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Identification of Differential Expression Cytokines in Hemolysis, Elevated Liver Enzymes, and Low Platelet Syndrome by Proteome Microarray Analysis and Further Verification

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Abstract

To screen the differential expression cytokines (DECs) in hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome, establish its differential cytokines spectra, and provide the clues for its diagnosis and pathogenic mechanism researches. Sera from four HELLP syndrome patients and four healthy controls were detected by proteome microarray. Then the analysis of Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and protein–protein interaction (PPI) network were performed and possible hub proteins were selected out, further verified by Enzyme Linked Immunosorbent Assay (ELISA) in sera from 21 HELLP syndrome patients and 21 healthy controls. Thirty DECs were defined according to *P*-value and fold change between HELLP group and control group. GO enrichment analysis showed that DECs were mainly involved in the regulation of inflammatory response and have relationship to growth factor binding, transmembrane receptor protein kinase, and cytokine receptor activity. Seven possible hub proteins were defined by PPI analysis, including IGFBP-3/Follistatin-like 1/FLRG/Fetuin A and MMP-13/Thrombospondin-5/Aggrecan. ELISA showed higher serum levels of Fetuin A/IGFBP-3/FLGR/MMP-13/Thrombospondin-5 in HELLP group than those in controls, while the levels of Follistatin-like 1 and Aggrecan were lower in HELLP patients (all $P < 0.05$ or <0.01).The serological DECs spectra of HELLP syndrome was established and seven possible hub proteins that may be more closely related to the disease have been verified, providing new clues for its pathogenesis, diagnosis, and clinical treatment.

Keywords

differential expression cytokines, HELLP syndrome, proteome microarray, hub proteins

Introduction

Hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome is a severe complication of pregnant women[1]. It often occurs in cases of preeclampsia (PE). Its clinical manifestations are various, typical clinical manifestations are fatigue, right upper abdominal pain and nausea and vomiting, sudden weight gain, pulse pressure widening, but a few patients without hypertension, proteinuria clinical manifestations[2]. The incidence being about 0.17% to 0.85% during pregnancy, while the maternal and perinatal mortality reaching up to 23.1% and 56.9%, respectively, HELLP syndrome poses a serious threat to the gravida and fetus[3].

Researchers have studied the pathogenesis of HELLP syndrome in many aspects, such as genetics, immunology, inflammatory response, etc., but the exact etiology still remains unclear[4–6]. The conventional view is that the main pathological changes of HELLP syndrome are basically the

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same as those of PE, while others consider it as a separate disease with its own unique etiology.

Cytokines play an important role in apoptosis, angiogenesis, innate immunity, cell growth, and differentiation. They are involved in many disease processes, including cancer and cardiovascular diseases[7]. Although the exact reason has not been fully understood yet, inappropriate activation of cytokines which lead to a series of pathophysiological changes seems to be the basis of HELLP syndrome[1].

In this study, sera from patients with HELLP syndrome and healthy pregnant women were detected by proteome microarrays, then HELLP syndrome high response differential cytokines were screened out. By bioinformatic analysis, possible hub proteins were found and tested further through more samples. The aim was to explore the pathogenesis of HELLP syndrome and provides theoretical clues for early diagnosis and clinical treatment.

Materials and Methods

Patients and Samples

This study was performed in accordance with the Declaration of Suzhou Affiliated Hospital of Nanjing Medical University, Suzhou, China and approved by the Ethics Committee Suzhou Affiliated Hospital of Nanjing Medical University, Suzhou, China. Ethical approval was obtained from the Ethics Committee of Suzhou Affiliated Hospital of Nanjing Medical University. All participants signed a consent form before the study.

Patients with HELLP syndrome admitted to Suzhou Municipal Hospital (Affiliated Suzhou Hospital, Nanjing Medical University) between 2018 and August 2019 were selected as HELLP group. The inclusion criteria of the HELLP group were in accordance with Mississippi diagnostic and classification Criteria platelets $< 150\times10^9$/l, coupled with an elevated lactate dehydrogenase > 600 IU/l; aspartate aminotransferase and/or alanine aminotransferase $\geq$ 70 U/l. Patients were divided into the class I to III based on the platelet count (class I: under 50×10^9/l, class II: 50–100 $\times 10^9$/l, and class III: 100–150 $\times 10^9$/l)[8]. Those with other hematological or hepatic diseases that also induced low platelets count or hepatic dysfunction were excluded. The normal control group (NC group) was from healthy pregnant women who were hospitalized for delivery at the same time, laboratory indicators of whom were all within the normal reference range.

Instruments and Reagents

For Microarray Analysis. G-Series Human Cytokine Antibody Array 440 kit (RayBiotech Inc., Guangzhou, Guangdong, China), a combination of 11 non-overlapping arrays to measure the relative expression levels of 440 human cytokines catalog, was used. The kit included Quantibody®Array glass chip, sample diluent, 20× wash buffer I, 20× wash buffer II, detection antibody cocktail, Cy3 equivalent dye-conjugated streptavidin, slide washer/dryer, adhesive device sealer, and manual. Additional main materials required were InnoScan 300 microarray scanner (Innopsys Company, Carbonne, France) and Thermo Scientific Wellwash Versa (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RayBio array data analysis tool (catalog number: GSH-CAA-440-SW, RayBiotech Inc.) was also used in the study.

For Enzyme Linked Immunosorbent Assay (ELISA). RayBiotech Human Fetuin A ELISA Kit, RayBiotech Human ACAN ELISA Kit, RayBiotech Human FLRG ELISA Kit, RayBiotech Human FSTL1 ELISA Kit, RayBiotech Human IGFBP3 ELISA Kit, RayBiotech Human TSP5 ELISA Kit, and RayBiotech Human MMP-13 ELISA Kit were used (RayBiotech Inc.). Biotek Elx800 ELISA Reader, Biotek Elx50 Microplate Washer (Biotek Instruments Inc., Burlington, VM, USA), and Sigmalplot 12.0 Analysis Software (Systat Software Inc., San Jose, CA, USA) were also used.

Methods

Venous blood samples were collected from patients and controls at the time of admission into hospital, before the initiation of any medical treatment. Two milliliters of venous blood samples were drawn into ethylenediaminetetraacetic acid tubes, let stand for 10 min, centrifuged at 3,000 r/min. Then sera were separated and packed, froze at -80°C for later use.

Microarray Analysis

Sera of four patients in HELLP group and four in NC group were detected by proteome microarrays, clinical information of which was shown in Table 1.

Microarray hybridization was carried out by RayBiotech Inc. The raw data obtained from the microarray scanning were processed by software for background removal and inter-microarray normalization, and then been analyzed. RayBio array data analysis tool (catalog number: GSH-CAA-440-SW) was used for data analysis. P value and fold change (FC) were used to screen differential proteins. Selection conditions were as follows: (1) FC = <0.83 or FC = >1.2, (2) fluorescence signal > 150, and (3) $P < 0.05$. Differential proteins were screened and visualized with volcano plot. The graph was drawn from the ggplot function and the packet was ggfortify from R/bioconductor.

Bioinformatics Analysis

Enrichment Analysis of Differential Proteins. With R package "clusterProfiler," protein function annotation Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were analyzed; $P < 0.05$ and the count $\geq$2 were set as threshold to indicate statistical significance. GO included biological process (BP), cellular component (CC), and molecular function (MF) of the proteins. By

Table 1. Basic Information of Samples for Proteome Microarrays (*$P < 0.05$; **$P < 0.01$).

	HELLP ($n = 4$)	Normal control ($n = 4$)	t/Z	P
Maternal age (years)	30.5 ± 4.8	26.5 ± 4.0	1.276	0.249
Gestational age at delivery (weeks)	35.2(31.5, 36.1)	39.5(36.1, 39.9)	−1.607	0.108
Nulliparity (%)	1 (25%)	4 (100%)	–	0.143
Systolic pressure (mmHg)	162.3 ± 26.7	122.3 ± 7.1	2.898	0.027*
Diastolic pressure (mmHg)	103.3 ± 12.5	73.3 ± 3.6	4.622	0.014*
Hemoglobin (g/l)	95.5 ± 15.1	113.0 ± 6.7	−2.061	0.085
Platelet count ($\times 10^9$/l)	69.0 ± 5.7	187.5 ± 25.0	−9.256	0.000**
ALT (U/l)	150.0 ± 44.5	11.3 ± 3.3	6.216	0.001**
AST (U/l)	212.2 ± 189.7	20.7 ± 6.4	2.018	0.137
LDH (U/l)	1185.0 (521.3, 8454.8)	187.5 (125.5, 268.3)	−2.309	0.021*
Singleton pregnancy (%)	3 (75%)	4 (100%)	–	1.000
Neonatal weight (g)	2120.0 ± 672.3	3275.0 ± 590.9	−2.696	0.031*
ART (%)	3 (75%)	2 (50%)	–	1.000
Cesarean delivery (%)	4 (100%)	2 (50%)	–	0.429

ALT: alanine aminotransferase; AST: aminotransferase; HELLP: hemolysis, elevated liver enzymes, and low platelet; LDH: lactate dehydrogenase; ART: assisted reproductive technology.

Table 2. Basic Information of Samples for ELISA (*$P < 0.05$; **$P < 0.01$).

	HELLP ($n = 21$)	Normal control ($n = 21$)	$t/Z/\chi^2$	P
Maternal age (years)	29 (26.5, 34.0)	30 (26.0, 31.5)	−0.543	0.587
Gestational age at delivery (weeks)	34.1 (31.1, 36.1)	39.3 (37.8, 40.1)	−4.379	0.000**
Nulliparity (%)	13 (61.9%)	13 (61.9%)	0.000	1.000
Systolic pressure (mmHg)	164.3 ± 17.5	123.2 ± 8.1	9.757	0.000**
Diastolic pressure (mmHg)	106.9 ± 14.9	74.5 ± 8.4	8.703	0.000**
Hemoglobin (g/l)	99.0 ± 17.2	113.4 ± 13.5	−3.027	0.004**
Platelet count ($\times 10^9$/l)	80.0 (55.5, 90.0)	203.0 (159.5, 252.5)	−5.372	0.000**
ALT (U/l)	104.0 (59.5, 248.0)	7.0 (6.0, 10.5)	5.556	0.000**
AST (U/l)	113.0 (61.5, 233.5)	16.0 (14.0, 19.5)	−5.514	0.000**
LDH (U/l)	1377.0 (1316.0, 1657.0)	186.0 (173.0, 202.0)	−5.548	0.000**
Singleton pregnancy (%)	17 (81%)	21 (100%)	–	0.107
Neonatal weight (g)	1923.8 ± 721.2	3226.2 ± 501.7	−6.793	0.000**
ART (%)	8 (38.1%)	2 (9.5%)	3.281	0.070
Cesarean delivery (%)	20 (95.2%)	3 (14.3%)	24.604	0.000**

ALT: alanine aminotransferase; AST: aminotransferase; HELLP: hemolysis, elevated liver enzymes, and low platelet; LDH: lactate dehydrogenase.

Metascape, the systematic analysis of gene functions supplied pathways which genes that encoded the differential proteins were performed.

Screening of Hub Proteins and Analysis. Protein–protein interaction (PPI) analysis was also conducted by Metascape database. By PPI analysis, hub proteins were screened and then analyzed further, including principal component analysis (PCA) and correlation analysis.

ELISA

Levels of proteins selected from differential expression cytokines (DECs) by bioinformatics analysis in sera of 21 HELLP patients as well as 21 normal pregnant women were measured by ELISA, according to the protocol. Clinical information of which was shown in Table 2.

Statistical Analysis

SPSS Statistics for Windows, Version 22.0 (Chicago, IL, USA) was used for statistical analysis. The chi-square test or Fisher's exact test was used to compare enumeration data. Measurement data were first analyzed by normality test to see whether they followed normal distribution. Data that followed normal distribution were expressed as mean ± standard deviation ($\bar{x} \pm S$) and compared by t-test, otherwise were expressed by median (percentile) [M (P_{25}, P_{75})] and compared by Mann–Whitney U test. Correlation was performed by Pearson correlation analysis. Values of $P < 0.05$ indicate statistical significance.

Results

Differential Expression Cytokines

According to the P value and FC between HELLP group and NC group, 30 DECs were defined as shown in Table 3 and

Table 3. Defined 30 Differential Expression Cytokines Between HELLP and NC Group.

Protein	Gene (encode the protein)	Mean signal expression		FC HELLP/NC	Log 2FC	*P* value
		HELLP	Normal control			
Upregulated						
RAGE	AGER	55344.94258	18739.06462	2.953452785	1.562351629	0.040081102
FLRG	FSTL3	93913.02936	32024.45818	2.932540773	1.55212148	0.0224501
MMP-13	MMP13	47920.05011	18380.14032	2.607164542	1.382433253	0.00920607
IL-21R	IL21R	1132.400811	456.4429825	2.48092501	1.308994303	0.047435471
MIP-3a	CCL20	2997.047174	1312.547301	2.283382223	1.190554931	0.047265659
Ferritin	FTL	181147.4191	103401.8874	1.751877298	0.808895744	0.011545903
TRAIL R3	TNFRSF10C	116488.3458	67269.51029	1.731666327	0.792151904	0.010531219
IL-10 Rb	IL10RB	1165.049139	716.1800722	1.626754477	0.70122128	0.039992873
PDGF Rb	PDGFRB	2604.193355	1703.319988	1.528892617	0.612194223	0.01026263
IGFBP-3	IGFBP3	24015.04982	15767.68579	1.523054819	0.606936449	0.022484868
CTACK	CCL27	11838.84087	7910.055316	1.496682437	0.581707626	0.013309029
Dtk	TYRO3	7711.249859	5156.771294	1.495363944	0.58040399	0.039567733
PECAM-1	PECAM1	10927.22475	7309.562196	1.494921919	0.580004798	0.006090797
GITR	TNFRSF18	2073.946451	1405.988786	1.475080365	0.560463275	0.011065063
VCAM-1	VCAM1	228590.7334	161450.2459	1.415858689	0.501674658	0.008462301
CD30	TNFRSF8	2025.891898	1522.673156	1.330483755	0.411715684	0.03346108
Fetuin A	AHSG	23390.20892	17631.93943	1.326581741	0.407693432	0.006653246
Trappin-2	PI3	111713.5764	89278.584	1.251291983	0.32341523	0.04207554
IL-1 RI	IL1R1	3803.074207	3070.026816	1.238775566	0.30882428	0.00354943
bIG-H3	TGFBI	33684.72848	27261.16512	1.235630551	0.305237356	0.012271485
Downregulated						
Thrombospondin-5	COMP	220155.9758	269378.5321	0.817273649	−0.291107679	0.012899927
Cathepsin S	CTSS	23238.77196	29614.4761	0.784709879	−0.349755367	0.006800493
ErbB4	ERBB4	1154.414913	1913.060203	0.603438884	−0.728225187	0.044980391
TRAIL	TNFSF10	1479.745172	2535.902518	0.58351816	−0.776744702	0.040624467
CD155	PVR	2907.491495	5426.298189	0.535814913	−0.899963091	0.031779621
Aggrecan	ACAN	1798.795306	4012.54303	0.448293088	−1.15704353	0.023068987
BMPR-IA	BMPR1A	1287.116107	3047.725698	0.422320194	−1.242943714	0.030161097
Follistatin-like 1	FSTL1	1190.962214	3239.408629	0.367648034	−1.442837253	0.034498459
Siglec-5	SIGLEC5	70458.43998	216640.9958	0.325231334	−1.620448021	0.044199358
BMPR-II	BMPR2	870.0932976	2823.872595	0.308120593	−1.697286651	0.018142872

FC: fold change; HELLP: hemolysis, elevated liver enzymes, and low platelet; NC: normal control.

presented as Volcano plot (Fig. 1). Compared with NC group, 20 DECs were upregulated in HELLP groups, while the other 10 DECs were downregulated. By Euclidean distance and complete cluster, dissimilarities between two groups were analyzed and heatmap was conducted on all DECs (Fig. 2).

Enrichment Analysis of the DECs

In terms of BP, CC, and MF, the GO analysis showed that the DECs are mainly distributed in nucleolar part of cell, plasma membrane, lysosomal lumen, vacuolar lumen, endoplasmic reticulum lumen, extracellular matrix, receptor complex, and fibrillar center (Fig. 3B). They participate in regulation of ERK1 and ERK2 cascade, aging, regulation of inflammatory response, regulation of lymphocyte activation, regulation of adaptive immune response and T cell–mediated immunity, positive regulation of chemotaxis, and cell adhesion (Fig. 3A). Their function mainly involved in S100 protein/peptide/amide/collagen/growth factor/amyloid-beta/fibronectin binding, also involved in transmembrane receptor protein kinase, cytokine/cargo/scavenger receptor activity (Fig. 3C).

The KEGG pathway analysis revealed that the DECs mainly focus on cytokine–cytokine receptor interaction, fluid shear stress and atherosclerosis, extracellular matrix organization, integrin 3 pathway, and bone morphogenetic protein. They also involved in the pathway about signaling by transforming growth factor beta (TGF-β) family members, interleukins, and post-translational protein phosphorylation (Table 4, Fig. 4).

Analysis of PPI Network

By analysis of PPI, interactions among DECs were investigated. Seven hub proteins were defined, including IGFBP-3/Follistatin-like 1/FLRG/Fetuin A and MMP-13/Thrombospondin-5 (TSP-5)/Aggrecan. The expression of these hub proteins between HELLP and NC groups showed significant differences (Fig. 5B). Two subsets were identified,

Table 4. Enriched Pathway Terms of Genes Encode the DECs.

Term	Count	*P*	Fold enrichment	Genes encode the DECs in the pathway
Malaria	3	0.043777875	49.29591837	COMP/PECAM1/VCAM1
Fluid shear stress and atherosclerosis	5	0.014762912	28.35093897	BMPR1A/BMPR2/IL1R1/PECAM1/VCAM1
Cytokine–cytokine receptor interaction	12	0.000019224	35.78518519	BMPR1A/BMPR2/TNFRSF8/IL1R1/IL10RB/PDGFRB/CCL20/ TNFSF10/TNFRSF18/TNFRSF10C/CCL27/IL21R
PID BMP pathway	3	0.038034467	57.51190476	AHSG/BMPR1A/BMPR2
NABA Matrisome associated	8	0.02115603	8.554227534	CTSS/MMP13/PI3/CCL20/TNFSF10/FSTL3/CCL27/ FSTL1
PID Integrin3 pathway	4	0.010046786	74.89922481	PDGFRB/PECAM1/PVR/TGFBI
Interleukin-10 signaling	3	0.042147959	51.39361702	IL1R1/IL10RB/CCL20
Post-translational protein phosphorylation	4	0.030740818	29.82098765	AHSG/IGFBP3/FSTL3/FSTL1
Signaling by TGF-beta family members	4	0.02871201	31.5751634	BMPR1A/BMPR2/FSTL3/FSTL1
Signaling by BMP	3	0.026168036	86.26785714	BMPR1A/BMPR2/FSTL1
Extracellular matrix organization	6	0.018148556	16.04983389	ACAN/COMP/CTSS/MMP13/PECAM1/VCAM1
Signaling by interleukins	8	0.014369523	10.15983176	AGER/ERBB4/IL1R1/IL10RB/PDGFRB/CCL20/ VCAM1/IL21R

DEC: differential expression cytokines; TGF: transforming growth factor.

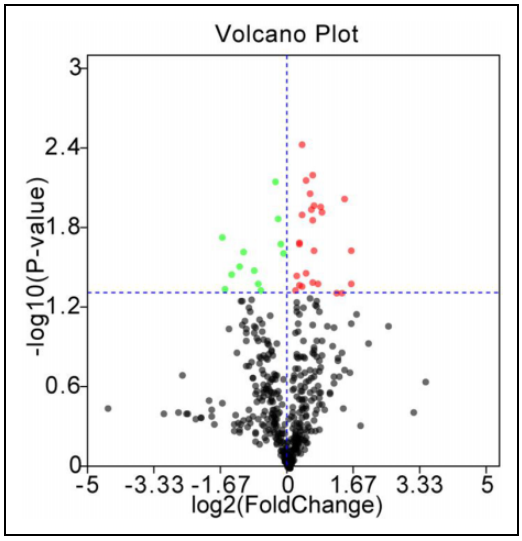

Figure 1. Volcano plot of differential expression cytokines (true presented DECs: red as upregulated and green as downregulated; black as false presented DECs). DEC: differential expression cytokine.

the first four proteins composed one subset (PPI-1), while the latter three composed another subset (PPI-2) (Fig. 5A).

According to the expression of these seven hub proteins, correlation analysis of the PCA was performed. Four proteins in PPI-1 were found to be highly correlated, while the other three proteins in PPI-2 were also highly correlated, as shown in Table 5 and the heatmap (Fig. 6).

Comparation of Levels of Seven Hub Proteins Between HELLP Group and NC Group by Further ELISA Test

ELISA results showed that the serum levels of Fetuin A/IGFBP-3/FLGR/MMP-13/TSP-5 in HELLP group were higher than those in NC group, while the levels of follistatin like 1 and aggrecan in HELLP group were lower, as shown in Table 6 and Fig. 7.

Discussion

Being a threat to the gravida and fetus, HELLP syndrome is a disease to which clinicians attach great importance. It is currently believed that a series of pathophysiological changes caused by improper activation of cytokines may be the basis of HELLP syndrome[1]. With the development of biological technology, proteome microarray analysis has been widely used in the screening of various disease markers, providing the possibility to study the pathogenesis of HELLP syndrome and search for more symbolic serological indicators of it.

In this study, a proteome microarray analysis related to 440 human cytokines was used to establish a serological differential cytokines spectra of HELLP syndrome including 30 potentially differential expression proteins.

Bioinformatics analysis screened out[7] possible hub proteins (IGFBP-3/Follistatin-like 1/FLRG/Fetuin A/MMP-13/TSP-5/Aggrecan) from the 30 DECs, and further verified them in more sera samples of HELLP syndrome patients and healthy pregnant women by ELISA. The results of ELISA were generally agree with those of the microarray analysis in earlier stage, except that ELSIA revealed that the expression of TSP-5 was higher in HELLP than NCs, while the microarray analysis showed it was lower in HELLP[8].

Recent exploration provided possible evidence for the concept that several placental derived factors are involved in hepatic injury and cause increased apoptosis of the liver sinusoidal endothelial cells (LSECs)[9]. Insulin-like growth factor-binding protein (IGFBP), containing IGFBP-1 to -6,

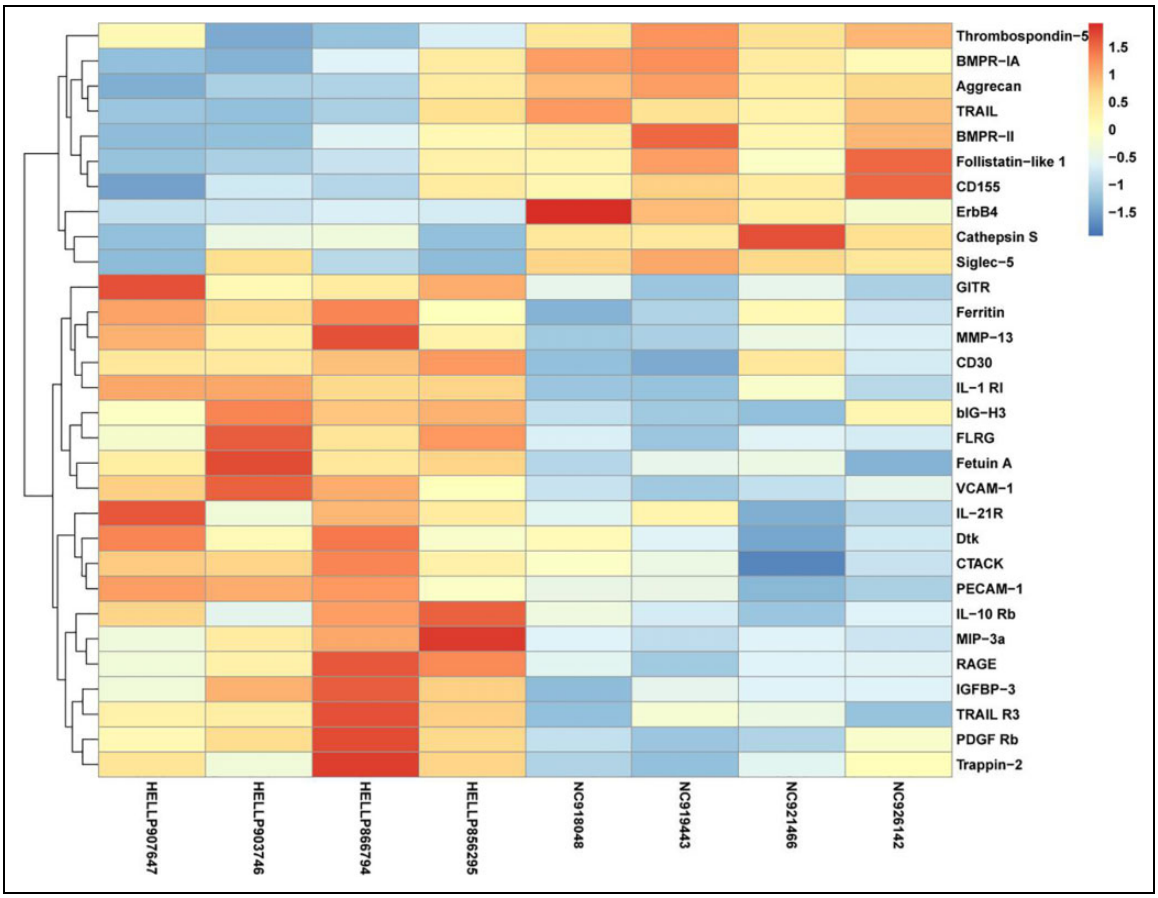

Figure 2. Clustering heatmap (red presented high expression; blue presented low expression. The first four specimen numbers on the horizontal axis presented HELLP group; the last four presented normal control group). HELLP: hemolysis, elevated liver enzymes, and low platelet.

is a super family of proteins that bind highly specifically to insulin-like growth factors (IGFs). IGFBP-3 is the most abundant IGFBP in serum and over 90% of IGF-1 in the body binds to it in the form of complex[10]. Human IGFBP-3 is mainly produced in LSECs and hepatocytes, which are also the main production sites of IGFs. It is also produced in other tissues, especially in the kidneys, intestines, uterus, and placenta[11].

Several studies have shown that both Kupffer cells regulating inflammation and HSCs regulating fibrosis can express IGF-1 R, which may affect the downregulation of serum IGF-1 level and upregulation of IGFBP-3[12]. It has been confirmed that IGFBP-3 is significantly upregulated and positively correlated with disease progression in serum of patients with nonalcoholic fatty liver disease (NAFLD)[13,14]. IGFBP-3 is possibly to activate the Smad pathway by binding to TGF-β receptors leading to apoptosis[15]. In this study, the expression of IGFBP-3 in the serum of HELLP syndrome patients was significantly higher than that of normal pregnant women. Therefore, it is speculated that the higher expression of IGFBP-3 increases the apoptosis of LSECs and leads to hepatocyte destruction, thus plays an important role in the pathogenesis of HELLP syndrome.

Fetuin A is a plasma glycoprotein with various functions synthesized by hepatocytes, adipocytes, osteoblasts and osteoclasts, involving in the regulation of calcium and phosphorus metabolism, ectopic calcium and phosphorus deposition as well as bone reconstruction[16,17]. It has been found that Fetuin-A can stimulate the expression of interleukin-6, monocyte chemotaxis protein-1, intercellular adhesion molecule-1, and selectin-D in human umbilical vein endothelium to promote local monocyte aggregation and inflammation progression[18–20]. And insulin resistance could lead to the decrease of nitric oxide synthesis and the increase of vasoactive mediators that promote vasoconstriction, further injury to endothelial function[21]. In this study, serum level of Fetuin A was found higher in HELLP patients than health gravidae. Combining existing research on Fetuin A, we infer that Fetuin A may cause the injury of vascular endothelial cells (ECs) by the promotion of insulin resistance and inflammatory response, thus induces the HELLP syndrome. But few studies have been found about the relationship between Fetuin A and HELLP syndrome.

Studies have shown FLRG may play a role in tissue damage repair[22–25]. And Mukherjee et al. found that FLRG-deficient adults had reduced visceral fat, increased glucose

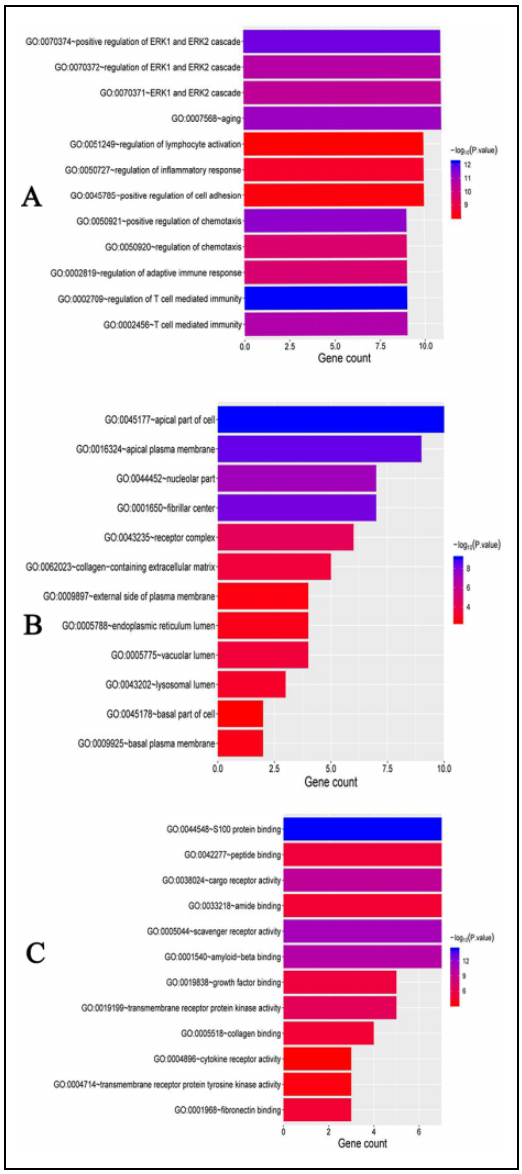

Figure 3. Results of GO analysis (A: BP, B: CC, and C: MF). BP: biological process; CC: cellular component; GO: Gene Ontology; MF: molecular function.

tolerance, and insulin sensitivity, speculating that FLRG might be related to insulin resistance[26]. Previous study found that protein and mRNA expression of FLRG in both serum and placenta of PE patients were significantly increased[27]. Another study showed that hypoxia culture significantly enhanced the expression of FLRG by trophoblast, while downregulation of it suppressed the proliferation, migration, invasion, and lipid storage but increased apoptosis of trophoblast, indicating its involvement in the pathogenesis of PE[28]. Earlier research that serum FLRG was positively correlated with tumor necrosis factor α and IL-6 levels, FLRG may play an important role in NAFLD by enhancing inflammatory response[29]. In this study, higher FLRG level was found in serum of HELLP patients. It can be speculated that by

Table 5. Correlation Analysis of Hub Proteins.

Protein1	Protein2	*R*	*P*
FLRG	MMP-13	0.610890999	0.107646372
FLRG	IGFBP-3	0.815711341	0.013564186
FLRG	Fetuin A	0.88519501	0.003464637
FLRG	Thrombospondin-5	−0.936187713	0.000618917
FLRG	Aggrecan	−0.591427531	0.122529535
FLRG	Follistatin-like 1	−0.571638324	0.138781641
MMP-13	IGFBP-3	0.809910691	0.014816648
MMP-13	Fetuin A	0.659310973	0.075319731
MMP-13	Thrombospondin-5	−0.757028397	0.029642575
MMP-13	Aggrecan	−0.884391368	0.003535684
MMP-13	Follistatin-like 1	−0.758014999	0.029306613
IGFBP-3	Fetuin A	0.786293941	0.020656365
IGFBP-3	Thrombospondin-5	−0.883302848	0.003633406
IGFBP-3	Aggrecan	−0.63171343	0.092928014
IGFBP-3	Follistatin-like 1	−0.50438264	0.202435971
Fetuin A	Thrombospondin-5	−0.868400959	0.005150126
Fetuin A	Aggrecan	−0.718833292	0.044509743
Fetuin A	Follistatin-like 1	−0.742328955	0.034930318
Thrombospondin-5	Aggrecan	0.713445412	0.046907137
Thrombospondin-5	Follistatin-like 1	0.718747913	0.044547142
Aggrecan	Follistatin-like 1	0.896837232	0.002536801

disturbing the TGF-β signal pathway, FLRG causes the injury of vascular ECs and liver by the participation in insulin resistance and inflammatory response, while suppresses the proliferation, migration, and invasion but increases apoptosis of trophoblast, thus plays a role in the etiology of HELLP syndrome.

Recently, it has been considered to be related to the autoimmune diseases, transplant rejection, cancer, and other diseases[30–33]. There are a few reports on FSTL1 and PE, but the conclusions are inconsistent. Ma et al. found that the expression level of FSTL1 in placenta tissues of PE patients was increased compared with normal pregnant women, as well as the serum FSTL1 levels of PE patients[34]. However, another study showed that the expression levels of FSTL1 in serum and placenta of pregnant women with PE were decreased compared with the pregnant women in control group[35]. In this study, the expression level of FSTL1 in serum of HELLP syndrome patients was lower than normal gravidae. According to available research results on FSTL1, we presume that FSTL1, as a proinflammatory mediator, plays an important role in maintaining and expanding the inflammatory response by regulation in signaling pathways such as TGF-β/Smad and NF-κB, thus participates in the pathogenesis of HELLP syndrome.

Aggrecan is the substantial proteoglycan of cartilage issues and is responsible for hydrodynamic functions, including weight bearing and elasticity[36]. Aggrecan degradation, cleaved by the reaction of matrix metalloproteinases and aggrecanses, respectively, plays a critical role in osteoarthritis (OA)[37]. Research results suggest that TGF-β1 can significantly promote aggrecan expression in vitro. Most of the existing studies on aggrecan are about bone and joint diseases[38]. In this study, the expression level of aggrecan

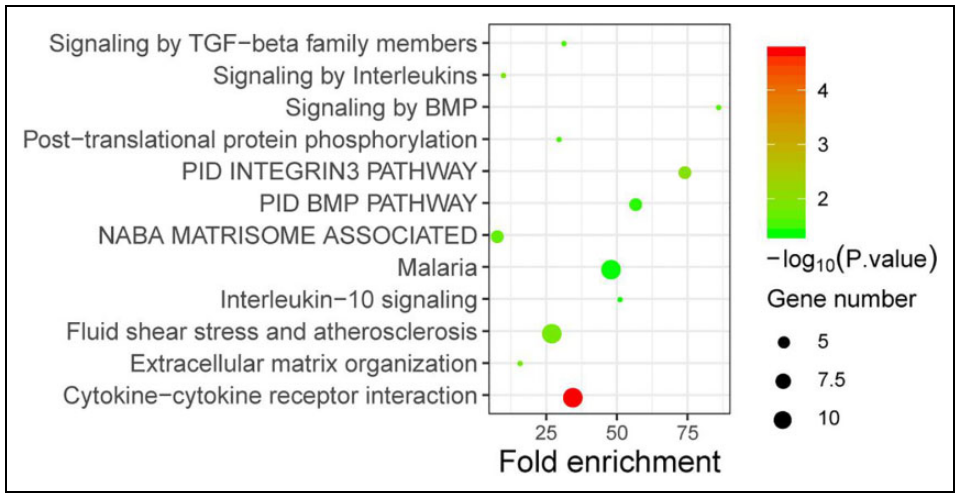

Figure 4. Results of pathway analysis.

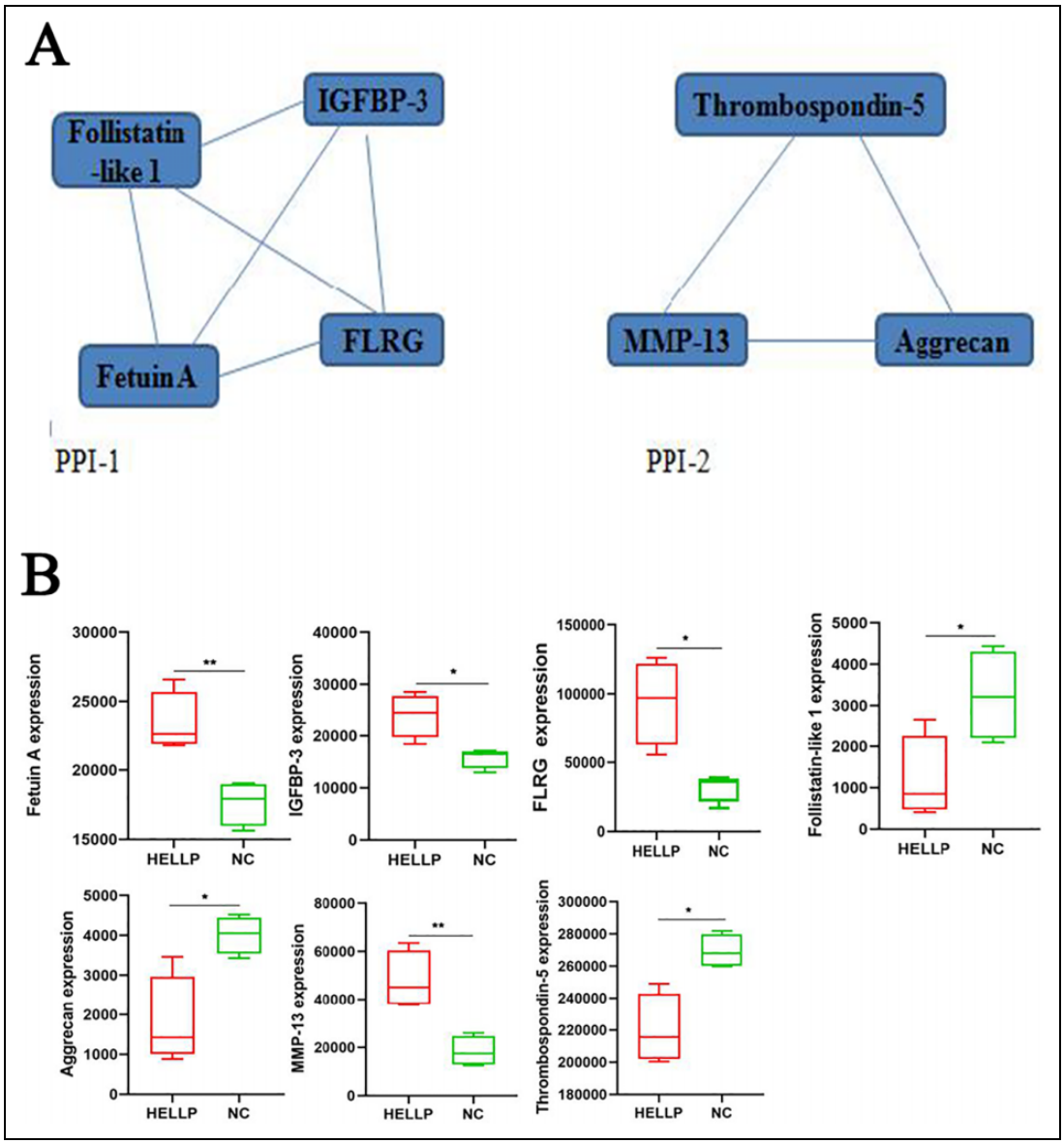

Figure 5. (A) Hub proteins and PPI subsets (PPI-1 and PPI-2). (B) Differential expression of hub proteins between HELLP and normal control group (*$P < 0.05$;**$P < 0.01$). HELLP: hemolysis, elevated liver enzymes, and low platelet; PPI: protein–protein interaction.

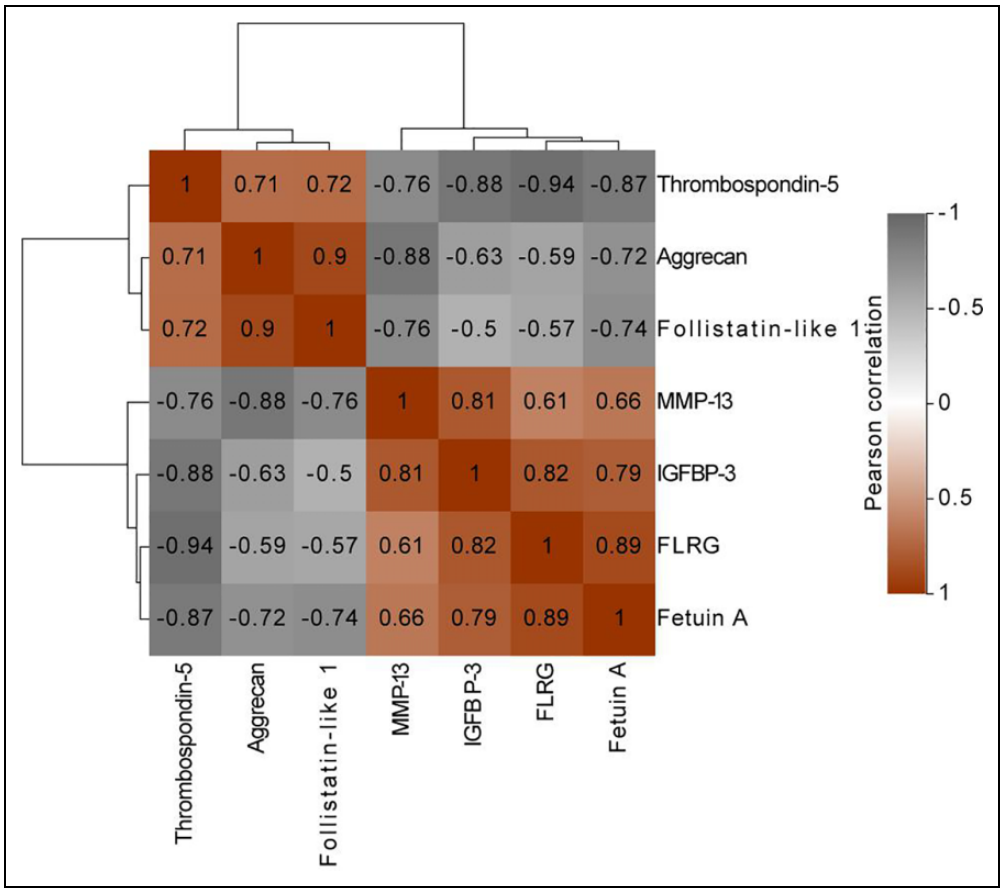

Figure 6. Correlation heatmap of hub proteins.

Table 6. ELISA Results of Seven Hub Proteins Between Two Groups (*$P < 0.05$; **$P < 0.01$).

Proteins	HELLP	Normal control	*t*/*Z*	*P*
Fetuin A (mg/ml)	3213.660 (1911.330, 4614.490)	2143.450 (1082.875, 2830.537)	−2.418	0.016*
IGFBP-3 (ng/ml)	75.163 ± 17.116	29.598 ± 9.973	−2.940	0.005**
FLGR (ng/ml)	259.977 (228.834, 404.123)	39.171 (7.772, 84.843)	−4.114	0.000**
Follistatin-like 1 (pg/ml)	916.400 (489.050, 1914.900)	3937.300 (1817.450, 5618.150)	−3.699	0.000**
Aggrecan (pg/ml)	383.440 (171.699, 479.191)	606.221 (399.294,910.708)	−3.010	0.003**
MMP-13 (ng/ml)	78.136 (51.081, 98.083)	17.648 (5.352, 40.094)	−4.765	0.000**
Thrombospondin-5 (ng/ml)	332.255 (215.251, 686.13)	102.882 (69.324, 150.871)	−4.760	0.000**

HELLP: hemolysis, elevated liver enzymes, and low platelet.

in serum of HELLP syndrome patients was lower than healthy pregnant women. There are no studies about relationship between aggrecan and HELLP syndrome. Aggrecan may also be involved in the pathogenesis of HELLP through TGF-β pathway, which needs further study.

It has been found that MMPs are not only considered as physiological mediators in maintaining of the extracellular matrix but also put forward as critical factors of the remodeling processes in pathological conditions. The expressions of MMPs have been found to increase in and associated with a variety of inflammatory diseases[39,40]. Previous studies have shown that MMP-13 was highly expressed in OA patients, suggesting that increased MMP-13 is related to cartilage degradation[41,42]. Studies showed that discoidin domain receptor 2 (DDR2) and MMP-13 may play an important role in the development of tumor angiogenesis. The expressions of DDR2 and MMP-13 upregulate in laser-induced choroidal neovascularization (CNV), suggesting MEK/ERK and P13K/Akt pathways suppress the development of CNV by regulating the expressions of DDR2 and MMP-13[43]. Studies have shown that when MMP-13 is overexpressed, it can degrade fibrinogen and affect cardiac systolic/diastolic functions, eventually leading to cardiac atrophy, suggesting that MMP-13 may be involved in the

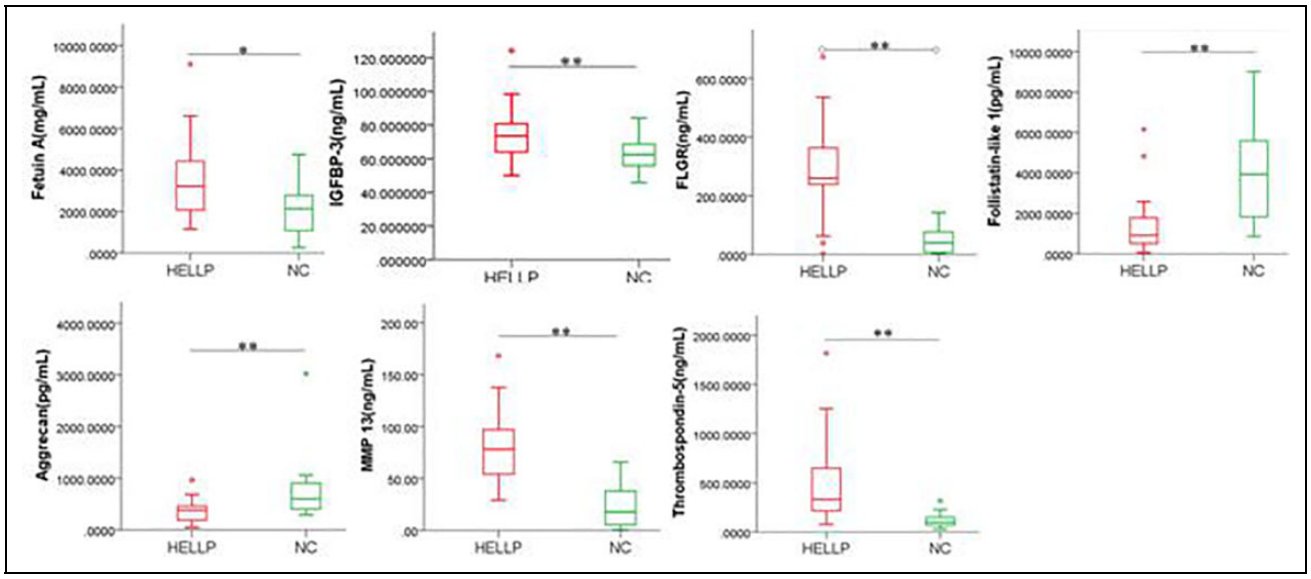

Figure 7. Differential expression of hub proteins between HELLP and normal control group in part 2 (*$P < 0.05$;**$P < 0.01$).

adverse remodeling of heart failure[44]. Increased expression of MMPs relate to pregnancy may play a role in vascular remodeling, angiogenesis, and changes in the vascular system. No previous study on the relationship between MMP-13 and HELLP syndrome was retrieved. In this study, the expression level of MMP-13 in serum of HELLP syndrome patients was higher than NCs. It can be inferred that MMP-13 may play an important role in vascular remodeling, angiogenesis, and inflammatory process, both in blood vessels and liver, through the MEK/ERK and P13K/Akt pathways, which may be related to HELLP syndrome.

TSPs are a family of oligosaccharide proteins distributed throughout vertebrates, containing TSP-1 to TSP-5, which impart a variety of functions by binding cytokines, proteases, and cell-surface receptors[45]. TSPs are critical mediators of angiogenesis and apoptosis in the process of vascular disease[46]. TSP-1 and TSP-2 are both described as strong inducers of EC apoptosis, which prevents angiogenesis, although TSP-2 has been less studied[47,48]. TSP-5 has been considered to be related to calcified vessels and vascular disease, while it has also been shown to promote vascular smooth muscle cell migration[49]. Studies have found that TSP-5 could suppress apoptotic genes and had a mixed effect on the angiogenic genes; it did not alter apoptosis but was proangiogenic. All these indicate that TSP-5 has a protective effect on ECs, and it may be beneficial for inducing angiogenesis in the setting of ischemia[50]. In a case-control study, Stenczer et al. found that the serum level TSP-2 in PE was higher than that in normal pregnant women. The study also pointed out that the increased expression level of TSP-2 may affect the function of endothelial growth factor in an independent way different from placental growth factor (PIGF) and soluble vascular endothelial growth factor receptor-1, thus played a role in angiogenesis by inhibiting EC proliferation and inducing EC apoptosis[51].

In this study, the serum level of TSP-5 in HELLP syndrome was higher than normal pregnant women as the result of microarray showed; however, the ELISA result showed the level of TSP-5 was lower in HELLP. The reason for the inconsistent results may be that the microarray analysis only tested four serum samples for each group, which could not fully reflect the true expression difference of TSP-5 between HELLP and controls. However, combining the decreased TSP-5 expression in HELLP syndrome revealed by ELISA, it is speculated that lower TSP-5 expression may weaken its protection on ECs, thus induces endothelial apoptosis of ECs and play a role in the reason of HELLP syndrome.

Conclusion

In conclusion, this study preliminarily constructed the serological DECs spectra of HELLP syndrome by protein microarray analysis, and some of the high-signal response proteins have potential application value as serological markers for early diagnosis, disease progression, and prognosis assessment. By bioinformatics analysis, seven possible hub proteins that may be more closely related to HELLP syndrome were screened out and been further verified. The specific functions of some differential proteins have not been fully understood, and studies on these seven possible hub proteins in HELLP syndrome are also extremely rare. According to previous studies, it can be speculated that through signaling pathway such as TGF-β/Smad, these proteins may play important roles in the pathological process of HELLP syndrome at the aspect of inflammation, injuries of ECs, apoptosis of LSECs, etc. In the future research, we plan to further explore these differential proteins to reveal the pathogenesis of HELLP syndrome and provides theoretical clues for early diagnosis and clinical treatment.

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Authors' Contribution

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

Availability of Data and Materials

The data are free access to available upon request.

Consent to Publish

All of the authors have consented to publish this research.

Ethical Approval

This study was approved by the Ethics Committee approved by Suzhou Affiliated Hospital of Nanjing Medical University.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with Suzhou Affiliated Hospital of Nanjing Medical University, Suzhou, China approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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