance (HOMA2-IR), updated homeostasis model assessment-beta cell (HOMA2-B), and updated homeostasis model assessment-insulin sensitivity (HOMA2-S%), were obtained using the HOMA Calculator v2.2.3 software based on FPG, C-peptide, and islet function. The non-HDL-C value was calculated by subtracting the HDL-C value from the TC value.

Label-free proteomics

Serum samples from five patients in the dapagliflozin group before and after dapagliflozin treatments for 3 mo were selected for the exploratory proteomics study. Individual differences such as diabetes duration, blood glucose levels, and body mass index (BMI) were minimized to ensure the reliability of the results. High-abundance proteins were removed from the samples using Pierce™ Top 12 Abundant Protein Depletion Spin Columns Kit following the manufacturer's instruction, and total protein concentration was determined by a BCA kit (Pierce). The proteins were resolved by 10% SDS-PAGE and visualized with Coomassie Blue staining. Each lane was excised and cut into bands containing proteins with different molecular weights. Each gel fraction was subjected to in-gel tryptic digestion. Peptide segments were classified by high-pH reverse HPLC with Agilent 300 Extend C18 (5 µm diameter, 4.6 mm inner diameter, 250 mm long).

   Digested peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS using a ThermoScientific Easy nLC-1000 in tandem with a Q-Exactive Orbitrap mass spectrometer. Each sample (5 µL) was resolved using a 60 min gradient (Buffer A: 0.1 formic acid in 2% acetonitrile; Buffer B: 0.1% formic acid in 90% acetonitrile) on a 2 cm Acclaim 100 PepMap Nanoviper C18 trapping column in tandem with a Thermo EASY-Spray column (PepMap® RSLC, C18, 3 µm, 100 A, 75 µm × 150 mm).

Secondary mass spectrometry data were retrieved using Maxquant (v1.5.2.8).

The database was SwissProt Human (20387 sequences), which included the counter-library in calculating the false positive rate (FDR) caused by randomly matching. The common contamination library was considered to eliminate contaminations. The enzyme cutting method was set to trypsin/P; the number of leakage sites was set to 2. The first-stage mother ion mass error tolerance to the first search and main search was set to 20 ppm and 5 ppm. The error tolerance of the secondary fragment ions mass was set as 0.02 Da. The alkylation of cysteine was set as fixed modification, while the oxidation of methionine and acetylation of protein N-terminus were set as alternative modifications. The FDR of protein identification and PSM identification was set as 1%.

   For data-dependent analysis, full scans were acquired at a 35000 resolution range of 400-200 m/z, while a 17500 resolution was used for MS/MS scans. Only the top 15 ions with +2 and +3 charges were selected for MS/MS with 10-s dynamic exclusion to prevent continuous reanalysis of abundant peptides. Following data acquisition, raw data files were compiled for each gel lane and searched with Proteome Discoverer 1.4's SEQUEST search algorithm using the reviewed, non-redundant homo sapiens complete proteome retrieved from UniProtKB.

Bioinformatics analysis

For protein functional enrichment evaluation, Gene Ontology (GO) and pathway enrichment analyses were carried out. GO annotations (including biological process (BP), molecular function (MF), and cellular component) were performed using the InterProScan database (v.5.14–53.0 http://www. ebi.ac.uk/interpro/) and according to an existing report[16]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis[16]. KAAS (v.2.0 http://www.genome.jp/kaas-bin/kaas_main) and KEGG mapper (v.2.5 http://www.kegg. jp/kegg/mapper.html) are the main tools used with the KEGG database. Prediction of subcellular localization was carried out using the wolfpsort software (v.0.2 http://www.genscript.com/ psort/wolf_psort.html). Cluster memberships were visualized using the heat map drawn by the function heatmap.2 in the R package gplots. For each annotation, Fisher's exact test was applied to

compare the enrichment of the differentially abundant protein against all identified proteins, and a *P* value < 0.05 was considered significant.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was performed for the quantification of alpha II B integrin, myeloperoxidase (MPO), and podocalyxin (PCX) using the kits from Bio-Rad Laboratories, United States (CSB-EL0118644HU for integrin, CSB-E08721h for MPO, and CSB-E09891h for PCX). All reagents were equilibrated to room temperature (18-25 °C) for at least 30 min and prepared according to the instructions of the relevant kits. The optical density of each well was measured sequentially at 450 nm with an enzyme-labeled instrument within 5 min after the termination of the reaction.

Statistical analysis

The statistical methods of this study were reviewed by Xue-Mei Wang. The quantitative data were described using means ± SD. The paired *t*-test was used to compare the indexes obeying the normal distribution before and after treatment. Variance analysis was used to compare the indexes obeying normal distribution, and a nonparametric rank-sum test was used to compare the indexes not obeying normal distribution. Pearson correlation analysis was used for the two variables obeying normal distribution, and Spearman grade correlation analysis was used for the variables not obeying normal distribution. The chi-square test was used to analyze the categorical data. For the enrichment test, Fisher's exact test was used to show the functional classification and pathway of significant enrichment of differentially expressed proteins ($P < 0.05$) using bubble diagrams. Two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS 22.0.

RESULTS

Dapagliflozin significantly improved the clinical manifestations in patients with T2DM

Twenty participants were treated from January 1, 2017 to December 1, 2018. Table 1 presents the characteristics of the participants in the dapagliflozin (after treatment) and control groups. Compared with the controls, the patients under dapagliflozin treatment had slightly higher FBP, lower FINS, and lower HOMA2-B, but no significant differences in HOMA2-S% and HOMA2-IR. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels. Table 2 presents the comparison before/after dapagliflozin. Compared with baseline, the participants after dapagliflozin treatment had significantly decreased BMI, waist circumference, and waist-hip ratio ($P < 0.05$) (Table 2). In addition, their blood glucose-related indexes, including HbA1c, FCP, FINS, FBP, and HOMA2-IR, also demonstrated significantly decreased levels ($P < 0.05$), while HOMA2-S% and HOMA2-B significantly increased ($P < 0.05$) (Table 2). Regarding the lipid metabolism-related indexes, non-HDL-C decreased ($P < 0.05$), while ApoA1 increased ($P < 0.05$) (Table 2). Moreover, RBP, HCY, and NEFA were significantly decreased levels after treatment ($P < 0.05$) (Table 2). Taken together, dapagliflozin substantially improved the clinical manifestation of patients with T2DM.

Identification of differentially abundant proteins by label-free proteomic

Next, we performed a label-free proteomics analysis of serum samples from five patients before and after dapagliflozin treatments. A total of 665007 sary spectra were obtained through mass spectrometry. A total of 4732 peptides were identified through spectral analysis, of which 3389 were specific peptides. We identified 534 proteins, all of which could be quantified (quantitative protein means that at least one comparison group has quantitative information). The evaluation of quantitative proteome reproducibility was performed by relative standard deviation (RSD) analysis, which showed that the biological replicates were statistically consistent (Figure 1A). The heatmap of the Pearson correlation coefficients from all quantified proteins between each pair of samples demonstrated that the linear correlation degree of the two metrics was not high (Figure 1B). Notably, 19 proteins exhibited significant differences before and after dapagliflozin treatments ($P < 0.05$; FC > 1.5), of which 18 were downregulated, and one was upregulated (Figure 1C).

In order to determine the characteristics of the differentially expressed proteins, we annotated the subcellular localization, Clusters of Orthologous Groups of proteins (KOG), Gene Ontology (GO), and KEGG pathway of the 19 differentially expressed proteins. The annotation of the subcellular localization showed that 31.6% of all differentially expressed proteins were localized to the cytoplasm, 26.3% to the extracellular space, 15.8% to the nucleus, 10.5% to the plasma membrane, 5.3% to the mitochondria, 5.3% to the endoplasmic reticulum, and 3.8% to the cytoskeleton (Figure 2A). In the GO analysis, the GO annotations are divided into three categories (biological process, cellular component, and molecular function), explaining the biological role of proteins from different perspectives. In the biological process classification of GO, the cellular process had the largest proportion, followed by the single-organism process and biological regulation. In the cellular composition classification, differentially expressed proteins were mostly expressed in organelles, followed by cells and extracellular regions. In the

Table 1 Analysis of clinical indexes between the dapagliflozin and control groups in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Dapagliflozin	Control	Wilcoxon W	*P* value
Age	39.80 ± 5.136	39.75 ± 5.990	397.000	0.724
Smoking history	16.55 ± 9.720	10.25 ±11.639	353.000	0.111
Height (m)	1.7670 ± 0.07255	1.7505 ± 0.090	389.000	0.569
Weight (kg)	74.950 ± 8.9176	72.525 ± 6.6718	381.500	0.440
BMI (kg/m^2)	24.3695 ± 1.82674	24.014 ± 1.6715	385.00	0.499
Waist circumference (cm)	96.350 ± 2.3402	93.525 ± 5.7502	325.000	0.021
Hip circumference (cm)	96.975 ± 2.4413	99.225 ± 6.4062	398.500	0.755
Waist-hip ratio	0.9937 ± 0.0132391	0.9494 ± 0.04815	308.000	0.006
SBP (mmHg)	124.80 ± 5.297	120.40 ± 5.423	323.500	0.018
DBP (mmHg)	78.85 ± 5.724	77.95 ± 5.969	394.500	0.671
Diabetes course (yr)	2.575 ± 1.2169	2.825 ± 1.8229	396.500	0.713
TG	1.884 ± 1.2172	0.979 ± 0.2376	1.2172203	0.002
LDL	2.8585 ± 1.0440	1.5975 ± 0.5079	1.0439867	< 0.001
HDL	1.0855 ± 0.2457	1.023 ± 0.1246	0.2457100	0.560
APOA1	1.3630 ± 0.2022	1.2625 ± 0.1882	0.2022271	0.010
APOB100	0.9425 ± 0.2651	1.0345 ± 0.1521	0.2651092	0.424
CRP	1.311 ± 0.9305	1.1635 ± 0.5462	0.9305511	0.967
HbA1c	6.7500 ± 0.6863	6.3700 ± 0.3827	0.6863327	0.032
THCY	12.030 ± 2.1451	12.4215 ± 1.2702	2.1450899	0.213
RBP4	37.150 ± 5.6033	35.800 ± 4.3842	5.6033	0.446
NEFA	0.8245 ± 0.5849	1.026 ± 0.1099	0.5849019	< 0.001
FCP	2.2770 ± 0.7903	2.231 ± 0.4151	0.7903104	0.525
TC	4.1950 ± 0.8378	3.7875 ± 0.4683	344.500	0.076
IR	2.7120 ± 1.7245	2.8440 ± .8424	358.500	0.163
FINS	8.4905 ± 4.4824	10.313 ± 3.2099	328.500	0.027
FBP	6.80 ± 1.105	6.15 ± 0.671	335.500	0.034
Non-HDL	3.05 ± 0.826	2.80 ± 0.410	364.000	0.142
HOMA2- insulin	75.6150 ± 20.339	89.460 ± 18.246	324.000	0.020
HOMA2-S%	61.6750 ± 21.1603	60.440 ± 14.789	400.000	0.787
HOMA2-IR	1.8185 ± 0.7285	1.7210 ± 0.3760	399.000	0.766

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

molecular function classification, binding molecules accounted for the largest proportion (Figure 2B). In terms of subcellular structure location, these differentially expressed proteins were mainly located in the cytoplasm, extracellular, nucleus, and plasma membrane. COG/KOG functional classification revealed that most of these differentially expressed proteins played a role in the cytoskeleton, extracellular structures, intracellular trafficking, secretion, vesicular transport, carbohydrate transport, and metabolism, as general function predictions (Figure 2C).

Table 2 Analysis of clinical indexes before and after dapagliflozin treatment in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Before dapagliflozin	After dapagliflozin	Wilcoxon W	*P* value
Weight (kg)	80.600 ± 10.4549	74.950 ± 8.9176	351.000	0.110
BMI	26.1875 ± 2.1889	24.3695 ± 1.8267	313.000	0.009
Waist circumference (cm)	98.300 ± 2.4570	96.350 ± 2.3402	310.50	0.007
Hip circumference (cm)	97.425 ± 3.1131	96.975 ± 2.4413	395.000	0.684
Waist-hip ratio	1.009350 ± 0.0172	0.9937 ± 0.0132	311.500	0.007
TG	2.3020 ± 1.7483	1.884 ± 1.2172	387.000	0.534
LDL	2.9310 ± 0.9873	2.8585 ± 1.0440	405.000	0.892
HDL	1.0525 ± 0.2722	1.0855 ± 0.2457	381.000	0.432
APOA1	1.2450 ± 0.2176	1.3630 ± 0.2022	334.500	0.041
APOB100	1.0345 ± 0.3400	0.9425 ± 0.2651	392.000	0.626
CRP	1.7090 ± 1.1454	1.311 ± 0.9305	349.000	0.098
HbA1c	8.0550 ± 1.0842	6.7500 ± 0.6863	277.000	< 0.001
THCY	14.6850 ± 3.0387	12.030 ± 2.1451	311.500	0.008
RBP4	43.310 ± 8.8547	37.150 ± 5.6033	315.500	0.010
NEFA	0.4525 ± 0.3246	0.8245 ± 0.5849	321.500	0.017
FCP	2.7980 ± 0.9510	2.2770 ± 0.7903	337.000	0.048
TC	4.6830 ± 1.1643	4.1950 ± 0.8378	347.500	0.091
IR	5.7385 ± 3.2238	2.7120 ± 1.7245	266.000	< 0.001
FINS	13.3500 ± 5.7057	8.4905 ± 4.4824	289.000	0.001
FBP	9.60 ± 2.437	6.80 ± 1.105	245.000	< 0.001
Non-HDL	3.70 ± 1.129	3.05 ± 0.826	337.000	0.037
HOMA2-insulin	53.4450 ± 21.1716	75.6150 ± 20.339	305.000	0.005
HOMA2-S%	44.7950 ± 16.2213	61.6750 ± 21.1603	310.500	0.007
HOMA2-IR	2.5185 ± 0.9686	1.8185 ± 0.7285	313.000	0.009

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

Functional enrichment analysis of differentially abundant proteins

For the annotation of all identified proteins and the screening of differentially expressed proteins, the differentially expressed proteins in our comparison groups were enriched at three levels: GO classi-fication, KEGG pathway, and protein domains. The purpose was to determine whether the differentially expressed proteins had significant enrichment trends in some functional types. GO functional classi-fication found that the homotypic intercellular adhesion pathway, lymphocyte activation pathway, and actin cytoskeleton regulation pathway were highly enriched in the classification of cell composition (Figure 3A). The pathways such as Cortex and Cytoskeleton were enriched significantly in the KEGG pathway (Figure 3B). In the function of molecular biology, the small GTP enzyme binding pathway and the Ras GTPase binding pathway were enriched significantly (Figure 3C).

We found that these proteins, such as actin, alpha II beta integrin, MPO, and PCX, were closely related by the KEGG pathway. Therefore, we performed a protein-protein interaction analysis and identified a network among the integrin protein, MPO, and PCX (Figure 4). The integrin protein was used to modulate the production of cytoplasmic actin by participating in the PKC and the FAK pathway to function on actin filament and vinculin. On the other hand, by participating in the PKC and FAK pathway to function on Rac family proteins, the integrin protein modulates the MAPK signal through the PAK pathway to eventually manipulate gene expression. Besides, the integrin protein can also act
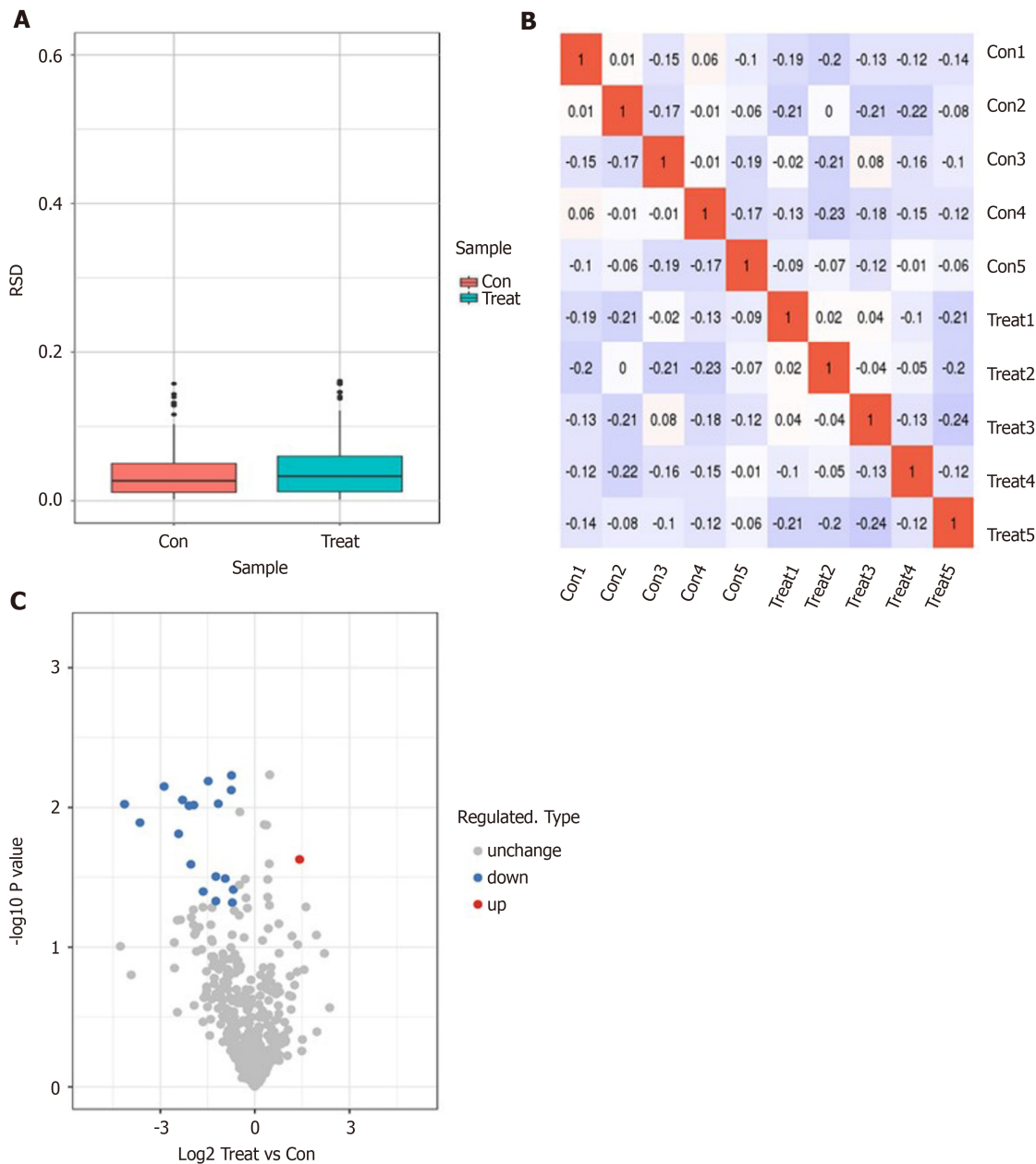
Figure 1 Identification of dapagliflozin treatment-associated differentially abundant proteins in serum samples from type 2 diabetic patients by label-free proteomics. A: Box plot of relative standard deviation (RSD) distribution of repeated samples using quantified proteins. A box plot drawn by the RSD of the quantitative protein value between replicate samples is shown. The smaller the overall RSD value is, the better the quantitative repeatability is; B: Heatmap of Pearson correlation coefficients from all quantified proteins between each pair of samples is shown; C: The volcano plot demonstrated differentially expressed proteins. The horizontal axis is the relative quantitative value of the protein after Log2 Logarithmic conversion, and the vertical axis is the value of the difference significance test *P* value after -Log10 Logarithmic conversion. The red dots in the figure indicate proteins with significantly differentially upregulated expression, and blue dots indicate proteins with significantly differentially down-regulated expression.

on the PI3K pathway through the SRC family, affecting Notch signaling pathway. The cytoplasmic actin is involved in the behavior of phagolysosome with coronin, and the MPO production is increased during the formation of autophagolysosome. PCX protein and actin are involved in regulating the actin skeleton, and the Notch signaling pathway also participates in this process (Figure 4).

Validation of three differentially expressed proteins and their correlations with clinical indexes

Based on the literature and our analysis of the biological function of candidate proteins that might be closely related to diabetes mellitus, three differentially expressed proteins (including MPO, alpha II B integrin, and PCX) were validated by ELISA. The results showed that the expression of MPO and alpha II B integrin protein in serum samples was significantly downregulated (*P* < 0.05), and the PCX protein levels were significantly upregulated (*P* < 0.05) after dapagliflozin treatment (Figure 5A). In addition, compared with the control group, the dapagliflozin group also had a significantly downregulated expression of MPO after treatment (*P* < 0.05) (Supplementary Figure 1). We also conducted a correlation

A

cytoplasm(31.58%)
extracellular (26.32%)
nucleus (15.79%)
plasma membrane (10.53%)
endoplasmic reticulum (5.26%)
cytoskeleton (5.26%)
mitochondria (5.26%)

B

C

[G] Carbohydrate transport and metabolism [W] Extracellular structures
[R] General function prediction only [Z] Cytoskeleton
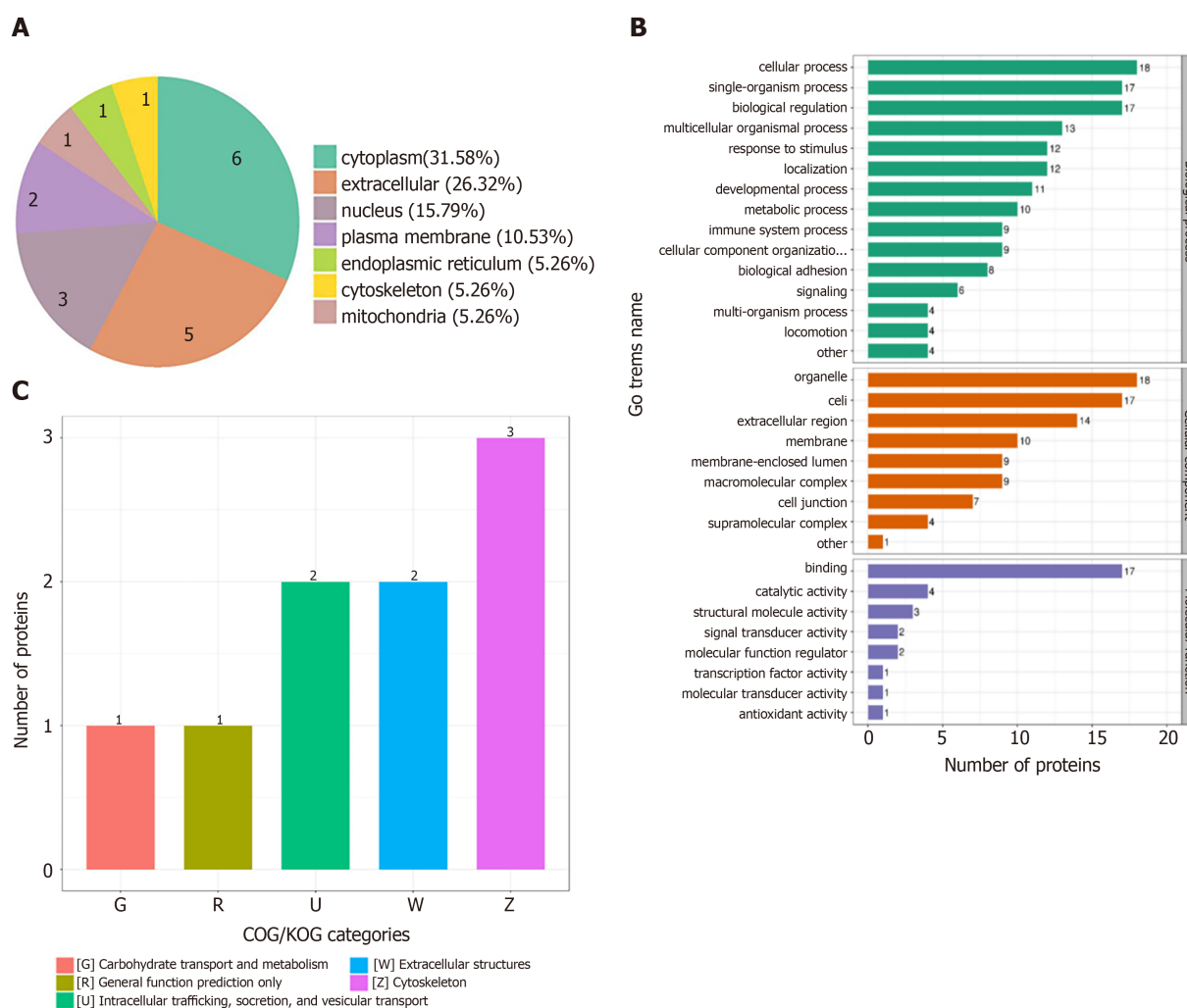[U] Intracellular trafficking, socretion, and vesicular transport

Figure 2 Annotation analysis of 19 differentially expressed proteins by subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations. A: Subcellular localization chart of differentially expressed proteins; B: Statistical distribution chart of differentially expressed proteins under each Gene Ontology category (2nd Level); C: EuKaryotic Orthologous Groups functional classification chart of differentially expressed proteins.

analysis between the expression levels of these three differentially expressed proteins and clinical indexes (Supplementary Table 1). We found that the serum alpha II B integrin protein levels were positively correlated with THCY (homocysteine) and FCP ($P < 0.05$, Figure 5B), while that of PCX was positively correlated with HOMA2-B and negatively correlated with HbAc1 and FBP ($P < 0.05$, Figure 5C). In addition, the serum protein levels of MCP were positively correlated with multiple parameters, including HbAc1, RBP, FCP, FINS, N-HDL, and HOMA2-IR, and negatively correlated with HOMA2-S% ($P < 0.05$, Figure 5D).

DISCUSSION

The SGLT-2 inhibitor dapagliflozin, as a new hypoglycemic drug, plays a role in lowering blood glucose by reducing the reabsorption of SGLT2 receptor glucose in renal tubular epithelial cells in patients with T2DM. Here, we performed label-free quantitative proteomics analysis of serum samples in patients before and after dapagliflozin treatments and identified differentially expressed proteins associated with dapagliflozin treatment. Notably, our function annotation and enrichment analysis suggested that three differential proteins (including αIIβ integrin, MPO, and PCX) potentially contribute to the renal and cardiovascular protective roles of dapagliflozin through participating in the regulation of multiple pathways. Furthermore, the serum differential expressions of these proteins were validated by ELISA, and their levels were correlated with some clinical indexes in patients with T2DM.

This study used dapagliflozin as the representative drug for SGLT-2 inhibitors. We found that the related indexes of islet function, such as FBG, HbA1c, FCP, FINS, and HOMA2-IR, decreased after dapagliflozin treatment. The lipid metabolism of T2DM patients was significantly improved after dapagliflozin treatment, as evidenced particularly by decreased non-HDL-C and increased ApoA1
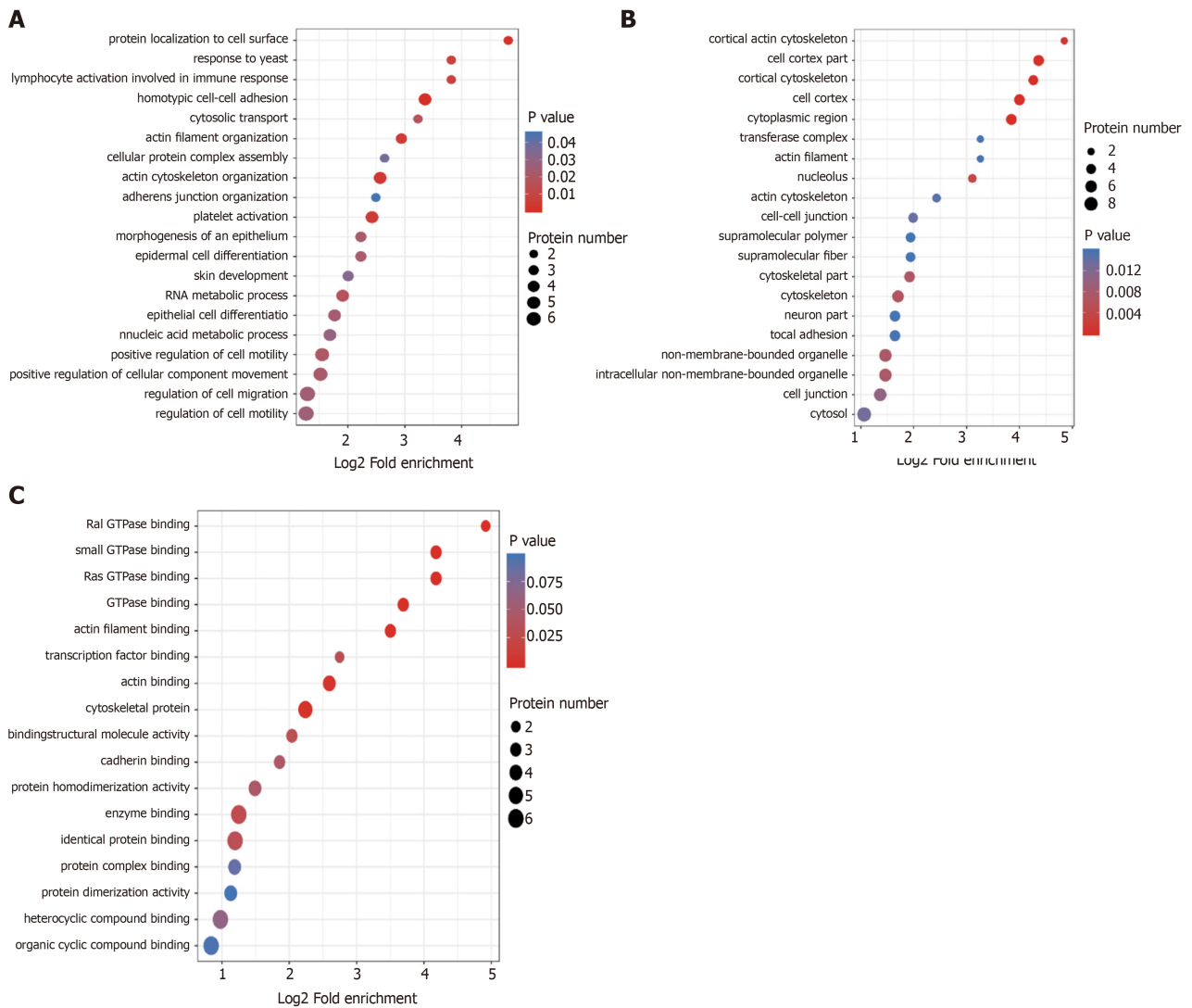
Figure 3 Gene Ontology enrichment bubble plots of differentially expressed proteins in three categories. A-C: The Gene Ontology enrichment bubble plots in the categories of biological process (A), cellular component (B), and molecular function (C) are shown. The bubble chart shows the results of the top 20 categories with the most significant enrichment. In the bubble chart, the vertical axis is the function classification or pathway, and the horizontal axis is the value after Log2 conversion of the ratio of the differential protein in the functional type compared to the ratio of the identified protein. The circle's color indicates the *P* value of enrichment significance, and the size of the circle indicates the number of differential proteins in the functional class or pathway.

Levels. Compared with baseline, the participants after dapagliflozin administration had significantly decreased BMI and hip circumference. We also found that RBP4, NEFA, and HCY were decreased after treatment with dapagliflozin. The controls were patients with mild T2DM in whom diet and exercise were sufficient to control their condition. Of note, dapagliflozin decreased FBG from 9.60 ± 2.44 to 6.80 ± 1.11 mmol/L, close to the value in controls (6.15 ± 0.67 mmol/L). Dapagliflozin also decreased FINS to lower levels than in controls, but HOMA2-B was lower than in controls, while there were no significant differences in HOMA2-S% and HOMA2-IR between the two groups. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels, also supporting the benefits of dapagliflozin. These results support the known effects of dapagliflozin in patients with T2DM[15]. RBP4, as a fat-derived factor, is closely related to obesity, insulin resistance, and other diseases[24]. Ost *et al*[25] found that RBP4 can prevent insulin-stimulated serine phosphorylation at position 307 of IRS1 by interfering with RBP4 and its antibodies in primitive adipocytes and correspondingly increasing the effective concentration of IRS1 tyrosine phosphorylation by half, as well as preventing the phosphorylation of ERK1/2. Therefore, it can be concluded that RBP4 might interfere with the Ras/MAPK signal of the insulin receptor by interfering with the phosphorylation of ERK1/2, thus participating in insulin resistance. In this study, after label-free quantitative proteomics, we also found that the differentially expressed proteins were significantly enriched in the Ras GTPase pathway. Since these two metabolic pathways share a common pathway, whether dapagliflozin could play a role through this pathway and the mechanism of RBP4 in dapagliflozin effectiveness need to be further studied.
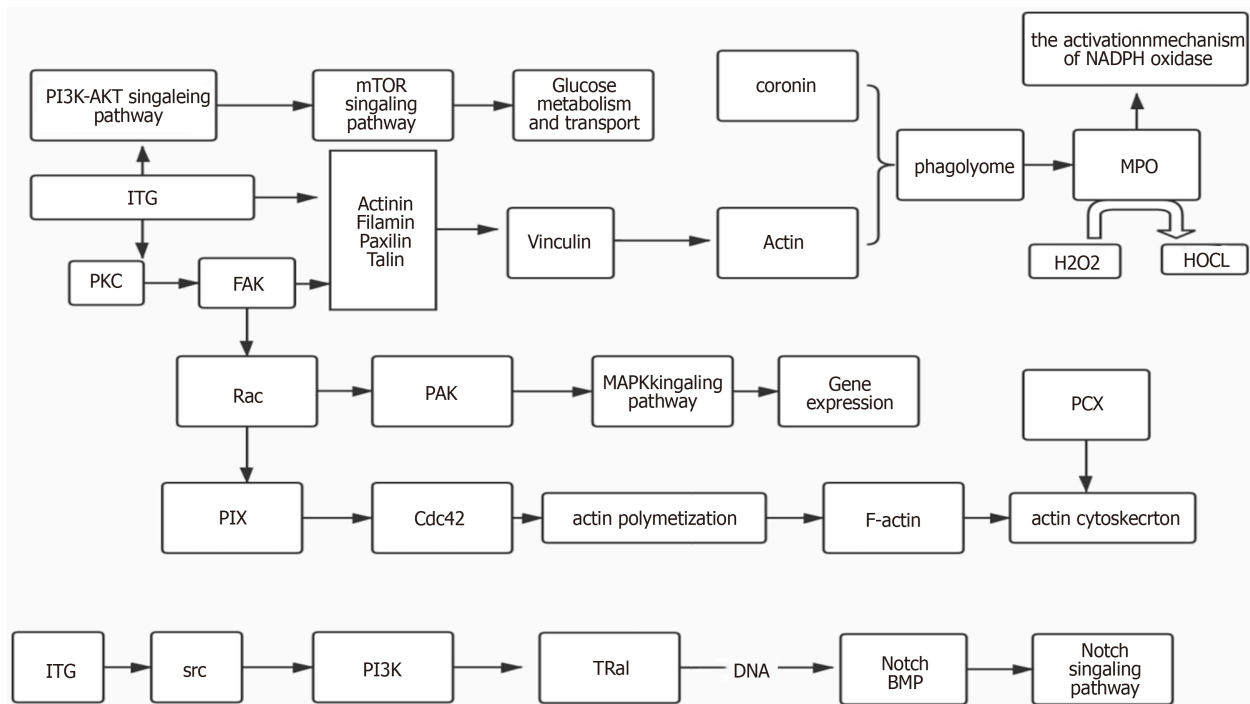
Figure 4 Treemap chart shows the interaction among differentially expressed proteins. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis identified a protein-protein interaction network among the integrin protein, myeloperoxidase, and podocalyxin.

Our comparative analysis with non-standard quantification demonstrated that 18 proteins were downregulated, and one was upregulated in serum samples of patients after dapagliflozin treatment. After consulting the literature, three candidate differential proteins closely related to T2DM were selected for ELISA validation. The differential expressions of these proteins were validated using serum samples from all the patients before and after dapagliflozin treatments. Notably, the correlation analysis suggested that the serum MPO protein levels were positively correlated with multiple clinical indexes, and the alpha II B protein levels were positively correlated with THCY and FCP levels. In addition, the serum PCX levels were negatively correlated with HOMA2-B and positively correlated with HbA1c and FBP. Based on these results and the literature, these three differential proteins might contribute to the beneficial roles of dapagliflozin in treating T2DM patients. Of note, PCX is the main surface antigen of podocytes and is normally expressed in renal podocytes, endothelial cells, and vascular endothelial cells and participates in maintaining the vascular endothelial cell barrier and reducing vascular inflammation. High glucose levels downregulate the expression of PCX in cultured podocytes *via* ERK1/2 MAPKs and inhibit the expression of PCX protein and mRNA by WT1 tumor protein and advanced glycation end-products[26], possibly resulting in the reduction of PCX in the blood. Second, we agree that integrins are cellular proteins that would not be expected to be found in circulation. Still, this study was not designed to determine the source of these proteins in the plasma. On the other hand, numerous studies report serum/plasma levels of various integrins as markers of diseases[27-29]. It could be hypothesized that the systemic inflammatory condition observed in T2DM increases cell death, releasing those proteins in circulation, but the present study cannot provide an answer regarding that point. Future studies will have to examine that specifically.

MPO is a heme enzyme and is the major protein in neutrophils and, to a lesser extent, in monocytes. MPO uses H_2O_2 to generate HOCl, a potent bactericidal agent, generating ROS[30]. MPO plays an essential part in the innate immune system by catalyzing the production of HOCl[31], but MPO has also been implicated as a very harmful agent in an increasing number of inflammatory-mediated disorders [32]. It has been reported that MPO is related to insulin resistance and inflammation parameters in overweight subjects with first-degree relatives of T2DM[31]. In addition, plasma MPO levels were positively correlated with the degree of coronary artery stenosis in T2DM patients, and increasing blood glucose might amplify the association between MPO and coronary artery disease[33]. Patients with uremic diabetic nephropathy with a low MPO level might be at a lower risk for any cardiac event than uremic patients with high MPO levels, suggesting that MPO might be a biomarker to predict coronary events in diabetic patients end-stage renal disease[34]. In this study, MPO levels in the dapagliflozin group decreased after treatment, related to the improvement of blood glucose control and inflammatory oxidation. Since our correlation analysis suggested that MPO was positively correlated with indexes including RBP, FCP, FINS, N-HDL, and HOMA2-IR, MPO very likely participated in oxidative stress regulation and acute, chronic inflammation of T2DM through a certain metabolic pathway. However, the specific mechanisms of MPO in contributing to the beneficial roles of dapagliflozin are still under
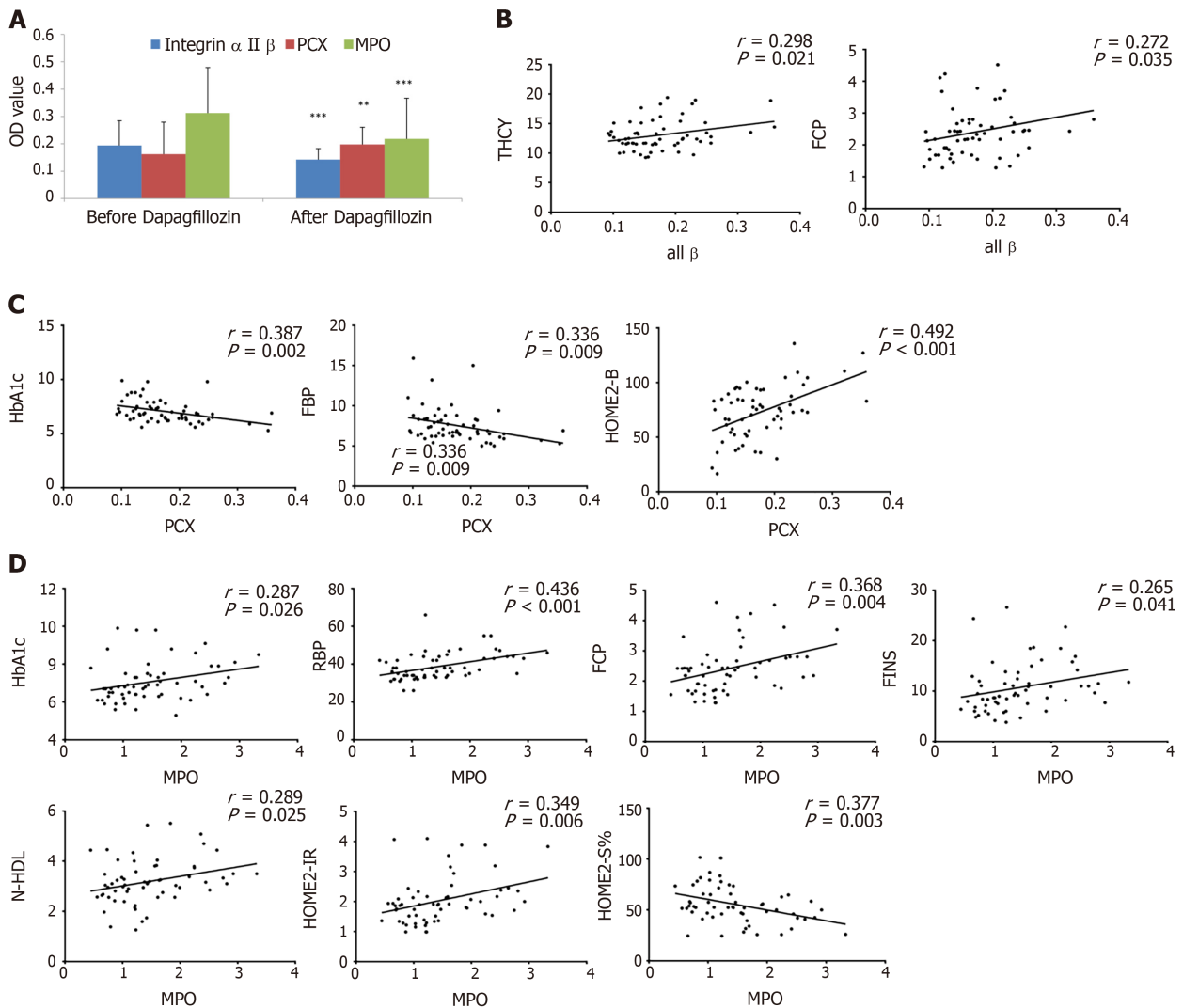
Figure 5 Correlation analyses of the expression levels of three differentially expressed proteins and clinical indexes of type 2 diabetic patients. A: The levels of three differentially abundant proteins, including myeloperoxidase (MPO), alpha II B integrin, and podocalyxin (PCX) proteins in serum samples of patients before and after dapagliflozin treatments were evaluated by enzyme linked immunosorbent assay. $n = 20$ for each group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, between the indicated groups; B-D: Dot plots show the Pearson correlations between the levels of alpha II B integrin (B), PCX (C), and MPO (D) proteins and some clinical indexes in patients before and after dapagliflozin treatments.

investigation.

Integrin is a transmembrane protein that exists on the cell membrane to mediate cell-to-cell interaction. It can interact with various growth factors at the receptor level and regulate cell adhesion, survival, growth, differentiation, proliferation, and migration[35]. There are 24 kinds of integrins in humans, formed by heterodimerization of 18 alpha subunits and eight beta subunits. Alpha II beta integrin is one of the important components of the integrin family[35]. Studies have confirmed that platelet apoptosis in patients with T2DM is higher than in normal patients, increasing alpha II beta integrin levels[36]. The mechanism might be that integrins participate in RAS/MAPK signal transduction through interaction with growth factors and synergism between integrins, related to insulin resistance and secretion deficiency[37,38]. It has not been reported through which pathway dapagliflozin can induce the downregulation of alpha II beta integrin. Our analysis indicated that the Ras GTP pathway enrichment might be related to the downregulation of alpha II beta integrin in the differential protein function enrichment pathway in this study, which needs further confirmation by additional experiments.

The normal expression of PCX can prevent negatively charged proteins from leaking into human urine, resist adhesion between adjacent foot processes, and prevent adhesion between parietal epithelial cells and capillary loops[39]. Through animal experiments, Qi *et al*[40] demonstrated that high glucose could activate the ERK1/2 MAPK pathway of podocytes and decrease PCX expression. Although hyperglycemia inhibits PCX expression during glomerular injury, it can aggravate podocyte injury, increasing PCX with urine excretion[40]. These results suggest that urinary PCX is increased in urinary nephropathy, but PCX expression decreases with high glucose levels. We found that PCX was

negatively correlated with HbA1c and FBP. The lipid metabolism disorders in diabetic nephropathy were higher than in diabetic patients without nephropathy, and there were obvious disorders in the early stage of diabetic nephropathy (Figure 5B). This study suggests that dapagliflozin might participate in the protective effect of PCX protein in kidney through lipid metabolism-related mechanisms, and there are many pieces of evidence. Dapagliflozin has a protective effect on kidney by affecting glomerular feedback, restoring renal blood flow and glomerular filtration rate, thus preventing the progression of diabetic nephropathy in the initial stage, and posing potential renal protective effect on patients with mild to moderate renal insufficiency[13-15]. SGLT2 inhibitors can also limit the glycotoxicity of the kidney itself and reduce renal hypertrophy[13-15]. Vallon *et al*[41] suggested that SGLT2 inhibitors could inhibit the expression of inflammatory markers and fibrotic markers, and whether PCX was involved in these processes needs further study.

This study has several limitations. Because it was an exploratory study, no power analysis was initially performed, and the participants were enrolled using convenience sampling. Five serum samples with relatively small heterogeneity were directly selected from the dapagliflozin group for LC-MS. The abundance of proteins in the different samples was analyzed by comparing the frequency of mass spectrometry analysis or the peak intensity of mass spectrometry. Because a single sample can only be analyzed separately, the analytical flux was relatively low, and repeated experiments are needed to improve the accuracy of the analytical flux. Moreover, the sample size of this study is insufficient. Due to limited time and funding, the mechanisms of the differentially expressed proteins associated with dapagliflozin treatments need to be further studied. Many proteins' bioinformatics database data collection might be needed for analyzing the interaction between identified differentially expressed proteins and predicting the correlation between these differentially expressed proteins and clinical indicators. In addition, the relationship between dapagliflozin treatments and the changes of isoproteins still needs to be further validated.

CONCLUSION

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients. Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

ARTICLE HIGHLIGHTS

Research background

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection.

Research motivation

The mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

Research objectives

This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM. The results could help understand the mechanisms of dapagliflozin in patients with T2DM.

Research methods

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

Research results

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin, and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay (ELISA) validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

Research conclusions

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients.

Research perspectives

Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

FOOTNOTES

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