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Original Article

Differential expressions of plasma proteins in systemic lupus erythematosus patients identified by proteomic analysis

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Lupus: 2D-gel electrophoresis;
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Abstract *Introduction:* Systemic lupus erythematosus (SLE) is a chronic and complex autoimmune disease with a wide range of clinical manifestations that affects multiple organs and tissues. Therefore the differential expression of proteins in the serum/plasma have potential clinical applications when treating SLE.
Methods: We have compared the plasma/serum protein expression patterns of nineteen active SLE patients with those of twelve age-matched and gender-matched healthy controls by proteomic analysis. To investigate the differentially expressed proteins among SLE and controls, a 2-dimensional gel electrophoresis coupled with high-resolution liquid chromatography tandem mass spectrometry was performed. To further understand the molecular and biological functions of the identified proteins, PANTHER and Gene Ontology (GO) analyses were employed.
Results: A total of 14 significantly expressed ($p < 0.05$, $p < 0.01$) proteins were identified, and of these nine were up-regulated and five down-regulated in the SLE patients. The functional enrichment analysis assigned the majority of the identified proteins including alpha 2 macroglobulin, complement C4, complement factor H, fibrinogen beta chain, and alpha-1-antitrypsin were part of the complement/coagulation cascade, which is an important pathway that plays a crucial role in SLE pathogenesis. In addition to these proteins the differential expressions of ceruloplasmin, transthyretin, and haptoglobin play a potential role in the renal system abnormalities of SLE.
Conclusion: Therefore, the identified differentially expressed proteins are relevant to SLE patient's cohort. Most importantly the up-regulated proteins might be the potential candidates

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for renal system involvement in SLE disease pathogenesis. In order to confirm the diagnostic/therapeutic potential of the identified proteins, future validation studies are required.

Introduction

Systemic lupus erythematosus (SLE) is a chronic and multifaceted autoimmune disease. It is characterized by the changes in both the innate and adaptive immune systems that lead to a loss of immunological tolerance, which brings about the stimulation of self-antigens and the production of excessive auto-antibodies.[1,2] Due to the involvement of multiple major organs in the disease pathogenesis, clinical symptoms vary widely among SLE patients.[2] Therefore, the diagnosis is multifactorial, and challenging due to the heterogeneous nature of the disease and it is hard to anticipate the exact disease status of a given patient or when flare-ups of SLE will occur. Emerging technologies and the rapid medical advancements have contributed to improving our understanding of SLE pathogenesis and helped with its management. In recent years, cutting-edge technology in the combination of different mass spectrometric approaches has been utilized intensively in numerous proteomic studies and several potential autoimmune disease biomarkers have been reported.[3,4]

The available specific serological prognostic/diagnostic markers are limited and the pathogenesis of SLE has not yet been fully elucidated. The major reported biomarkers till now are monocyte chemoattractant protein-1 (MCP-1),[5] the tumor necrosis factor-like weak inducer (TWEAK),[6] transferrin,[7] various interleukins and TNF-α.[8] However, these organ-specific biomarkers are not specific enough to serve as measures of SLE disease activity and severity. The limited sensitivity and specificity of these established biomarkers means that there remains a need to explore further and identify more disease indicators or predictive markers that will help to identify disease symptoms, thus facilitating diagnosis, rather than identifying all the characteristics of the illness.

Human plasma represents the deepest version of the human proteome and it is a rich source of proteins by an order of ten magnitude concentration. Therefore, alterations in plasma protein expression levels have been found to correlate with disease severity and manifestations.[9] Thus, these proteins may also hold significant diagnostic/prognostic potential, and this can allow to serve as a biomarker for SLE. Thus, the goal of this study is to identify differentially expressed proteins present in the plasma of SLE patients compared with healthy controls using a gel-based proteomic analysis coupled with high-resolution electrospray ionization liquid chromatography and tandem mass spectrometry (LC-ESI-MS/MS) have been employed. To further understand the biological, molecular and potential functions of the identified proteins with changed levels of expression in SLE patients, gene ontology (GO) functional enrichment analysis, and protein analysis through evolutionary relationships (PANTHER) were performed.

Material and methods

SLE and healthy samples

Nineteen active SLE patients (28–62 years of age) with nephritis, and twelve age and gender-matched healthy control (age ranges from 25 to 62 years) plasma samples were collected at Cathay General Hospital (CGH), Taipei, Taiwan. The classification criteria of the American College of Rheumatology were used for the diagnosis of SLE.[10] In order to determine SLE disease activity, the Systemic Lupus Erythematosus Disease Activity Index renal domain (SLEDAI-R) scoring method was used.[11] According to the disease activity index parameters, all of the recruited SLE patients had active disease with nephritis. The average SLEDAI-R score of the nineteen SLE samples was >10 and the Urine Protein-to-Creatinine Ratio (UPCR) was >3 g/l with high titers of anti-dsDNA (>4.2 IU/mL) and antinuclear antibodies (ANA test of ≥1:640) were collected according to Wang et al.[12] All the patients were suffering from proteinuria (UPCR >3 g/l) with low complement levels. Table 1 showed the detailed demographic characteristics of the patients and the matched controls. This study had approval

Table 1 General and demographic characteristics of SLE patients and healthy control samples.

	SLE patients	Healthy individuals
Number of samples	19	12
Female:male (% female)	18:1 (94.7%)	9:3 (75%)
Age (years)	32.1 ± 1.5[a]	32.9 ± 2.1[a]
SLEDAI-R Score (average)	10.25 ± 2.8[a]	N/A
UPCR	>3 g/l	N/A
Anti-ds DNA antibodies		
<30 (n = 19)	2 (10.52%)	N/A
30 to <60 (n = 19)	2 (10.52%)	N/A
60–200 (n = 19)	7 (36.8%)	N/A
>200 (n = 19)	8 (42.1%)	N/A
Anti-nuclear antibodies (ANA)	≥1:640	N/A
Disease status	All patients showed active disease condition	N/A

[a] Data are represented as mean ± standard deviation.
N/A: Not applicable.

from the Institutional Review Board (IRB) (Approval Code CT-099005) for Research Ethics of the Cathay General Hospital, Taiwan. Informed consent was provided by all of the blood donors.

Depletion of high abundance serum albumin protein

Plasma samples were collected in heparin-containing tubes from whole blood samples of SLE patients and controls.[13] All the samples were processed to deplete serum albumin using a dye-based proteo-prep blue serum albumin removal kit (Thermo Co. USA). By following the manufacturer's protocol, the provided suspended slurry was used to prepare the spin columns by centrifugation at 8000×*g* for 10 s. Next, 0.1 ml of plasma samples were added to a spin columns, and incubated for 10 min and centrifuged at 8000×*g* for 60 s, and the same step was repeated twice to remove additional albumin.[14] The depleted plasma samples protein concentration were determined by Bradford assay[15] and the samples were stored at −80 °C until further analysis.

Two-dimensional gel electrophoresis (2-DE)

Acetone/TCA precipitation

The albumin depleted plasma samples were studied using three technical replicates by 2-DE analysis. Specifically, 100 μl samples were mixed with 500 μl of 10% TCA in acetone and the mixture was allowed to precipitate overnight incubation at −20 °C. The precipitated samples were centrifuged at 8000×g for 15 min. Then the supernatant was discarded and the pellet was washed with 500 μl of 90% ice-cold acetone; this was followed by air drying for 5 min. Next, the pellet was suspended in 100 μl of lysis buffer containing 7 M urea, 2.5 M thiourea, 100 mm dithiothreitol (DTT), 40 mM Tris-HCI, 4% w/v 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonates (CHAPS), and 0.5% v/v Igepal CA-630.

Isoelectric focusing (IEF)

The protein samples were resolved by IEF using immobilized linear pH gradient (IPG) strips (7 cm strips, pH 3—10 NL, BIORAD, USA). In total, 350 μg of each plasma sample was rehydrated in a 200 μl final volume using freshly prepared rehydration buffer contained 8M urea, 100 mm DTT, 4% CHAPS), 0.5% carrier ampholytes pH 4—7, 0.01% bromophenol blue and 40 Mm Tris. The samples were allowed to rehydrate on the 7 cm IPG strips for 12—16 h at 20 °C. The rehydrated strips were then focused by IEF using a Protein IEF cell (Bio-Rad) according to the following protocol: 500 V for 1 h, 1000 V for 1 h, 6000 V for 2 h, then 6000 V to give a total of 40,000 V h. Then the strips were washed with milliQ water and equilibrated with equilibration buffer (30% glycerol, 6 M urea, 2% SDS, 75 mM Tris—HCl pH 8.8, 0.001% BPB) containing 1% DTT by shaking for 20 min. This was followed by alkylation using the same equilibration buffer, without DTT, and containing 25% iodoacetamide (IAA); this was carried for 15 min with shaking in the dark.

Second dimension SDS-PAGE

Each equilibrated IPG strips were placed on top of separate 12%—14% polyacrylamide gels and fixed in place using 0.5% agarose. Molecular weight markers were separated simultaneously to the protein samples and the two sets of gels were run in the vertical Protein II Xi Cell (Bio-Rad) at 10 mA/gel for 30 min and 25 mA/gel using 190 mM Tris-glycine running buffer (pH 8.3) The gels were run until the bromophenol blue dye reached the bottom of the gels. Then, the gels were fixed and stained with Coomassie Brilliant Blue R-250 (CBB-R-250) staining solution (0.1% CBB R-250, 50% methanol and 10% glacial acetic acid). In order to visualize the protein spots, the gels were placed in freshly prepared destining solution (40% methanol and 10% glacial acetic acid), and this step was repeated twice until the gels are fully resolved.

Image analysis

CBB stained gels were scanned by an image scanner (Biorad GS-800) and the images were saved as TIFF files for further analysis. In order to carry out comparisons of the 2-DE protein patterns between SLE patients and controls, PD Quest advanced 8.0.1 software (Biorad, CA, USA) was used. Detection of each protein spot, its measurement, background subtraction, and spot matching were carefully performed. To match the protein spots between the two study groups, a healthy control gel image that had the maximum number of spots was used as the reference gel. The automatic spot detection wizard in the PD-Quest software was used to subtract the background and match each protein spot on the various gels with the reference gel. Manual editing or deleting was performed when necessary. Each protein spot volume/intensity calculated automatically by the software and these were matched with the reference gel. The amount in each protein spot was expressed as a volume, which was defined as the sum of the intensities of all the pixels that made up the detected spot. Protein spots with >2 fold difference in expression level were selected, and these spot volumes were then normalized as a total valid spot intensity/volume. A local regression model was used by the PD-Quest software to normalize the obtained data. Next, the acquired dataset from the image analysis was quantified by "quantity one" software (Biorad CA, USA) in order to assess the qualitative and quantitative variations in the identified protein spots when the two study groups were compared. All three gel images obtained from the plasma/serum samples of the patients and controls were subjected to statistical analysis. Finally, the spots found to differ between patients and controls with a class ratio of greater than 2 fold or less than or above 0.5 fold, each of which fulfills the statistical criteria of either $p < 0.05$ and/or $p < 0.01$ were subjected to liquid chromatography tandem mass spectrometry analysis.

In-gel digestion

The protein spots showing statistically significant differences in expression level between the SLE and control subjects were manually excised from gels and transferred to a 1.5 ml Eppendorf tubes. In-gel digestion was performed

as described in a previous report.[16] Briefly, the gel pieces were destained using 100 mmol/L NH_4HCO_3 in 30% acetonitrile (ACN) and then lyophilized. Next, they were rehydrated in 30 μl of 50 mmol/L NH_4HCO_3 containing 50 ng trypsin solution (Promega, USA). After digestion, the peptides were extracted three times using 100 μl 0.1% trifluoroacetic acid in 60% ACN. Subsequently, multiple extracts of the same protein were pooled together and freeze-dried. The resulting lyophilized tryptic peptides were processed for mass spectrometric analysis.

Protein identification by electrospray ionization liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS)

The tryptic digested peptides were analyzed using the electrospray ionization liquid chromatography technique. The tandem mass spectrometric analysis was performed on a nano-ACQUITY UPLC coupled Q-TOF, Synapt-HDMS mass spectrometer (Waters Corporation, Milford, MA, USA). Approximately 5 μl of peptide samples were separated using a C_{18} reverse phase column (1.7 μm × 75 μm × 250 mm) (Waters Corporation, Milford, MA, USA) at 5 μl/min flow rate. Peptides were initially pre-concentrated and desalted at a flow rate of 5 μl/min using a 5 μm symmetry C_{18} trapping column (internal diameter 180 mm, length 20 mm) (Waters Corporation, Milford, MA, USA) using 0.1% FA, and 0.1% FA in 90% ACN buffer. After the injection, the peptides were eluted into the Nano-LockSpray ion source at a flow rate of 5 μl/min using a gradient of 2%—40% for 50 min. The peptides were ionized in the electrospray ionizer in the liquid phase and then they consecutively allowed to enter the ion trap, were fragmented (MS/MS) and then detected. The data-dependent mode was used to analyze the peptide ions. Each sequence of scans consisted of one full MS scan followed by 4 MS/MS scans of the most abundant ions. The peak lists were generated using MassLynx software and each generated MS/MS spectrum of the peptide spectral peaks lists (.pkl files) was processed automatically using in-house Mascot (http://www.matrixscience.com) protein identification software.

Protein identification and quantification

For the protein identification Mascot version 2.2 (http://www.matrixscience.com) software was used with the following parameters: the Swiss-Prot, and National Center for Biotechnology non-redundant (NCBInr) with human taxonomy database specified; trypsin digestion with two missed cleavages, followed by carbamidomethyl as fixed and oxidation (M) as variable modifications; mass values of MH+ monoisotopic, peptide charges of 1 H+; peptide mass tolerances of 100 ppm and 0.6 Da; and a false discovery rate (FDR) of <1. Consistently identified proteins from all the three technical replicates or from at least two of the three proteomic analysis that fulfilled the statistical criteria ($p < 0.05$) were considered to be a true protein hit. In addition to this, all the observed proteins had to have a mascot sequence threshold assignment score of >70, a reported probability score of $^{-10}\log(p)$, and peptide sequence coverage of at least 15% in order to be matched with a theoretical sequence. Finally, the pI values of the selected spots from gels with more than two unique peptides were taken into consideration as a way of testing the best match of the protein to the selected protein spot on the gel.

Bioinformatics analysis

The gene ontology (GO) (http://www.geneontology.org/) and PANTHER database were used to interpret the biological processes, molecular functions and cellular components of the identified proteins.[17] Furthermore, to understand the pathway enrichment analysis of the identified proteins, the KEGG (Kyoto Encyclopedia of Genes and Genomics) database was interrogated.[18]

Statistical analysis

The statistical analysis was performed using IBM SPSS statistics software 2.0 (SPSS16, SPSS Ltd., Working, Surrey, UK) for Windows. The results are presented as the mean ± standard deviation (SD). To differentiate the plasma proteome changes in SLE patients compared to control individuals, the analyzed triplicate dataset was quantified using a two-tailed unpaired Student's t-test (95% confidence interval) and single factor analysis of variance (ANOVA). To compare the differences in protein expression levels between the patient and control groups (mean ± SD), all of the protein spots volume ratios were compared between the SLE patients and controls. In order to detect the statistically significant proteins, a filtering condition of at least a 50% difference in the ratio was used. A protein with a $p < 0.05$ was considered statistically significant, while a protein with $p < 0.01$ was considered as highly significant difference.

Results

Proteins identified using 2-DE analysis

The comparative gel-based proteomic profiling was performed to identify the differentially expressed proteins from SLE patients and healthy controls. Both the samples were studied in triplicate to increase the reliability of the data. The 2-DE gels were well resolved and showed a reproducible pattern within the protein profiles. Representative gel images of SLE and controls are shown in Fig. 1. The images were quantified using PD-Quest Version 4.0 and every matched spot was assigned a unique sample spot protein (SSP) number by the software. The identified differentially expressed protein spots as were quantified based on their change in expression volume/intensity. After automated detection, and matching with the reference gel, approximately 158 ± 3 from SLE, and 149 ± 1 from controls protein spots were identified. To identify the differentially expressed proteins the data from the patient's group were compared with the control group using the expression values of each spot. This resulted in 54 protein spots being identified as differentially expressed; these all had a fold change of more than two or a fold change of less than 0.5

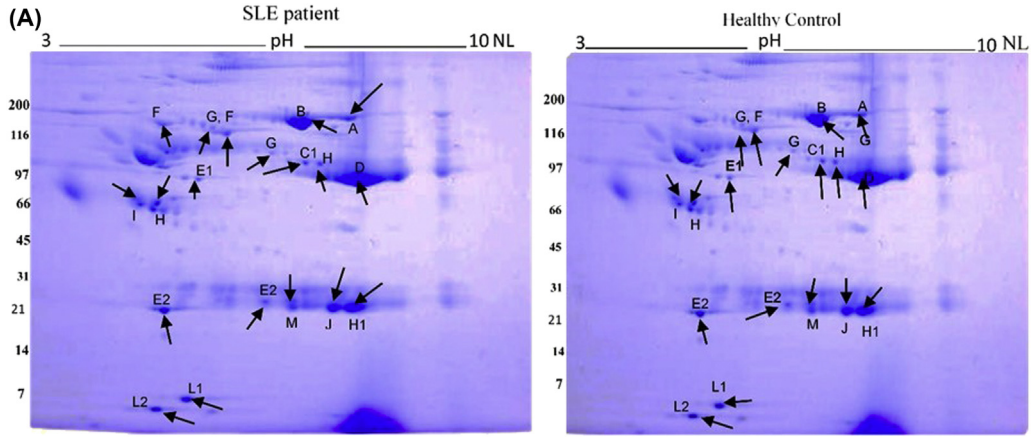

Figure 1. Differential expressions of protein spots from SLE patients compared to healthy controls identified by 2D gel electrophoresis. A) Representative 2-DE images of the serum albumin depleted SLE patients samples (n = 19) compared with the healthy control samples (n = 12) focused using non-linear, pH; 3—10 IPG strips (7 cm). A: Apolipoprotein B-100, B: Alpha-2-macroglobulin, C1: Complement Factor H, E2: Alpha-1-antitrypsin, E1: Complement C4, H: Haptoglobin, F: Ceruloplasmin, I: Serotransferrin, L1: Hemoglobin subunit alpha-1, L2: Hemoglobin subunit beta, M: Retinol binding protein, J: Transthyretin: G:Fibrinogen gamma chain, D: Serum albumin.

fold. Based on the statistical analysis, out of the 54 spots, 14 protein spots consisting of nine up-regulated and five down-regulated were fulfilled the statistical criteria ($p < 0.01$ or <0.05). These differentially expressed protein spots were subjected to LC-ESI-MS/MS analysis.

A comparative enlargement (zoom) view of each protein spots from 2-DE gels were shown in Fig. 2 will provide a clear view of differential expression pattern between SLE patients and healthy control samples. The nine up-regulated proteins from our analysis were Complement factor H (CFH; spot C1), Alpha-2-macroglobulin (A2M; spot B), Ceruloplasmin (CP spot F), Haptoglobin (Hp; spot H), Hemoglobin subunit alpha-1 (HBA-1; spot L1), Hemoglobin subunit beta (HBB; spot L2), Apolipoprotein B-100 (APOB-100; spot A), Retinol-binding protein (RBP4; spot M), and Alpha-1-antitrypsin (A1AT; spot E1); On the other hand, Transthyretin (TTR; protein spot J), Serotransferrin (TF; protein spot I), Fibrinogen beta chain (FGB; spot G), Complement C4 (C4; spot E1), and Serum albumin (ALB; spot D) were found to have down-regulated expression in SLE patients compared to the controls. The bar graph clearly illustrates the protein spots differences between the SLE patients and healthy controls groups (Fig. 3). The data is expressed as the mean ± standard deviation with statistical

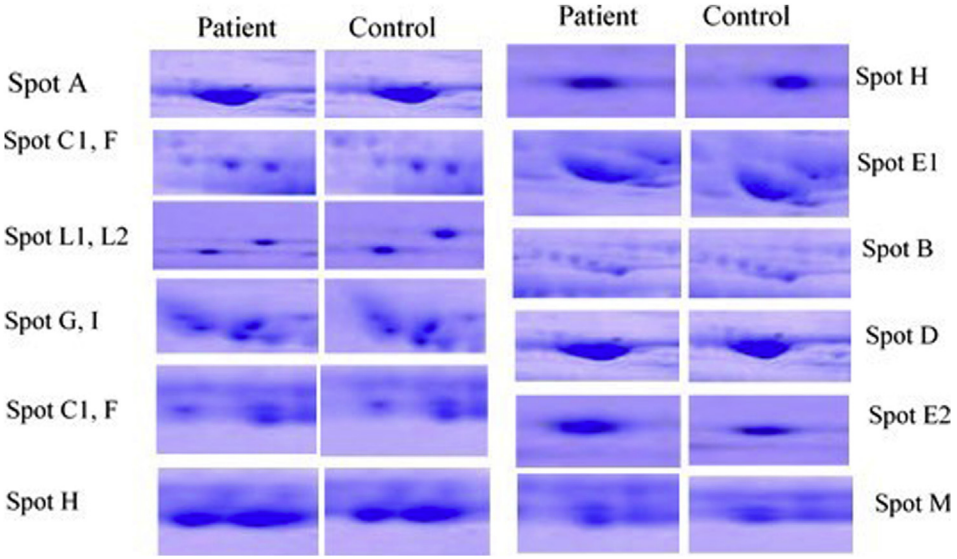

Figure 2. Enlargements of the 2D electrophoresis zoom view gel images (pH range 5—7) covering the region encompassing all the identified protein spots when the SLE patients and healthy controls are compared. A: Apolipoprotein B-100, B: Alpha-2-macroglobulin, C1: Complement Factor H, E2: Alpha-1-antitrypsin, E1: Complement C4, H: Haptoglobin, F: Ceruloplasmin, I: Serotransferrin, L1: Hemoglobin subunit alpha-1, L2: Hemoglobin subunit beta, M: Retinol binding protein, J: Transthyretin: G: Fibrinogen gamma chain, D: Serum albumin.

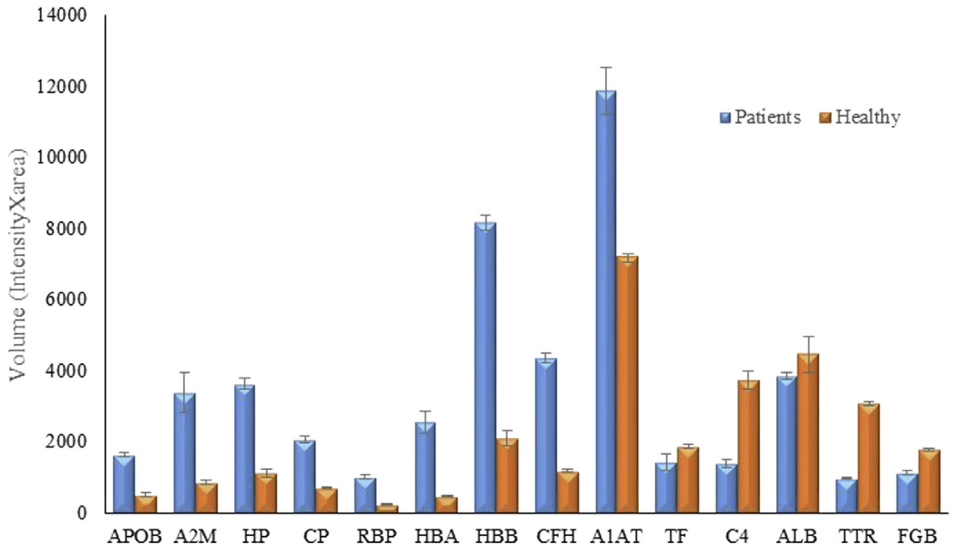

Figure 3. Quantitative comparison of protein spot volumes (spot intensity × area) of the fourteen differentially expressed proteins by triplicate 2-DE analysis when SLE patients and healthy controls are compared. The orange colored bar plot represents the healthy controls, while the blue bar plot represents the patients. The data are expressed as mean ± standard deviation. Comparisons of the means of protein volume/intensity in the plasma of the SLE patients and healthy controls were made by Student's t-test with p values of <0.05 and 0.01 as the limits of significance.

criteria of $p < 0.05$ or <0.01. The complete protein list of differentially expressed proteins from SLE patients compared to healthy controls are shown in Table 2.

Functional annotations of protein profile

The PANTHER and the GO ontology enrichment analysis was able to provide valuable insights to this protein cohort in terms of cellular component, molecular function, and biological process. Under the molecular function the identified proteins were involved in five major areas of binding (36%), transporter (14%), catalytic (29%), receptor (14%), and signal traducer activity (7%) (Fig. 4a). For biological process, the identified proteins were involved in various metabolic (10%), immune system (10%), cellular (17%), localization (23.30%), and inflammatory mediated responses (10%) (Fig. 4b). On component analysis, 75% of identified proteins were found to be in the extracellular region and 25% were membrane related proteins (Fig. 4c). For protein classification category, the identified proteins were classified into transporters (6%), transfer/carriers (25%), signaling molecules (16%), defense or immunity (9%), enzyme modulators (13%), hydrolases (10%), an oxidoreductase (3%), calcium binding (3%), a cell adhesion molecule (3%) and receptors (9%) (Fig. 4d).

KEGG pathway analysis

In order to obtain pathway information, the identified proteins were analyzed using the KEGG database which was assigned to four pathways ($p < 0.05$) were shown in Table 3. Among fourteen proteins, five proteins (A2M, C4A, CFG, FGB, and A1AT) belonged to the complement and coagulation cascades (mmu 04610), which is an important pathway that plays a crucial role in SLE pathogenesis. The second highest hit pathway, consisting of two proteins (C4A, CFH), was *Staphylococcus* infection (map 05150), it is an important pathway that may play a significant role in SLE pathogenesis due to the disease's involvement of the skin, particularly rashes and redness symptoms which are regularly reported by SLE patients. The third highest joint protein hits were associated with the African trypanosomiasis (map 05143) with two proteins (C4A, CFH), and malaria (map 05144), with two further proteins (HBA1, HBB). Based on PANTHER and GO functional enrichment analysis three proteins CP, CFH, and A2M from this protein cohort were majorly involved in the cellular and biological processes and immune system regulation. Therefore, the base peak chromatogram of these three proteins were shown in Fig. 5.

Discussion

Human plasma proteome is the largest and deepest source of the complex proteomic sample comprised of over ten orders of magnitude concentration of proteins and tissue leakages.[9] The changes in the abundance of these proteins have been playing an important role in various human diseases. In recent times, the proteomics approach has been successively identified various biomarkers in skin, kidney, and serum of SLE patients.[19,20] However, to the best of our knowledge, this is the first SLE plasma proteomic study from Taiwan. In this study we attempted a comparative proteomic approach using 2-DE combined with LC-MS/MS analysis successfully identified 14 significantly altered expressions of proteins ($p < 0.01$ and <0.05) with nine up-regulated (CFH, A2M, CP, APOB, Hp, HBA-1, HBB, A1AT, and RBP4) and five down-regulated proteins (TTR, C4, TF, FGB, and ALB) from active SLE patients compared with the healthy controls.

Table 2 List of identified up and down-regulated proteins from 2-DE triplicate analysis of SLE (n = 19) and healthy (n = 12) samples.

Protein expression classification	Band number	Protein name	Accession	Gene name	AAs	Molecular mass (Da)	Protein spot volume		Fold change* Patients/ healthy	Sequence coverage %	Matched peptide sequences	pI	*p*-value U-Test ANOVA	MS/ MS score
							Patients	Healthy						
2-DE														
Up-regulated proteins	A	Apolipoprotein B-100	P04114	APOB-100	1670	515,605	1631.35	508.03	3.2	28	18	6.9	<0.0001	217
	B	Alpha-2- macroglobulin	P01023	A2M	1474	163,281	3379.73	868.13	3.8	30	16	6.12	0.0001	1048
	C1	Complement Factor H	P08603	CFH	1231	139,09	4364.5	1183.55	3.6	15	16	6.21	<0.01	424
	F	Ceruloplasmin	P00450	CP	1065	122,205	2075.41	707.23	2.9	18	14	6.44	<0.001	437
	H	Haptoglobin	P00738	Hp	406	45,205	3627.16	1127.73	3.2	18	7	6.53	<0.01	97
	L1	Hemoglobin subunit alpha-1	P69905	HBA1	142	15,258	2557.4	476.8	5.3	15	3	4.7	0.002	78
	L2	Hemoglobin subunit beta	P68871	HBB	146	15,998	8158.23	2102.39	3.8	16	4	4.2	0.001	96
	M	Retinol binding protein	P02753	RBP4	201	23,016	1020.69	243.93	4.1	11	3	6.06	0.001	107
	E2	Alpha-1- antitrypsin	P01009	A1AT	418	52,21	21866.23	4364.5	3.04	18	21	5.89	0.0011	124
Down-regulated proteins	I	Serotransferrin	P02787	TF	698	75,36	1427.44	1871.71	0.7	45	14	4.54	0.01	253
	E1	Complement C4	P0C0L4	C4	1744	192,78	1391.37	3738.37	0.3	14	9	5.18	0.0001	91
	D	Serum albumin	P02768	ALB	609	63,09	3853.56	4469.06	0.8	38	16	7.1	0.0001	290
	J	Transthyretin	P02766	TTR	147	15,887	993.01	3068.67	0.3	33	6	6.92	0.0146	227
	G	Fibrinogen beta chain	PO2675	FGB	453	55,928	1145.13	1783.71	0.6	18	21	6.37	0.0021	84

AA: Amino acids MW: Molecular weight, pI: Iso-electric point. Fold change: * The average (triplicate analysis) patient protein spot volume/average (triplicate analysis) healthy protein spot volume, *p*-value: probability of significance <0.05, 0.0.

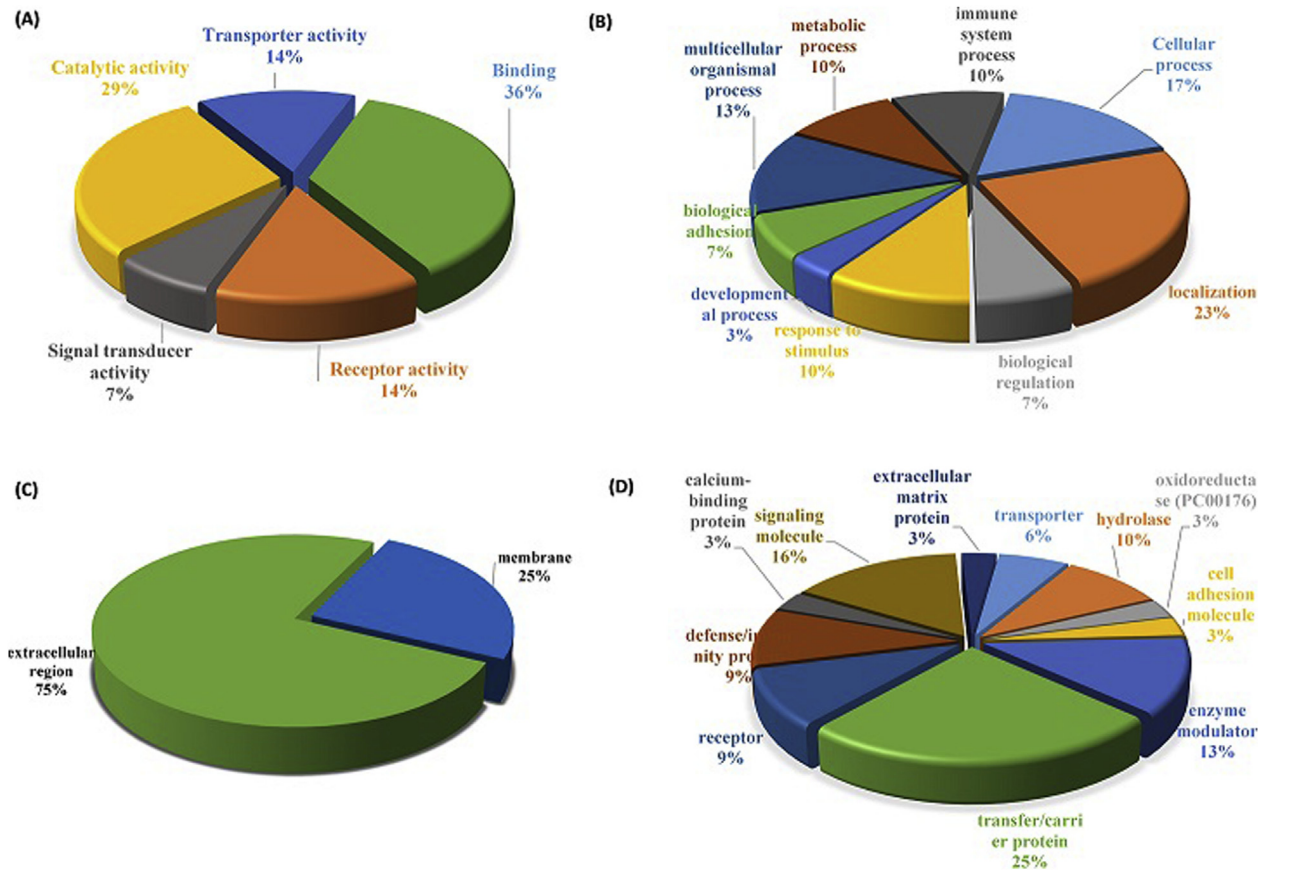

Figure 4. The functional annotations and protein classification by PANTHER analysis of the proteins with altered expression. (A) Biological process, (B) Molecular Function, (C) Cellular components and (D) Protein classification system of the identified proteins.

The effect of the renal system leads to lupus nephritis (LN) is up to 60% in SLE.[21] Thus, during our proteomic analysis majority of the identified proteins were majorly reported as important markers for SLE disease activity and severity. The elevated level of RBP from this study has been reported as a marker for renal disease activity in lupus patients.[22,23] In addition to this, TTR and Hp proteins also show differential expressions in our analysis have been reported previously with similarly altered expressions in urine samples of active LN patients compared to inactive LN.[24,25] The higher level of Hp is associated with disease severity and regulation of the immune system in autoimmune diseases.[26] Numerous studies have been pinpointed the involvement of TTR in amyloid diseases, including systemic amyloidosis, familial polyneuropathy, and familial amyloid cardiomyopathy.[27,28] Therefore, the differential expression level of TTR identified by our study not only resonates with nephritis but also illustrates a possible link to the development of amyloidosis. However, to understand the actual mechanism behind TTR with SLE further studies are necessary.

A great deal of evidence has demonstrated that extreme oxidative stress leads to severe inflammation. Moreover, SLE is known to cause severe persistent inflammation in many of the major organs of the body. Our analysis suggests that some of our identified proteins are involved in inflammation and regulation of the immune system. APOB have been shown to play a significant role in immune system regulation and inflammation. It was previously identified in the lupus patients with cardiovascular diseases and myocardial infarction, along with changes in low and high-density lipoproteins (LDL, HDL).[29] The up-regulated expressions of APOB from this study is reporting for the first time in plasma samples of SLE without any cardiac complications. Another interesting protein that showed elevated expression in this study is CP. It was previously identified in the urine samples of LN patients and its differential expressions were proposed as a promising biomarker for LN and glomerulopathy.[30] Hence, the similar findings during our study could indicate a contribution to the kidney dysfunctions and kidney abnormalities in SLE. Additionally, a non-immunoglobulin protein, A2M, which is also up-regulated in our study, has been reported previously to be abnormally glycosylated in autoimmune disease patients.[31] To the best of our knowledge, it has never been mentioned in any earlier SLE proteomic studies.

Complement proteins are key components involved in various aspects of SLE pathogenesis.[32] This study has identified elevated levels of CFH, and the dysregulated expression of C4 among our SLE patients. Earlier studies have suggested that CFH and its related genes are associated with SLE disease susceptibility and are also involved in

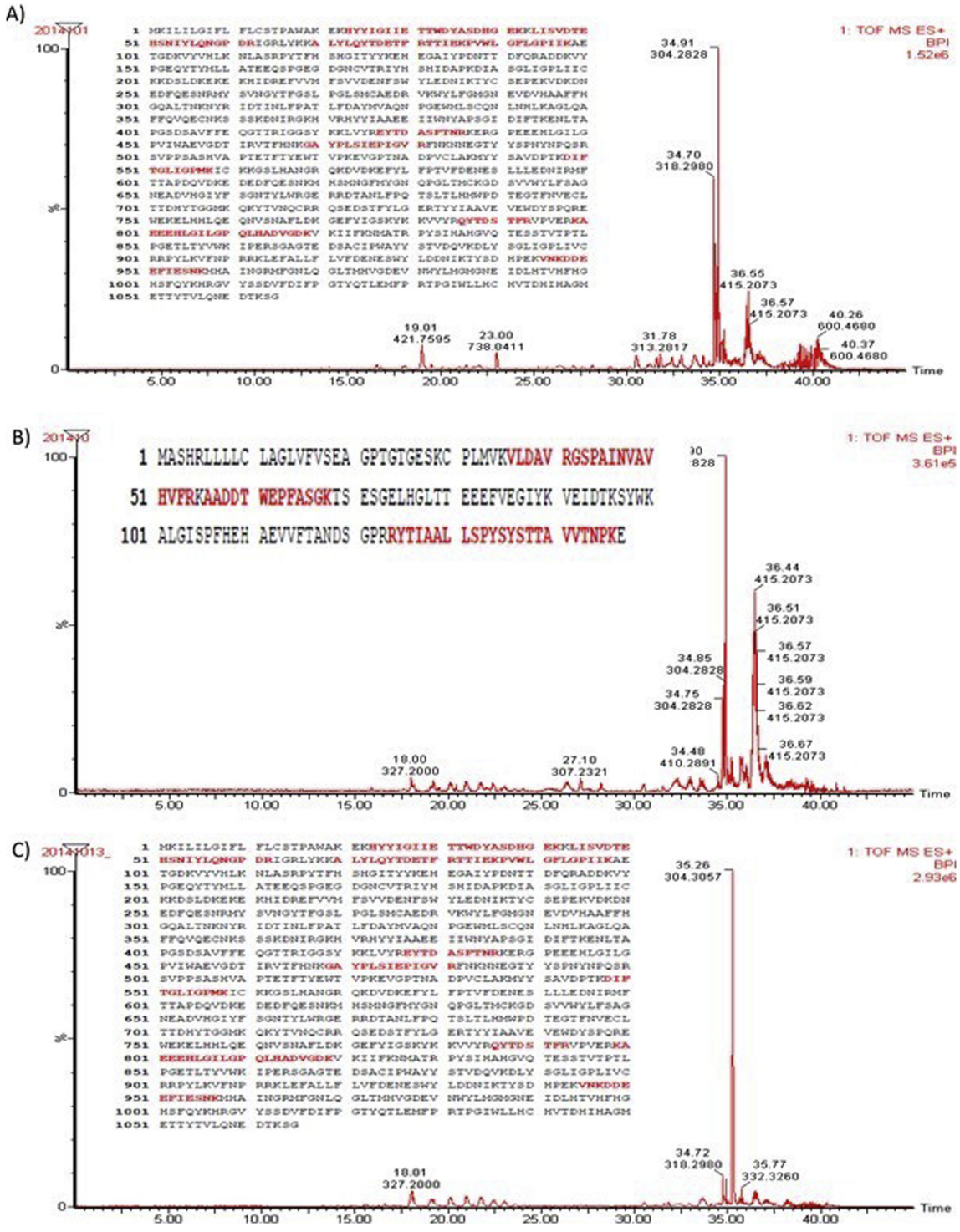

Figure 5. Identification of protein spots by LC-ESI-MS/MS analysis. A) Base peak chromatograms of the protein spot 'F' Ceruloplasmin, B) spot 'B' Alpha-2-macroglobulin and C) the protein spot 'C' Complement factor H. The database searching tool identified these proteins with the highest protein identification score along with the matched peptide sequences in red color.

LN.[33] Moreover, a decreased expression of C4 has been suggested to indicate disease severity or activity.[34] In addition to this, the C3/C4 differential levels may also help in the differential diagnosis including lupus enteritis.[35] The Carlsson et al. reported the down-regulated expressions of complement proteins reflect SLE disease severity and, these proteins could be potential markers for disease activity.[36] Therefore, the identified CFH, and C4 during our analysis probably indicate significant SLE disease activity and a susceptibility to develop LN and enteritis.

Another up-regulated protein is A1AT. Recent evidence has suggested that it is a promising biomarker for SLE renal

Table 3 List of top four identified pathways of the identified differentially expressed proteins from KEGG pathway analysis.

KEGG ID	Pathway name	Protein counts	*p*-value*
mmu04610	Complement and coagulation cascades	5	1.3E−7
map05150	*Staphylococcus aureus* infection	2	4.6E−2
map05143	African trypanosomiasis	2	2.8E−2
map05150	Malaria	2	4.2E−2

*Statistical test: hypergeometric test or Fisher's exact test, $p < 0.05$.

flares and LN activity.[20,37] A similar increase in expression of A1AT in SLE patients during our study suggests the renal system of our patients may be involved in the pathogenesis. Last but not the least, a hemoglobin derived protein, HBA-1, and HBB were consistently up-regulated during our analysis. These proteins are involved in the oxygen transport from the lungs to various tissues of the body and have never been mentioned in any previous studies of SLE.

The down-regulated protein cohort is also worthy of attention regarding SLE. Firstly, TF has been reported previously to be a biomarker for SLE disease activity.[38] In addition, a coagulation pathway related protein FGB is also down-regulated in this study has been reported previously to be involved in thrombosis among Hispanic lupus patients.[39] Since thrombosis is a serious complication of SLE, the altered expressions of FGB may help with identifying early thrombotic symptoms among lupus patients. ALB also showed decreased expression during our analysis, which implies an active SLE disease condition.[40] There has been increasing evidence to suggest that an altered expression of ALB is able to help to predict disease activity, including glomerular disease and nephrotic syndrome.[41] Therefore, our findings are consistent and to a large part agree with the prior studies.

Conclusion

In this comparative plasma proteomic study, we successfully identified some significantly altered proteins which are relevant to an SLE patient's cohort. Most importantly, the up-regulated proteins, namely HBA, HBB, CP, TTR, CFH, A1AT, RBP, and Hp, might be the potential candidate biomarkers for renal system involvement in SLE disease pathogenesis. In order to confirm the diagnostic/therapeutic potential of the identified proteins, future validation investigations such as ELISA or Western blotting, and/or high-throughput microarray analysis need to be carried out. Unfortunately, this study has limitations that it was difficult to obtain samples from patients with non-active lupus and other autoimmune diseases that could then be used to discriminate quantitative protein expression changes affects these two sub-groups. Furthermore, the role of these proteins as prognostic markers in SLE with nephritis needs further investigation.

Data statement

All the data which explains our research goal has been included with in this article. There is no unavailable or confidential data for this project.

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Ethical approval and consent to participate

This study obtained the approval from the Institutional Review Board (IRB) for research ethics at Cathay General Hospital, Taiwan. (IRB-Approval code CT-099005).

Competing interests

The authors declare that they have no competing interests.

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