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Research Article

Itraq Labeling Coupled With LC-MS/MS Screens Differentially Expressed Protein in Serum of Children with Type 1 Diabetes Mellitus

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Abstract

Background: Proteomics is of great value in improving the understanding of the pathological mechanism of Type 1 Diabetes Mellitus (T1DM) and searching for diagnostic biomarkers and therapeutic targets.

Objective: This study was to screen Differentially Expressed Proteins (DEPs) that are closely related to T1DM in the serum of children with T1DM and normal children.

Methods: Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) coupled with Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) was performed on 40 serum samples of children with T1DM and 40 normal serum samples of normal controls. DEPs were identified and their functions were analysed based on GO and KEGG analysis.

Results: A total of 107 DEPs related to T1DM were obtained, including 65 up-regulated proteins and 42 down-regulated proteins. GO analysis found that most of the DEPs were mainly involved in biological processes including cellular processes, single biological processes, biological regulatory processes, and metabolic processes. Most of the DEPs belong to the intracellular protein, organelles protein, and membranes protein. Pathway analysis found that the DEPs played a role in the occurrence of T1DM through the immune system, signal transduction, signaling molecules and interaction, and infection.

Conclusion: iTRAQ coupled with LC-MS/MS technology identified several serum DEPs related to T1DM in children, which were involved in a variety of biological processes, laying a foundation for further verification of biomarkers and contributing to the screening, detection, and follow-up of T1DM development.

Keywords: Type 1 diabetes mellitus, Children, Serum biomarker, Proteomics, Differentially expressed protein

Introduction

Type 1 Diabetes Mellitus (T1DM) is an autoimmune and endocrine dyscrasia disease and is characterized by immune-mediated destruction of pancreatic β cells [1]. The prevalence of T1DM in children increased significantly worldwide [2-4], becoming one of the most common chronic diseases in children [5]. Although insulin therapy is a mature treatment strategy for T1DM, children with T1DM still face serious complications, which is the most important burden for patients [6]. Novel biomarkers with high sensitivity and

specificity may contribute to the development of diagnostic and therapeutic approaches for T1DM. iTRAQ labelling coupled with liquid chromatography and mass spectrometry (LC-MS/MS) is a proteomic assay that combines iTRAQ stable isotope labelling with LC-MS/MS to achieve a high-throughput proteome analysis focusing on whole protein molecules of tissue, cell, and body fluid [7,8]. This provides a powerful technique for studying the global differential proteomics associated with disease and for finding protein markers for disease. *Zhang, et al.,* found 106 differentially expressed peptides in pancreatic tissues of T1DM mice and healthy controls based on LC-MS [9]. *Muhadas Tursunyiming, et al.,* conducted quantitative proteomics detection on serum samples from three generations of Uyghur early-onset diabetes and preliminarily identified 32 serum proteins related to Uyghur early-onset diabetes. Further comparison among 12 proteins in serum of T1DM, type 2 diabetes, Uyghur early-onset diabetes, and healthy subjects found that serum protein, such as SERPINA7, APOC4, LPA, C6, and F5, is involved in protein characteristics that are associated with Uyghur early-onset diabetes [10]. *Wen Dejia, et al.,* used iTRAQ labelling coupled with LC-MS/MS quantitative proteomics technology to find a total of 26 differentially expressed proteins in the vitreous fluid of diabetic retinopathy patients, including 7 up-regulated proteins and 19 down-regulated proteins [11]. However, there are few reports on proteomics studies of T1DM serum in children using iTRAQ labelling coupled with LC-MS/MS technology.

In this study, iTRAQ labelling coupled with LC-MS/MS was intended to explore the serum DEPs between children T1DM patients and healthy controls, as well as to perform the functional analysis of DEPs, to find target proteins closely related to T1DM. This study provides a basis for exploring genetic biomarkers and improving the diagnosis of T1DM in children.

Materials and Methods

Objects and Groups

Forty patients (20 Uygur and 20 Han) diagnosed with T1DM in our hospital were included in this study, and 40 healthy non-diabetic participants hospitalized for other reasons were selected as controls. The age and sex of children in the case group and control group were matched. The inclusion criteria were participants met the new diagnostic criteria proposed in the report of the Diabetes Society of the Chinese Medical Association. The diagnostic criteria were patients with (a) and two or more (b)-(f) as follows:

a) a short medical history lasting 3-4 weeks,

b) ketonuria,

c) nocturnal urine >3-4 times,

d) a significant trend of weight loss,

e) a first-degree relative with diabetes who requires insulin treatment, and

f) past medical history of autoimmune disease. Exclusion criteria were patients with diabetic ketoacidosis, no fever in recent 1 week, less than 37°C of body temperature, and no stress or infection.

Forty children with T1DM were divided into 4 groups, including Han nationality patient group 1 (HB1), Han Nationality patient group 2 (HB2), Uygur nationality patient group 1 (WB1), and Uygur nationality patient group 2 (WB2), with 10 patients in each group. Forty healthy controls were divided into 4 groups: normal Han Nationality group 1 (HZ1), normal Han Nationality group 2 (HZ2), normal Uygur Nationality group 1 (WZ1), and normal Uygur nationality group 2 (WZ2), with 10 persons in each group. General data of the case group and the control group were collected, including age, gender, nationality, etc. Data related to diabetes in the case group and control group were recorded, such as K, Na, Ca, HbA1c, Fasting Plasma Glucose (FPG), High-Density Lipoprotein Cholesterol (HDL-c), Low-Density Lipoprotein Cholesterol (LDL-c), Total Cholesterol (TC), Triglyceride (TG).

Medical Ethics

Informed consent was issued to participants before the study and the purpose and methods in detail were explained. The study was carried out after obtaining the consent of patients. The whole study was completed under the supervision of the Ethics Department of Urumqi People's Hospital in Xinjiang Uygur Autonomous Region.

Serum Samples

Fasting blood was taken from all subjects after fasting for 12 h. Serum samples were obtained at two centrifuges (3000rpm for 15 minutes) and stored at -80°C until biochemical analysis. K^+, Na^+, Ca^{2+}, TC, TG, HDL-c, LDL-c were measured by colorimetric method.

Serum Protein Extraction

Serum proteins were extracted using reductive alkylation. Serum was added with protein lysate supplementary with phenylmethyl sulfonyl fluoride, ethylenediamine tetra acetic acid and dithiothreitol. The supernatant was taken after ultrasonic centrifugation. The supernatant was added with the corresponding reagents and centrifuged again. The precipitate was dissolved in tetraethyl ammonium bromide by ultrasound and centrifuged to obtain the supernatant for protein concentration measurement using Bradford quantification. Protein was added with trypsin and was bathed at 37°C for 24h, and then the mixture was added with another 1μg trypsin and based 37°C for 12h. The digestive solution was drained by a vacuuming instrument, and TEAB solution was added to the sample in a 1:1 ratio with water to redissolve the digested peptide segment. The peptide samples were pooled in the serum same group for proteomic analysis.

iTRAQ Labeling and Strong Cation Separation

Isopropanol was added to iTRAQ to produce a labeling reagent. Samples of HB1, HB2, WB1, WB2, HZ1, HZ2, WZ1, and WZ2 were

labelled with different sizes of isotopes. The labelled peptides were mixed and separated by cation exchange chromatography.

MS Analysis

Based on the Q-Exactive mass spectrometer, the primary mass spectrometry and secondary mass spectrometry were completed.

Data Processing and Analysis

SPSS 21.0 statistical software was used for data processing and analysis. The counting data between the case group and the control group were expressed as a rate (%), and the measurement data were expressed as mean±standard deviation. The independent sample t-test was performed to compare the difference between the groups. A P value less than 0.05 was considered statistically significant.

Proteins were identified by Mascot and PD software and the corresponding proteins were searched from the protein database Uniport. PD software was used to complete the relative quantitative experimental processing of iTRAQ labelling. After quality control, the identification information of protein and peptide, the molecular weight distribution of protein, and coverage distribution of protein were counted by Microsoft Office Excel 2017 and visualized to visually display the distribution of protein and peptide quantity and length, and other information.

The screening criteria of proteins in this study were: upregulated protein abundance Fold Change (FC)>1.2 times or down-regulated protein abundance FC<1.2 times, and P<0.05 was considered a DEP. Finally, the DEPs between the two groups were identified according to the judicial principle of the results, and the Volcano plot and heat map were drawn using R software. The total gene expression was reflected using a Volcano plot. The volcano map was drawn using the g plots packages in R. The volcano map had four columns of ID, log2FC, P-value, and Significant (the labelled protein is up-regulated, down-regulated, and no different). The significantly up-regulated protein was logFC>log21.2 and P<0.05 and the significantly down-regulated proteins were logFC<log21.2 and P<0.05, and the rest were not significantly changed proteins. Heat maps were used to measure the similarity of expression between samples or proteins. Cluster analysis was performed on the selected proteins, and heat maps were drawn using g plots and heat map in R language to visually show the global expression changes of multiple proteins in various scripts.

GO analysis was divided into three parts, including Molecular Function, Biological process, and Cellular component. After collecting the DEPs screened by the iTRAQ method, all the DEPs were classified on the website according to their biological functions and physiological reactions involved in the human body, and the corresponding three parts were analysed and represented by a bar graph.

KEGG Pathway can be divided into seven major categories: Metabolism, Genetic information processing, Environmental Information processing, Cellular processes, biological systems, Human disease, and Drug development. In this study, the pathway of KEGG was searched on the website to find the corresponding database (KEGG pathway), and KEGG functional annotation statistics were performed on the DEPs, and the functional classification of the pathway or pathway that the protein participated in was clarified at the functional level, which was represented by a bar graph.

Results

General Characteristics of Subjects

A t-test was conducted on the general data between the two groups, and the results showed that the P values of K^+, Na^+, and Ca^{2+} between the two groups were 0.832, 0.077, and 0.055, which were all >0.05, indicating that there was no significant difference between the two groups. The t-test results of TC, TG, HDL-C, and LDL-C between the TIDM group and control group showed that P values were 0.903, 0.983, 0.075, and 0.564, which were all >0.05, indicating that there was no significant difference between the two groups (Table 1).

Table 1: Comparison of general data between T1DM patients and healthy controls.

Comparison	T1DM Group	Control Group	T Value	P Value
K^+	4.05±0.36	4.03±0.44	0.213	0.832
Na^+	140.86 ± 2.48	139.54±3.99	1.793	0.077
Ca^{2+}	2.26±0.14	2.34±0.23	-1.95	0.055
TC	1.77±0.68	1.75±1.24	0.122	0.903
TG	4.45±0.99	4.45±1.03	-0.022	0.983
HDL-c	1.03±0.32	1.23±0.63	-1.81	0.075
LDL-c	2.73±0.84	2.84±0.83	-0.579	0.564

Serum Sample Quality Testing

The serum samples contained high- and low-abundance proteins. In the actual experimental operation, high-abundance proteins would affect the detection of low-abundance proteins, so the low-abundance protein enrichment system was used in this study to reduce the concentration of high-abundance proteins in complex

serum samples. Polyacrylamide gel electrophoresis was performed after the concentration of high protein abundance was reduced. The electrophoretic results are shown in Figure 1. On the left were the electrophoretic results of the samples before the removal of high protein abundance and showed that all the 8 groups of samples present dark gray bands at about 60-70KD, which were different from other positions. The graphic on the right of Figure 1 was the electrophoresis results of the samples with high protein abundance removed and showed that the black-gray bands of the 8 groups of samples at about 60-70kD were lighter than those in the left, and the gray bands within the range of 70-180KD were distributed, and the edges of different protein bands were neat and clear.

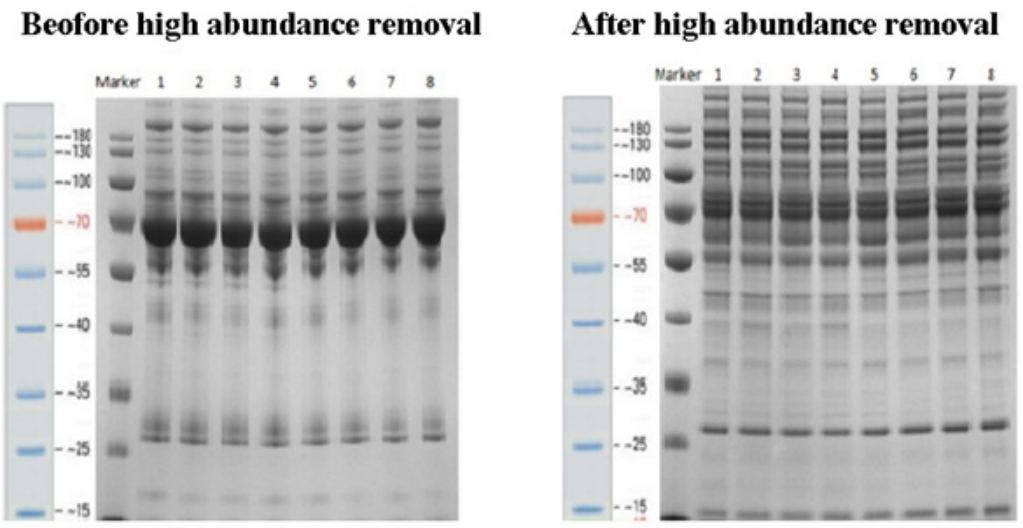

Figure 1: Comparison of protein electrophoresis results before and after high abundance removal using polyacrylamide gel electrophoresis experiment.

Identification of Qualitative Results of Proteins

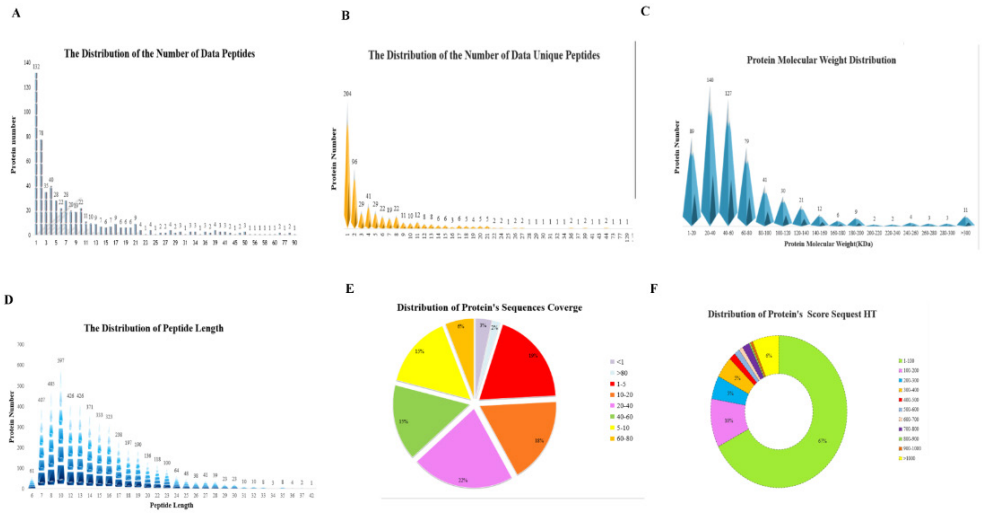

Figure 2: Identification results by MS. (A) A map of the number distribution of peptides. (B) Number distribution of specific peptides. (C) The molecular weight distribution of the protein. (D) Peptide length distribution. (E) Pie chart of protein coverage. The larger the sector area is the more proteins have coverage in this region. The numbers in the sector represent the coverage range and the proportion of proteins distributed in this region. (F) Pie chart of protein Score Sequest HT.

Peptide number, specific peptide number, protein molecular weight, protein coverage, and other corresponding protein number were described. Figure 2A showed that proteins with peptide numbers of 1-21 had a large number of proteins, among which peptide number of 1 was the most number (132 protein) and peptide number more than eleven had 123 proteins. Figure 2B showed that proteins with the peptide number of 1 had the largest number (204 proteins), and proteins with the peptide number of 2 were 96

proteins and the peptide number of 11 was 84. Figure 2C shows that the highest proportion of proteins was that with a molecular weight greater than 100KDa, followed by the range of 10-60KDa, containing 356 proteins. Figure 2D showed that the peptide with 10 amino acids was the most, with 596 peptides, followed by the peptide with 8, 12 and 13 amino acids, with 485, 426 and 426 peptides, respectively. Figure 2E showed that proteins with coverage of more than 10 accounted for 63% of all proteins, among which the number of proteins with coverage of 10-20 and 20-40 were more, corresponding to 18% and 22%. Figure 2F showed that 1-100 proteins accounted for 67% of all proteins, followed by 100-200 proteins accounted for 10% of all proteins, and more than 200 proteins accounted for 23% of the total protein (Figure 2).

Deps Screening between Groups

Five groups of samples were compared for DEPs screening, and the groups were HB-VS-HZ, WB-VS-WZ, HZ-VS-WZ, HZ-VS-WZ, HWB-VS-HWZ. T-tests and multiple differences were performed between the two groups, and the following results were obtained. Serum proteins of HWB and HWZ groups were compared, and 107 DEPs were screened out, including 65 up-regulated proteins and 42 down-regulated proteins (P<0.05). Comparing HB and HZ groups, 57 DEPs were screened out, including 32 up-regulated proteins and 25 down-regulated proteins (P<0.05). By comparing the serum proteins of HB and WB groups, 25 DEPs were obtained, including 6 up-regulated proteins and 19 down-regulated proteins (P<0.05). Comparing WB and WZ groups, 72 DEPs were screened out, including 46 up-regulated proteins and 26 down-regulated proteins (P<0.05). By comparing serum proteins of HZ and WZ groups, 14 DEPs were screened out, including 4 up-regulated proteins and 10 down-regulated proteins (P<0.05) (Table 2). Figure 3 showed the volcano plot of DEPs. In the figure, the blue dots were up-regulated proteins, the red dots were down-regulated proteins, and the green dots were proteins with no significant change (Figure 3).

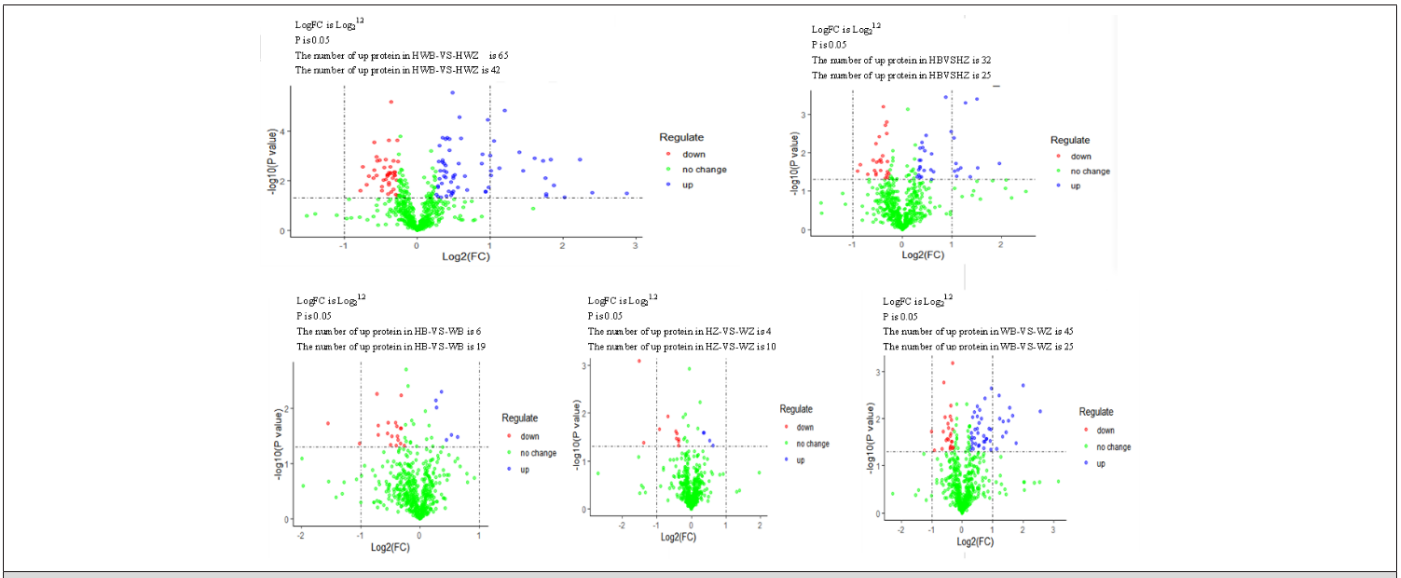

Figure 3: Volcano plot of DEPs between T1DM and healthy control groups.

Table 2: DEPs between groups by iTRAQ coupled with LC-MS/MS

Comparable Group	Up-regulated DEPs	Down-regulated DEPs	Indifference Protein	Total Protein
HB-VS-HZ	32	25	522	57
WB-VS-WZ	45	25	509	70
HZ-VS-WZ	4	10	565	14
HB-VS-WB	6	19	554	25
HWB-VS-HWZ	65	42	472	107

Note*: HB-VS-HZ represents Han patient group and Han normal group; WB-VS-WZ represents Uygur patient group and Uygur normal group; HZ-VS-WZ represents Han normal group and Uygur normal group; HB-VS-WB represents Han patient group and Uygur patient group; HWB-VS-HWZ represents the patient group and the normal group.

Cluster Analysis of DEPs

A total of 579 proteins were identified by LC-MS/MS. Proteins in eight groups of HZ1, HZ2, WZ1, WZ2, HB1, HB2, WB1, and WB2 were analyzed. Each set of samples was represented by a vertical column and a protein by a row. The colors in the figure indicated the relative expression levels of proteins in this group of samples.

The clustering tree among proteins was at the far left of the picture, the clustering tree among each group of samples was at the top of the picture, and the sample group name is marked at the bottom of the picture (Figure 4).

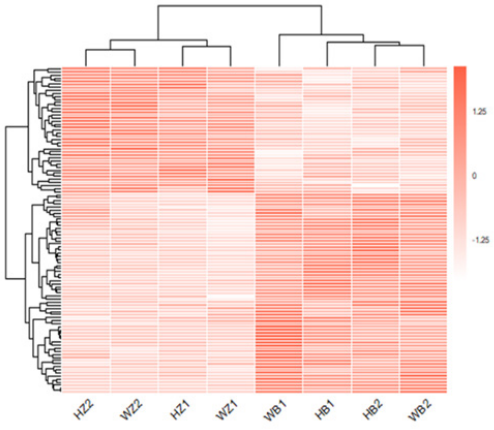

Figure 4: Cluster analysis of DEPs.

GO Annotation Analysis of DEPs

A total of 107 DEPs related to T1DM were screened and their functions were analysed by bioinformatics. GO analysis classified the DEPs into three categories: biological processes (green), molecular functions (blue), and cellular components (red) (Figure 5). DEPs participated in many biological processes, among which proteins involved in cell processes were the most, with 99 related proteins. The second was a single biological process with 98 related proteins. Next in line were biological regulatory processes (with 95 related proteins), Metabolic process (with 92 related proteins), Localization (with 79 related proteins), Cell tissue formation or biosynthesis (with 73 related proteins), Positive or negative regulation of biological processes, (with 69 related proteins), growth processes (with 62 related proteins), Developmental processes, immune system, locomotion, biological adhesion, multicellular biological processes, reproductive processes, growth, cell killing, detoxification, etc. The results also showed that most of the proteins belonged to intracellular, organelle, and membrane proteins. In the functional classification of DEPs, most proteins were involved in binding, followed by catalytic activity, transporter activity, signal sensor activity, transcription factor activity, nucleic acid binding transcription factor activity, etc. (Figure 5).

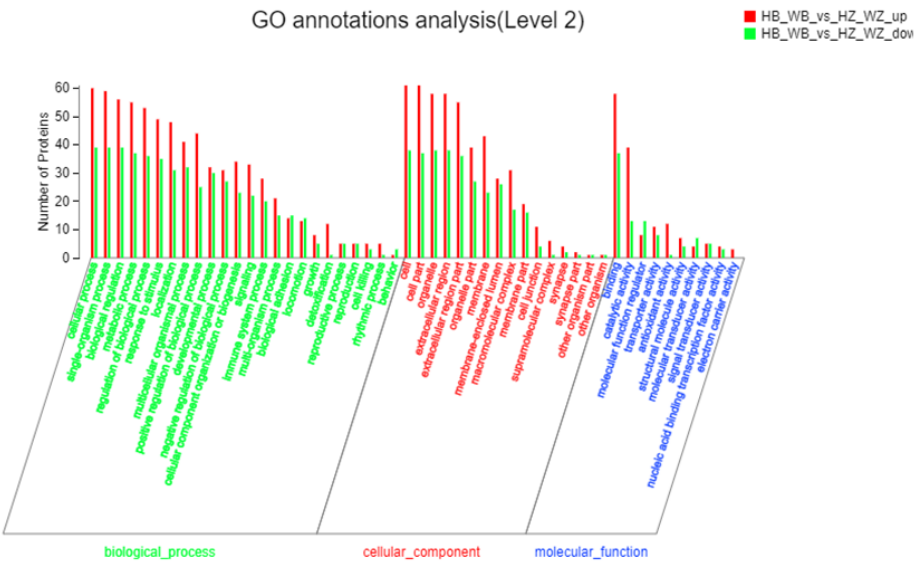

Figure 5: GO annotation of DEPs between T1DM and control groups.

Pathway Annotation Analysis of DEPs

Figure 6 illustrated the DEPs associated with nucleotide metabolism, immune system, substance dependence, cell growth and death, amino acid metabolism, carbohydrate metabolism, transshipment and catabolism, circulatory system, energy metabolism, translation, signal transduction, different biomass biodegradation and metabolism, signal molecule and the interaction, aging, infectious diseases: viral, folding, classification and degradation, infectious diseases: parasites, nervous system, neurodegenerative diseases, cell motility, cell community-eukaryotes, endocrine and metabolic diseases, drug resistance, anti-tumour, metabolism of cofactors and vitamins and other amino acid metabolism, autoimmune diseases, infectious diseases: bacteria (Figure 6).

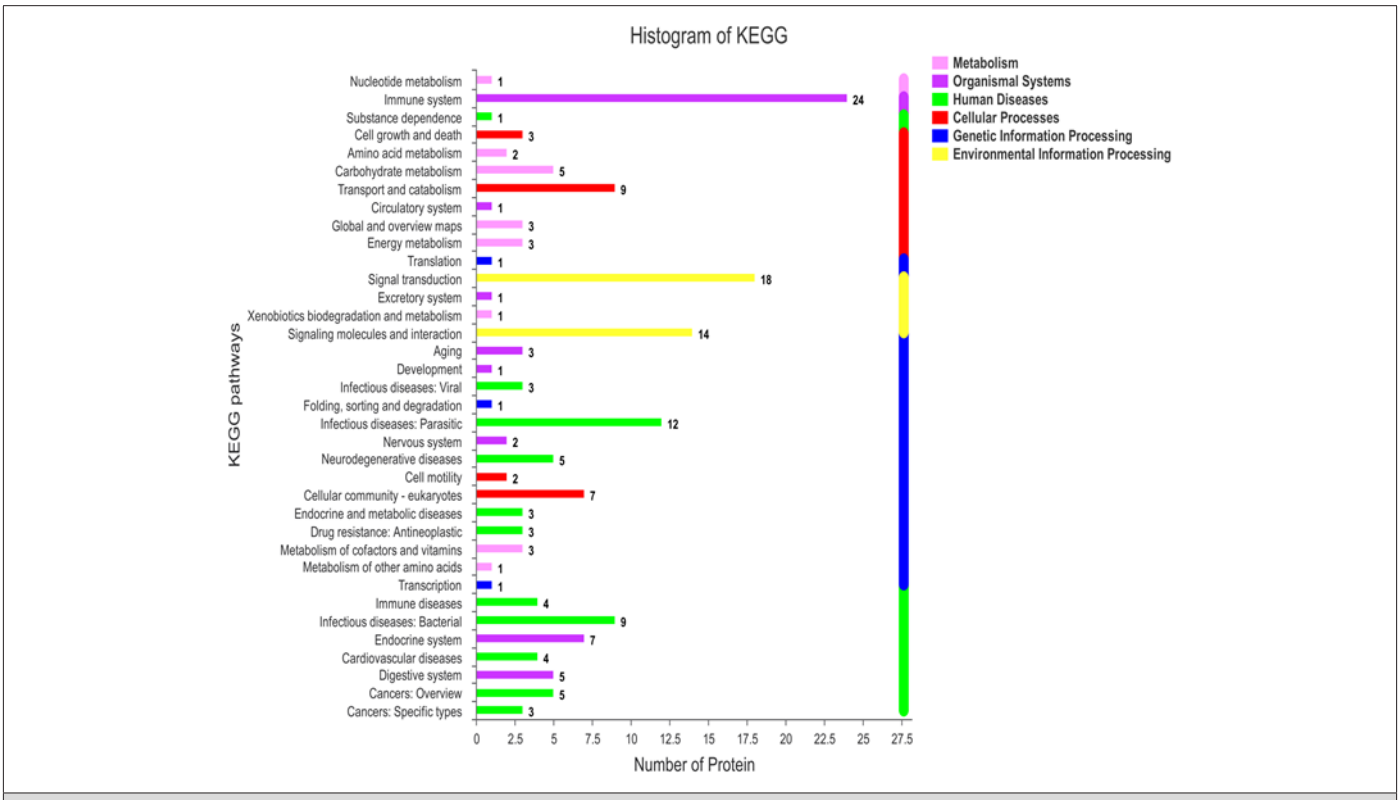

Figure 6: KEGG distribution of DEPs in T1DM and control group.

Discussion

Changes in serum protein levels were significantly correlated with T1DM progression and T1DM-related diseases [12,13]. Using label-free mass spectrometric analysis, differential serum proteins were found to be involved in T1DM-related pathology in serum protein comparison between TDM1 and healthy controls, such as immune system, lipid metabolism, and pathways of coagulation [14]. *Overgaard AJ, et al.,* compared plasma proteomics of T1DM patients with healthy controls, and 39 that had been identified as T1DM-related plasma proteins showed significant changes in protein expression levels [15]. The rapid progression in mass spectrometry (MS)-based proteomics has found some unrecognized proteins, which is helpful for the exploration of biomarkers of disease diagnosis and pathological mechanism. This may contribute to screening and follow-up for the development of T1DM [16]. In The present study, iTRAQ labelling coupled with LC-MS/MS identified 107 serum DEPs correlated to T1DM in children, which are involved in a variety of biological processes.

T1DM is characterized by hyperglycaemia and in the chronic stage, hyperglycaemia is associated with abnormalities in lipids, proteins, carbohydrates, and oxidative stress [17]. In this study, by comparing and analysing the basic information of T1DM patients and the normal controls, there was no significant difference in calcium, sodium, potassium, TC, TG, HDL-c, and LDL-c between the two groups. The underlying reason could be the subjects in this study were younger paediatric patients and most of diabetes they suffer from is newly developed recently or diagnosed at an early stage. In this case, the patient is at a lower stage of clinical diabetes, which has not involved other organs and the pathological manifestations are mostly changes in blood glucose, but no changes in TC, TG, HDL-c, and LDL-c.

Previous studies on serum proteomics of adult T1DM showed that functional DEPs were related to binding and catalysis, and

were involved in biological processes related to regulation, cellular, localization, stimulus response and immune system [14]. We compared serum proteins in the case group and the control group and identified a total of 107 DEPs related to the serum of children with T1DM, including 65 upregulated proteins and 42 proteins down-regulated proteins. GO annotation analysis revealed that these proteins are involved in a variety of biological processes and signal transduction pathways, including cellular processes, single biological processes, bioregulatory processes, metabolic processes, localization, cell tissue composition, or biosynthesis. The results of this study found that DEPs involved in cell process and single biological process were the most, which was the same as the research result of researcher Avaguri Tohoti [18].

Insulin-producing β cells suffer sustained autoimmune destruction by T cells and macrophages infiltration in the islets. The autoimmune destruction of insulin may be a consequence of a sequence of inciting events such as genetic susceptibility, infection, diet, microbiota, environment, etc at least months, but most likely several years [19-21]. In general, genetic, and environmental factors (including microorganisms and parasites) interact to promote processes such as metabolic changes and islet immune responses before clinical symptoms, involving multiple proteins and signalling pathways [22-24]. Our DEPs functional classification results show that DEPs are involved in cell functions related to T1DM progression, focusing on the immune system, signal transduction, signalling molecules and interaction, infection disease: parasitic and bacterial, transport and catabolism, cellular community. MS-based proteomics supports the interaction between genetics and environment in the pathogenesis of T1DM and the responsibility of the pathological mechanism.

In conclusion, this study combined iTRAQ labelling with LC-MS/MS to screen differently expressed serum proteins, suggesting that this technique can be used for serum proteomic studies of diabetes biomarkers and disease progression. However, the role of these proteins in the occurrence and development of T1DM is not fully understood, and further evaluation of the reliability of serum differential proteins as candidate markers for the diagnosis of diabetes and their participation in the pathogenesis of T1DM is needed.

Conflict of Interest

Authors declare there is no conflict of interest.

Acknowledgement

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