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Identification and investigation of protein-related molecules in patients with hyperlipidemia using label-free combined with bioinformatics analysis

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ABSTRACT

This study aimed to identify proteins associated with high-fat diet patients and investigate their relationship with this dietary pattern. Five hyperlipidemia female patients and five normal individuals were included as the experiment and control groups, respectively. Blood samples were collected from both groups, and bioinformatics tools were employed for gene ontology annotation, KEGG pathway annotation, GO enrichment analysis, pathway enrichment analysis, and protein clustering to pinpoint genes, proteins, and pathways relevant to high-fat diet patients. Mass spectrometry analysis was subsequently used to confirm these proteins. The results indicated that bioinformatics analysis identified several proteins (P09871, P01019, P48740, P02654, P02649) potentially involved in the high-fat diet process by regulating downstream pathways. Label-free analysis revealed 3915 peptides in both groups, with 16 protein expression levels up-regulated in the experiment group, 13 of which showed significant differences. In contrast, 12 protein expression levels were down-regulated in the experiment group, with two showing significant differences. Notably, the proteins highlighted by bioinformatics analysis aligned with those identified through mass spectrometry. In conclusion, label-free analysis combined with bioinformatics can effectively identify proteins linked to high-fat diet patients. This research provides a fresh perspective on addressing high-fat diet-related issues using this approach.

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Introduction

Hyperlipidemia, as a common disease that causes obesity, has a complicated etiology and involves liver abnormalities, gut microbiota, circadian clock, fat content in food, and nucleotide biological function (1,2). Hyperlipidemia is characterized by remarkable overweight, excessive internal fat deposition, and chronic imbalance between body caloric intake and energy expenditure, which is a serious challenge for patients with obesity (3). Currently, the detailed mechanism underlying hyperlipidemia is still unclear. Clinically, hyperlipidemia can lead to organ injuries in patients, such as cardiovascular system, kidneys, liver, retina, lungs and so on, and these organ injuries would be easily health-threatening. A previous systematic study by Zhang et al. (4) reported that the incidence of hyperlipidemia throughout the world is up to 40%. Clearly, hyperlipidemia is a disease that threatens people's health, especially for the elderly. Hyperlipidemia is a disease induced by multiple protein targets (5). Therefore, finding these targets involved in hyperlipidemia plays a key role in preventing or relieving the development process of hyperlipidemia. Interestingly, label-free, as a common technology used for detecting protein, has been widely utilized for a series of protein targets from patients who have all kinds of diseases including hyperlipidemia (6,7). However, the detailed protein targets linked to hyperlipidemia in these studies are still limited, and further identification and investigation of protein targets in hyperlipidemia patients are required to clarify.

Previous studies have confirmed that bioinformatics analysis technology can screen the protein targets associated with hyperlipidemia by regulating cell biological and cell pathological processes, and these protein targets play an important role in cell apoptosis, cell cycle regulatory, cell proliferation, oxidative stress, inflammatory response (8-10). At present, the role of these proteins in patients with hyperlipidemia is still unclear, a study by Liu Y (11) points out that YAP (Yes-associated protein) and TGF β (transforming growth factor β) levels in hyperlipidemia are significantly increased and can accelerate the process of hyperlipidemia. Moreover, some studies (12) have predicted that interleukin-1βcan participate in the hyperlipidemia by mediating downstream responding genes to control the cell biological and cell pathological process of hyperlipidemia. However, information provided by these studies is still limited.

In our study, the hyperlipidemia patients enrolled in this trial, and we aim to investigate the differences in protein expression in blood samples from the hyperlipidemia

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patients and normal people. Protein, genes, and pathways relating to high-fat diet patients are established. Bioinformatics analysis including gene ontology (GO) and enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, differentially expressed proteins KEGG enrichment analysis, and protein cluster analysis are utilized to screen and identify the differentially expressed proteins and genes. The proteins and genes screening results are further identified by label-free technology to measure the predicted proteins and genes relating to the hyperlipidemia.

Materials and Methods

Patients

A total of 5 patients with hyperlipidemia from our hospital from January 2020 to December 2021 were included in this clinical study and were set as an experiment group. During the same period, 5 normal people were also enrolled in this trial and were set as the control group. All people enrolled in this clinical study were willing to be detected using label-free technology. All protocols used for this study were prospectively reviewed and approved by the Use Committee of our hospital, and written informed consent forms from all patients were obtained. Blood samples from two groups were taken and analyzed by label-free technology.

Inclusion criteria for all patients are as follows: the patients for the experiment group, patients are female; patients have a triglycerides value of more than 2.26 mmol/L; patients have a cholesterol value of more than 6.22 mmol/L; patients have a low high-density lipoprotein value of less than 1.04 mmol/L; the people for control group are female and have no symptoms like high-fat diet; all patients are willing to conduct the label-free measurement.

Exclusion criteria for this study are as follows: patients have an age of less than 18 years old; patients who have secondary hypertension disease; and patients who disagreed with receiving the label-free measurement.

Blood sample analysis

The blood samples in two groups were collected and immediately separated by centrifugation at 3000 rpm at 4°C for 15 min, and then serum samples were obtained. The highest abundant protein in serum samples was removed by Agilent multiple affinity removal LC column human 14 according to Agilent protocol, and then low-abundance protein solutions were collected. An ultrafiltration concentration was conducted using a 5 kD ultrafiltration device, and one volume of SDT lysis buffer, and then boiling water bath for 10 min and centrifugation at 14000g for 15min. The protein concentration of serum samples was determined by the BCA method and these serum samples were stored at -80°C for further experiments.

SDS-PAGE electrophoresis

A 20 μg of protein in each group was dissolved with a six-volume loading buffer, and a boiling water bath for 5 min, and then electrophoresis by 12% SDS-PAGE at 250 v of constant pressure for 40 min.

Mass spectrometry analysis

An easy nLC chromatographic system (Thermo Fisher Scientific, Waltham, MA, USA), which consists of an Agilent 1260 infinity II HPLC (Agilent Technologies, Inc. CA, USA) and Q Exactive Plus mass spectrometer (acclaim pepMap RSLX 50 μm × 15 cm, Thermo Fisher Scientific, Waltham, MA, USA), was used to analyze the serum samples by a chromatographic column (Thermo Fisher Scientific, Waltham, MA, USA). Buffers A consists of 0.1% aqueous solutions of formic acid and Buffers B consists of 0.1% aqueous solutions of zoate acetonitrile (acetonitrile accounts for 80%). we separated the serum sample at a flow velocity of 300 nL/min. Following a concentration gradient was set (3% buffers B for 0~5 min, 3% buffers B to 28% buffers B at linear gradient from 5min to 45 min; 28% buffers B to 38% buffers B at linear gradient from 45min to 50 min; 38% buffers B to 100% buffers B at linear gradient from 50min to 55 min; 100% buffers B from 50 min to 55 min.). After that, a positive ions scan ranging from 350~1800 m/z was analyzed using Q Exactive Plus. Mass-to-charge ratios of peptides and peptide fragments were also analyzed.

Gene ontology annotation

Firstly, we compared the target protein term with the relative protein term database using the NCBI BLAST+ database in a Linux server, and the top 10 rated protein sequences with an E-value ≤1×e-3 were obtained to use for subsequent analysis. Secondly, target protein term and the top 10 rated protein sequences were extracted using the Blast2 GO Command Line (download link: www.geneontology.org). To further identify the GO annotation, an annotation augmentation by interProScan was conducted to investigate the motif between the EBI database and target protein and aimed to improve the accuracy of Gene Ontology.

KEGG pathway annotation

To screen the KEGG pathway relating to the target protein term, the KEGG GENES database was used to compare the target protein term using the KOALA software (KEGG Orthology and links annotation), and then we classified the target protein term by KEGG Orthology. Finally, pathways relating to the target protein term were obtained.

GO and pathway enrichment analysis

GO annotation and pathway annotation were performed to analyze using enrichment analysis. GO term enrichment with Fisher's exact test was conducted and pathway term enrichment with Fisher's exact test was performed, and we aimed to obtain the significant level of a GO term or a KEGG pathway by comparing the distribution of GO term or KEGG pathway in target protein term and total protein term.

Protein cluster

After GO and pathway enrichment analysis, quantitative information in the target protein term was collected using normalization method. After that, the target protein expression level in hyperlipidemia patients using matplotlib software that consists of distance algorithm and average linkage. Finally, hierarchical cluster heatmap for protein terms was obtained.

Western blotting

The HSC-3 cells protein was subjected to 10% SDS-

PAGE and loaded, transferred to PVDF membrane, and washed with TBST for 5 min. Primary antibody (1: 2000; Bioworld Techmology, Inc., Nanjing, China) and GAPDH (1: 1000; Bioworld Techmology, Inc., Nanjing, China) were added and incubated overnight at 4°C. After washing with TBST 3 times (10 min/time), a secondary antibody (1:10 000; Bioworld Techmology, Inc., Nanjing, China) was added and blocked for 2 h at room temperature. After washing with TBST 3 times (10 min/time), ECL luminescent reagent was added for development, and the gray value of the bands was analyzed.

Real-Time PCR (RT-PCR)

TRIzol reagent (15596026, Invitrogen, Carlsbad, CA, USA) was incubated with cell lines and then centrifuged to obtain the resultant supernatant. Next, total RNA was obtained from the resultant supernatant 200 μL of chloroform chloroform. An equal volume of isopropanol was added to the resultant supernatant and centrifuged, then the supernatant to obtain the precipitate that was rinsed using 1 mL of 75% absolute ethanol. Next, the RNA was dissolved using DEPC water and RT-PCR Kit (TaKaRa, Dalian, China) was utilized to reverse mRNA transcription by RT-PCR system (ABI 7300, Foster City, CA, USA). The mRNA relative level was detected using the $2^{-\Delta\Delta Ct}$ calculation method and repeated the assay three times. Finally, Primer3 V.0.4.0 was performed to design the primer sequences.

Statistical analysis

Statistic Package for Social Science (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) is used for statistical analysis. Measurement data are expressed by mean ± standard deviation (x±s), and the t-test is used to compare the difference between the two groups. $P<0.05$ shows the difference is significant.

Results

Go functional analysis

To clarify the function of differentially expressed genes**,** we investigated the biological process, molecular function and cellular component involved in hyperlipidemia. Go annotation result by level 2 statistic is shown in Figure 1. These proteins related to the biological process are involved in the cellular process, biological regulation, response to stimulus, immune system process, and localization. Similarly, these proteins linked to molecular function mainly refer to binding, catalytic activity, molecular function regulator, structural molecular activity, and molecular transducer function. Compared with the control group, the number of proteins and percentage of proteins relating to binding, catalytic activity, molecular function regulator, structural molecular activity, and molecular transducer function are remarkably decreased, and these results show that these proteins probably participate in the development of hyperlipidemia by controlling molecular function. Finally, these proteins mediate cellular components including the cell part, extracellular region part, protein-containing complex, organelle part, and membrane.

Go enrichment analysis

We utilized the Go enrichment analysis to further investigate the relationship between these proteins and hyperlipidemia. As is presented in Figure 2, the GO enrichment analysis results show that the cellular component mainly involved in extracellular region, endoplasmic reticulum, endocytic vesicle lumen, extracellular matrix, secretory granule, haptoglobin-hemoglobin complex, hemoglobin complex. Followed by molecular functions relating to hyperlipidemia, we find that these molecular functions refer to selenium binding, extracellular matrix binding, polysaccharide binding, SH3 domain binding, extracellular matrix structural constituent conferring compression resistance, heme binding, and biological processes are involved in defense response, female pregnancy, response to hydrogen peroxide, negative regulation of extrinsic apoptotic signaling pathway via death domain receptors, regulation of immune response, positive regulation of cell death, cellular oxidant detoxification, nitric oxidant detoxification, positive regulation of activation of Janus kinase activity, regulation of protein complex assembly.

KEGG pathway function and enrichment analysis

To further investigate the relationship between KEGG signal pathway and these proteins relating to hyperlipidemia, we selected signal pathways for further signal pathway annotation and pathways enrichment analysis to identify the significant signal pathway. As is depicted in Figure 3. These significant signal pathways have been associated with the renin-angiotensin system, in malaria. KEGG pathway function and enrichment results can predict potential signal pathways involved in the renin-angiotensin system, malaria participates in the process of female patients with hyperlipidemia.

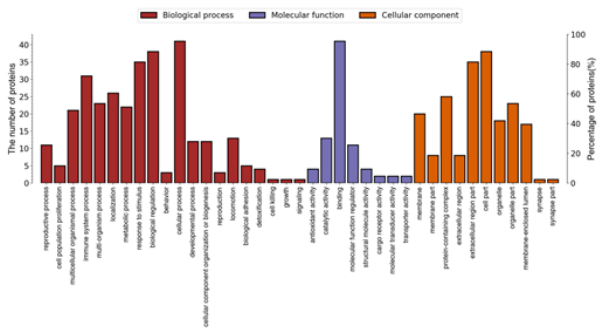

Figure 1. Go functional analysis results, and these results consist of biological processes, molecular function, and cellular components.

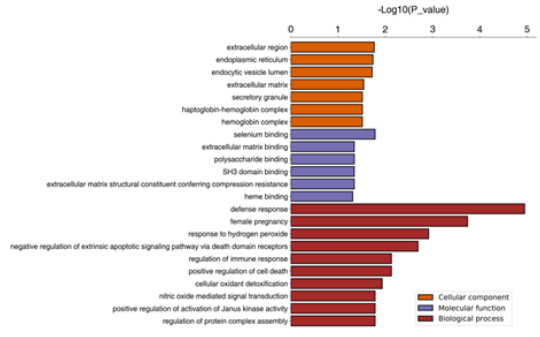

Figure 2. Go functional analysis results are shown in Figure 2, and these results consist of biological processes, molecular function, and cellular components.

Protein clustering analysis

To further find the potential protein group which has similar regulation function involved in the same signal pathway, we conducted the protein clustering analysis. As is shown in Figure 4. Lines in Figure 4 represent proteins, and the characters on the right in Figure 4 are protein IDs. The column in Figure 4 represents the sample detected, and the characters below the column are the sample abbreviation. The tree structure above the graph boundary represents the protein classification and the tree structure on the left of the graph represents the sample classification. We can conclude that 28 proteins were clustered into 4 categories, and we can see that the protein in each category has a consistent expression level. In addition, 10 patients in our study were divided into 2 categories according to the clustering protocol, and these results were consistent with the groups we designed.

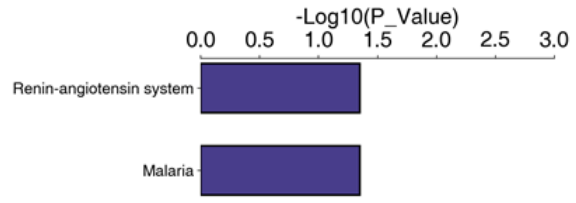

Figure 3. KEGG pathway function and enrichment results depicted, pathways relating to HFD refer to renin-angiotensin system, malaria.

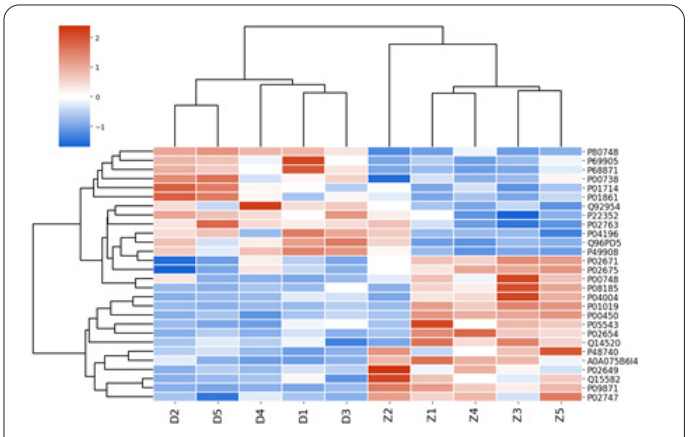

Figure 4. Protein clustering result depicted in Figure 4, tree structure above the graph boundary represents the protein classification and tree structure in left of the graph represents the sample classification.

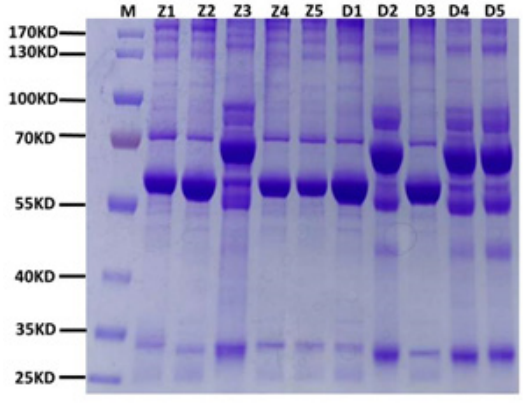

Figure 5. SDS-PAGE electrophoresis results between control group and experiment group are shown in Figure 5.

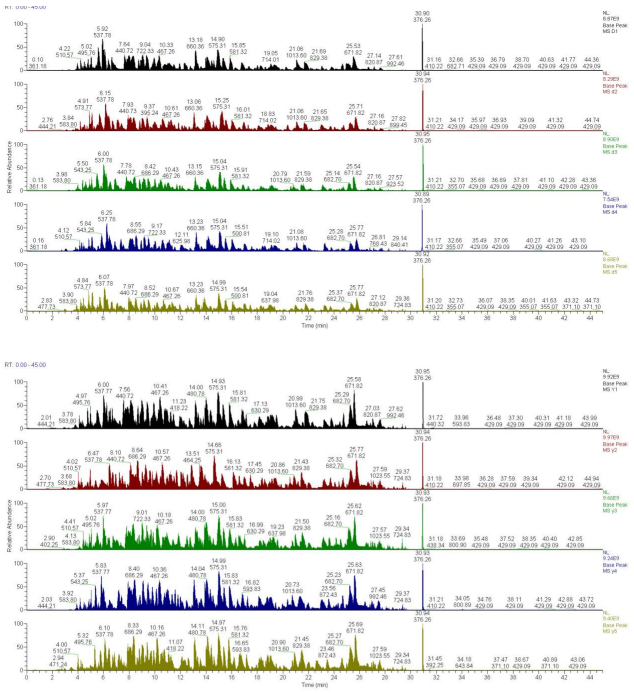

Figure 6. LC-MS/MS mass spectrum results between the control group and experiment group are shown in Figure 6. Peak waves with retention time ranging 0~45 min are leptokurtic and the highest peak occurs at 30.92 min.

SDS-PAGE electrophoresis analysis

The SDS-PAGE electrophoresis result is shown in Figure 5. The samples in the control group and hyperlipidemia group have a bright band at 170 KD, 130 KD, 100 KD, 70 KD, 55 KD, 40 KD, 35 KD, 25 KD, respectively. Compared with the control group, expression levels at 170 KD, 130 KD, 100 KD, 70 KD, 55 KD, are significantly increased and expression levels at 40 KD, 3 5KD, 25 KD are remarkably decreased. These results show that differentially expressed proteins probably have been associated with the development of hyperlipidemia. Protein mass satisfies experimental needs. In addition, total protein can be used to conduct more than two experiments.

LC-MS/MS mass spectrum analysis

To identify the protein and peptides relating to the hyperlipidemia, LC-MS/MS mass spectrum is used to analyze the relative protein involved in hyperlipidemia. LC-MS/MS mass spectrum results are presented in Figure 6. Peptide features are recognized and qualitative analysis was performed using Maxquant software. Then quantitative analysis is conducted by label-free quantitation (LFQ). To identify the protein, Protein IDs, Gene name, fasta headers, number of proteins, peptides, unique peptides, sequence coverage, Mol. Weight, LFQ intensity X are analyzed and a number of proteins range 1~5, the peptides ranging 1~128, and unique peptides ranging 1~114. To identify the peptide, sequence, length, missed cleavages, mass, proteins, leading razor protein, unique groups, charges, pep, score, intensity, and intensity X were also analyzed according to the mass spectrum analysis instruction manual, and our outcomes show that the peptide length ranging 7~45, the mass ranging 699~4595, the pep ranging 0~0.084, the score ranging 16~508.

Peptides analysis

To further investigate the protein relating to the hyperlipidemia, protein weight distribution is performed, and the protein weight distribution result is depicted in Figure 8A, and we totally identify 333 protein groups from two groups of samples, and the horizontal axis in Figure 8A represents the protein molecular weight and the vertical axis represents the number of proteins. We can see that protein weight distribution that has a value of 10~20, 20~30, 30~40, 40~50, 50~60, and 60~70 has significantly increased number of proteins. To further identify the number of proteins, the protein ratio distribution is also utilized to investigate the protein weight distribution. As is shown in Figure 8B. The horizontal axis in Figure 8B represents the fold change (log2) and the vertical axis represents the number of proteins, these results show that proteins mainly distribute from -1 to 1. To further clarify the relationship between the protein and peptide. A spindle chart regarding protein and peptide is established to identify the protein. These outcomes are shown in Figure 8C. The horizontal axis represents protein expression the fold change (log2) and the vertical axis represents peptide LFQ intensity. These results show that 16 protein expression levels in the experiment group are clearly up-regulated compared to those in the control group, of which 13 protein expression levels are significantly difference. However, our outcomes also show that 12 protein expression levels in the experiment group are clearly down-regulated compared to those in the control group, of which 2 protein expression levels are significantly difference.

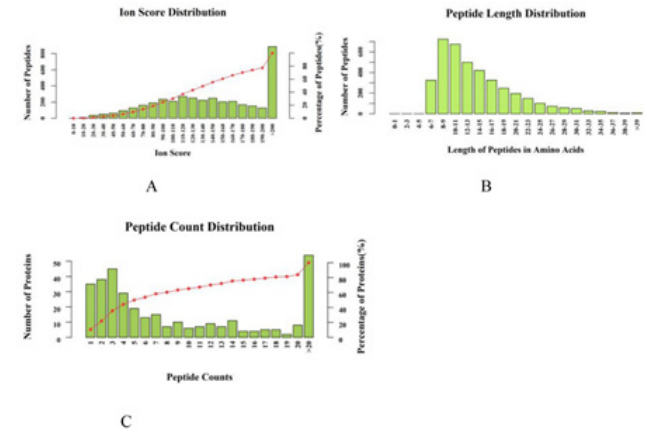

Figure 7. The peptide ion score distribution, peptide length distribution, and peptide count distribution between the control group and experiment group are shown in Figures (A), (B), and (C), respectively.

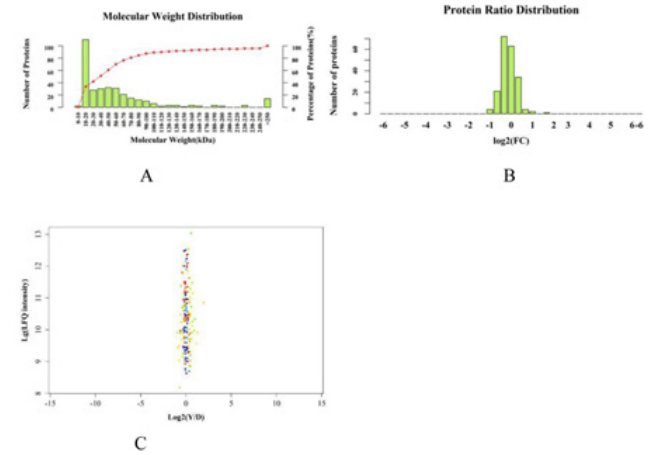

Figure 8. The molecular weight distribution, protein ratio distribution, and spindle chart regarding protein and peptide between the control group and experiment group are shown in (A), (B), and (C), respectively.

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Protein analysis

To further investigate the protein relating to the hyperlipidemia, protein weight distribution is performed, and the protein weight distribution result is depicted in Figure 8A, and we totally identify 333 protein groups from two groups of samples, and the horizontal axis in Figure 8A represents the protein molecular weight and the vertical axis represents the number of proteins. We can see that protein weight distribution that has a value of 10~20, 20~30, 30~40, 40~50, 50~60, and 60~70 has significantly increased number of proteins. To further identify the number of proteins, the protein ratio distribution is also utilized to investigate the protein weight distribution. As is shown in Figure 8B. The horizontal axis in Figure 8B represents the fold change (log2) and the vertical axis represents the number of proteins, these results show that proteins mainly distribute from -1 to 1. To further clarify the relationship between the protein and peptide. A spindle chart regarding protein and peptide is established to identify the protein. These outcomes are shown in Figure 8C. The horizontal axis represents protein expression the fold change (log2) and the vertical axis represents peptide LFQ intensity. These results show that 16 protein expression levels in the experiment group are clearly up-regulated compared to those in the control group, of which 13 protein expression levels are significantly difference. However, our outcomes also show that 12 protein expression levels in the experiment group are clearly down-regulated compared to those in the control group, of which 2 protein expression levels are significantly difference.

Western-blotting results

Western blotting was utilized to assess the expression level of C1S, AGT, HBB, and HBA1 protein, and these results show that the expression levels of C1S, and AGT in the model group were significantly higher than those in the control group ($P<0.05$). Expression levels of HBB, and HBA1 in the model group were significantly lower than those in the control group ($P<0.05$) (Figure 9).

RT-PCR results

RT-PCR was utilized to assess the expression level of C1S, AGT, HBB, and HBA1 mRNA, and these results show that the expression levels of C1S, and AGT mRNA

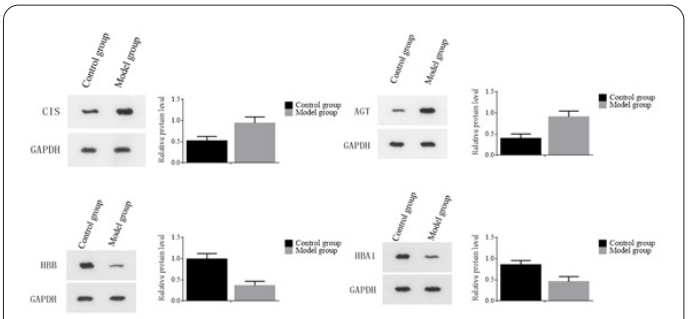

Figure 9. The expression level of C1S, AGT, HBB, HBA1 were determined using Western-blotting.

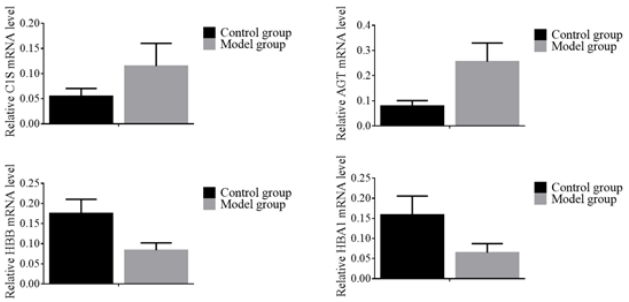

Figure 10. The mRNA level of C1S, AGT, HBB, HBA1 were determined using RT-PCR.

in the model group were significantly higher than those in the control group (P<0.05). The expression levels of HBB and HBA1 mRNA in the model group were significantly lower than those in the control group (P<0.05) (Figure 10).

Discussion

Clinically, a high-fat diet is a kind of disease that has a close correlation with multiple diseases such as stroke, atherosclerosis, and diabetes, and these diseases have seriously threatened human lives (13,14). It is urgent to take effective actions to relieve or prevent the process of hyperlipidemia. Currently, many drugs have been used to treat patients with hyperlipidemia, but the therapeutic effect is still limited (15,16). Generally, previous studies have reported that patients with hyperlipidemia aged from 22 to 55 years old, the patients in our study have a similar age (17). To find the therapeutic methods of treating hyperlipidemia, we need to conduct a series of studies to investigate the detailed pathogenesis of hyperlipidemia, especially finding the key protein target that can control the development of disease (18). Interestingly, bioinformatics analysis is a type of systematic method that can indicate the genes, proteins, and downstream signal pathways that participate in the development of disease. Therefore, bioinformatics analysis including gene ontology (GO) and enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in our study is used to indicate the genes, proteins, and pathways. In addition, to verify these proteins involved in hyperlipidemia, label-free technology in our study is used to test proteins (19,20). This paper indicates the genes, proteins, and signal pathways involved in hyperlipidemia by bioinformatics analysis. Followed by a label-free technology to test the proteins by bioinformatics analysis.

Initially, these outcomes in our study indicate differentially expressed genes by GO functional analysis. In addition, we also have indicated some proteins involved in hyperlipidemia, proteins such as P09871, P01019, P48740, P02654, and P02649, these proteins in hyperlipidemia patients have clearly up-regulated. Our results show that these proteins related to the biological process refer to binding, catalytic activity, molecular function regulator, structural molecular activity, and molecular transducer function. Abnormal expression of these proteins possibly participates in the development by regulating these biological functions. Some previous studies have pointed out that PPARγ1 or γ2 up-regulation accelerates the development of hyperlipidemia by regulating the binding function, PON1 up-regulation can relieve the process of hyperlipidemia by controlling the catalytic activity function, enteroendocrine cells can accelerate the disease progress by molecular transducer function (21-23) we can conclude that our results are consistent with those study result reported by regarding studies. After that, GO enrichment analysis is used to obtain the significant level of a GO term by comparing the distribution of GO term, and GO enrichment analysis in our study is utilized to analyze the differential protein functions, such as selenium binding, extracellular matrix binding, polysaccharide binding, and these differential proteins have associated with hyperlipidemia pathogenic progression by regulating these protein molecular function, and these results are consistent with responding previous studies (24,25).

Besides, we further identify the differentially expression protein downstream regarding the signal pathway involved in the hyperlipidemia pathogenic progression by KEGG pathway function and enrichment analysis. These outcomes in our study indicate differential pathways by KEGG pathway function analysis. These pathways' functions mainly refer to the phagosome, pathogenic Escherichia coli infection, tuberculosis, hematopoietic cell lineage, amoebiasis, and these pathways in hyperlipidemia patients have aberrant activation. Abnormal activation of these pathways is possibly associated with hyperlipidemia pathogenicity development by regulating these biological functions. Some previous studies have reported that phagosome participates in the hyperlipidemia process by maintaining cellular homeostasis, pathogenic Escherichia coli infection can regulate the process of hyperlipidemia by the mitochondria-mediated pathway, and up-regulation of tuberculosis can accelerate the hyperlipidemia progress (26-28) we can see that our results are consistent with those result reported by responding previous studies. Then KEGG enrichment analysis is used to obtain the significant signal pathways, and KEGG enrichment pathways including renin-angiotensin system, malaria. These different pathways have been associated with hyperlipidemia pathogenic progression by regulating these pathways and downstream molecular targets, and these results are consistent with responding to previous studies (29).

To further identify the proteins relating to hyperlipidemia, a protein cluster is used to investigate the significant proteins, and our results show that P80748, P69905, P68871, P00738, P01714 have close correlation with the hyperlipidemia process. In addition, label-free method including Mass spectrometry analysis is utilized to identify the peptides and protein. For the peptides, we mainly identify sp|P05154|IPSP_HUMAN, sp|P0C0L4|CO4A_HUMAN, sp|P0C0L5|CO4B_HUMAN, sp|Q92954|PRG4_HUMAN, sp|P0C0L4|CO4A_HUMAN; sp|P0C0L5|CO4B_HUMAN. We identify in protein section IGLV4-69, IGLV8-61, IGLV4-60, IGLV10-54 and so on. These proteins may contribute to the pathogenesis of hyperlipidemia.

In conclusion, the findings in this study have indicated that some genes, proteins, and downstream pathways can participate in the hyperlipidemia process. This research could provide a potential therapeutic target in hyperlipidemia and it may provide some suggestions for the treatment and diagnosis of hyperlipidemia by regulating genes, proteins, and downstream pathways. However, the sample size is relatively small in this study, followed by a large sample study needs to be conducted to identify the

detailed proteins. The specific regulation mechanisms of abnormal proteins in hyperlipidemia patients are required to be explored. Our findings provide a new perspective for studies on the treatment of high-fat diets using label-free combined with bioinformatics analysis. Thus, we can accurately find these proteins involved in the process of hyperlipidemia and conduct targeted therapy of hyperlipidemia and effectively slow or prevent the development of hyperlipidemia.

Ethics approval and consent to participate

The ethics approval was obtained from the Ethic Committee of The Wuxi Maternal and Child Health Hospital, Wuxi School of Medicine, Jiangnan University and written informed consent was obtained from all patients.

Conflict of Interests

The authors declared no conflict of interest.

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