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Identification and validation of differentially expressed proteins in serum of CSU patients with different duration of wheals using an iTRAQ labeling, 2D-LC-MS/MS

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Abstract. Chronic spontaneous urticaria (CSU) is one of the most common types of chronic urticaria (CU), with symptoms that recur easily, migrate and are refractory. It is unclear whether association between the differentiation of protein expression levels in the serum of CSU patients and the different duration of wheals exists. In the present study the samples were divided according to the duration of the wheals into group A (wheal duration <2 h) and group B (wheal duration 12-24 h). Differentially expressed proteins in sera of CSU patients with different durations of wheals were identified and validated with isobaric tags for relative and absolute quantitation (iTRAQ) in combination with two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS). Three hundred and seventy CSU serum-related proteins were initially identified. Among these proteins, ~30 had significant differences between the groups. According to the classification of biological functions and upregulated/downregulated values, serum amyloid A (SAA), CFL1, TPM4 and monocyte differentiation antigen (CD14) were chosen and validated by enzyme-linked immunosorbent assay (ELISA). The expression levels of CD14 in sera were not significantly different among the groups. SAA, CFL1 and TPM4 were associated with the wheal duration in CSU patients and therefore could be considered as new potential inflammatory biomarkers associated with CSU.

Introduction

Chronic urticaria (CU) is a common distressing skin disorder characterized by recurrent wheals and/or angioedema lasting for >6 weeks. CU can be divided into chronic spontaneous urticaria (CSU), inducible urticaria and other types of urticaria according to different etiology. CSU is a mast cell-driven disease that is defined as the recurrence of wheals, angioedema or both for >6 weeks due to known or unknown causes, excluding chronic inducible urticaria and urticaria vasculitis (1). The incidence of CSU and the etiology is unclear, the prevalence in the general population is ~0.5-1%, and the annual incidence is ~1.4% (1,2). Lapi *et al* (3) found that the annual prevalence of CSU in Italy increased from 0.02 to 0.38% from 2002 to 2013. CSU is the most common type of CU, accounting for ~2/3 of all cases (4), which is the same as that found in China (5). Due to the lack of specific treatment, the symptoms easily recur and are difficult to cure. CSU seriously affects the patients' quality of life and the stability of the immune environment. Antihistamine is the most commonly used drug for CSU treatment. However, this is far from satisfactory. Due to its complicated disease mechanism, most clinicians diagnose and treat CSU according to their symptoms. The objective and measurable indicators of disease activity are absent, so it is hard to evaluate the degree of disease, treatment efficiency and prognosis correctly. Thus, further investigation is required.

Although a large number of studies have been conducted on the pathogenesis of CSU, many hypotheses on CSU still have no conclusive evidence to confirm its mechanism. The specific allergen can not be found in most CSU patients. So the mechanism can not be well elaborated through IgE-mediated classic type I hypersensitivity. It is also difficult to explain with autoimmune theory, or genetic factors. Several researchers have suggested that blood parameters may indicate disease activity and duration. This would assist monitor treatment and could provide potential prognostic biomarkers of CSU (6-9). Some strong evidence has shown significant differences between patients with CSU and healthy controls in blood levels or values of D-dimer, C-reactive protein (CRP), matrix metalloproteinase-9 (MMP-9), mean platelet volume (MPV), factor VIIa, prothrombin 1+2 (F1+2), tumur necrosis factor, dehydroepiandrosterone sulphate and vitamin D (10). Data have shown that plasma FDP, D-dimer and serum CRP may be well associated with each other and significantly associated with the disease severity of CU, but not with the skin reactions of the autologous serum (11). However, Korean scholars Kim *et al* (12) have found that

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the expression level of serum clusterin increases in patients with a positive autologous serum skin test (ASST) than a negative one. Fujii *et al* (13) further found that, after the urticaria subsided the elevated circulating thrombin-antithrombin (TAT) III complex level recovered to within the normal range, and so did the elevated D-dimer level in all cases except two. This may explain the clinical symptoms of wheals in urticaria patients that vanished gradually by themselves. Researches (11,14) have shown that C3, C4 and CRP are closely related with the severity of CSU in the diagnosis of CSU and/or prognosis. Some authors have studied the inflammatory and cytokines in serum and lesions of CSU patients. This can not clearly explain the exact relationship with the pathogenesis of the disease. Conflicting evidence might also be explained by different patient populations and differences in the analysis of the results (e.g., difference in standardization between the various producers of the assay kits, the existence of various cut-offs and distinct methodological measurements of blood parameters).

With the further development of genomics and proteomics research, as well as the continuous accumulation of protein research data, proteomics research will lead to breakthroughs in the pathogenesis, diagnosis, treatment and new drug development of various skin diseases. Increasing number of proteomic techniques have been applied to skin diseases (15-17), including some allergic skin diseases (18-22). So far, proteomics technology has not been applied in the field of urticaria and is rarely reported. Isobaric tags for relative and absolute quantitation (iTRAQ) technology is a new relative and absolute quantification technique of *in vitro* isotope labeling of peptides developed by ABI Scientific, Inc. (Sterling, VA, USA) in 2004. Proteomics research based on the iTRAQ labeling method is a powerful tool for discovering protein markers. This technique can quantitatively compare proteins in 8 different samples at the same time. Therefore, it can distinguish the protein changes in quality and quantity between normal and disease samples, pre- and post-treatment, disease progression and cell culture under different conditions.

In this study, iTRAQ labeling combined with two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS) technique was used to study the protein profiling of sera from CSU patients with different durations of wheals and normal subjects. A total of 370 serum proteins associated with CSU were initially identified. Among those identified proteins, ~30 were found to have significant differences between the groups. According to the classification of biological functions and upregulated/downregulated values, SAA, CFL1, TPM4 and monocyte differentiation antigen (CD14) were chosen and validated by enzyme-linked immunosorbent assay (ELISA).

The results showed that in CSU patients with wheal duration of 12-24 h the expression level of SAA increased (P=0.047767), while CFL1 and TPM4 levels decreased (P=0.049229 and P=0.0049, respectively) in comparison with the healthy control group. Compared with the group of patients with wheal duration of <2 h, the expression level of SAA in CSU patients with 12-24 h wheal duration had no significant difference, while the expression levels of CFL1 and TPM4 decreased (P=0.01684 and P=0.0186, respectively). However, there was no significant difference in serum levels of SAA, CFL1 and TPM4 between CSU patients with wheal duration of <2 h and the healthy control group. The expression level of CD14 in serum was no significantly different among the three groups. SAA, CFL1 and TPM4 proteins were associated with wheal duration in CSU patients and might be considered as new potential inflammatory biomarkers associated with CSU.

Materials and methods

Specimens and sample collection. A total of 20 CSU patients were selected and divided into group A and B according to the duration of the wheals. Group A: wheal duration <2 h, 10 cases; group B: wheal duration 12-24 h, 10 cases. All the serum samples were collected before the wheals subsided. All patients conformed to the diagnostic criteria of CSU in EAACI/GA (2) LEN/EDF/WAO guidelines (1).

At the same time, group D, whose outpatient physical examination was positive, served as a healthy control group. The 10 cases comprising the control group underwent blood tests. Liver and kidney function, blood glucose and blood lipids were normal, and there were no previous allergic and systemic diseases.

The study was approved by the Ethics Committee of Pudong New Area People's Hospital (Shanghai, China). Signed informed consents were obtained from the patients or the guardians.

After the differentially expressed CSU serum-associated proteins were identified among the groups, 42 CSU patients were re-selected: 21 patients with wheal duration <2 h (group Y) and 21 patients with wheal duration of 12-24 h (group G), and 21 healthy controls (group J) were selected. The level of differentially expressed serum protein was examined by ELISA test.

The proteins that flowed through the depletion column were precipitated using ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. After precipitation, protein pellets were re-suspended in dissolution buffer using the iTRAQ experiment kit (AB Sciex, Framingham, MA, USA). The total protein concentration of each sample was determined by Bradford protein assay (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described (7). A total of 100 μg aliquots of each of the 3 samples were then reduced, alkylated, digested with trypsin, and labeled individually with one iTRAQ tag (Applied Biosystems: Thermo Fisher Scientific, Inc., Foster City, CA, USA) according to the manufacturer's instructions. Group A, B and D samples were labeled with 113, 114 and 115 tags, respectively. The labeled samples were then pooled and dried by centrifugal evaporation (Christ Alpha 1-2 and Christ RVC 2-25; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

2D-LC conditions. Chromatographic separation of the pooled samples was performed on an Acquity Ultra Performance LC system (Waters Corp., Milford, MA, USA). Tryptic digested and labeled peptides were first fractionated by a strong cation exchange (SCX) liquid chromatograph using a 0.5x23 mm, 5 μm, 300 Å column (Waters Corp.). Samples were loaded

Table I. Differentially expressed proteins of group A and B.

Accession no.	Entry name	Protein names	Ratio B/A
P02743	SAMP_HUMAN	SAP-component	1.719164386
P67936	TPM4_HUMAN	Tropomyosin α-4 chain (TM30p1)	0.55495
P02741	CRP_HUMAN	CRP	2.707280979
P35527	K1C9_HUMAN	CK-9	0.378568685
P04264	K2C1_HUMAN	CK-1	0.458821195
P01861	IGHG4_HUMAN	Ig γ-4 chain C region	0.654559002
P08567	PLEK_HUMAN	Pleckstrin	0.605110697
P35908	K22E_HUMAN	CK-2e	0.490515564
P23528	COF1_HUMAN	Cofilin-1	0.283736309
P36980	FHR2_HUMAN	Complement factor H-related protein 2	0.56605
P02790	HEMO_HUMAN	Hemopexin	0.6199
P02533	K1C14_HUMAN	K14	0.664
P08571	CD14_HUMAN	Monocyte differentiation antigen CD14	1.66208565
Q8NFI4	F10A5_HUMAN	Putative protein FAM10A5	1.5919
P58546	MTPN_HUMAN	Myotrophin (protein V-1)	0.5265
O94985	CSTN1_HUMAN	Calsyntenin-1	0.5115
P01608	KVD33_HUMAN	Immunoglobulin κ variable 1D-33	0.6313
P63267	ACTH_HUMAN	Actin	0.66

Group A: wheal duration <2 h, 10 cases; group B: wheal duration 12-24 h, 10 cases. SAP, serum amyloid P; CRP, C-reactive protein; CK, cytokeratin; K14, keratin-14.

onto the column and eluted stepwise by injecting salt plugs of 10 different molar concentrations of 25, 50, 75, 100, 150, 200, 300, 400, 500, 1,000 mM of NH_4Ac. Ten fractions were collected from the SCX column. Each of the fractions was then loaded onto a reverse phase (RP) column, ZORBAX 300SB-C18 column (5 μm, 300 Å, 4.6x50 mm; Agilent Technologies, Inc., Santa Clara, CA, USA). The flow rate used for separation on RP column was 0.4 μl/min. Buffer A was 5% acetonitrile, 95% water, 0.1% formic acid and buffer B was 95% acetonitrile, 5% water, 0.1% formic acid. Elution was performed using a gradient ranging from 5 to 45% buffer B for >90 min.

MS/MS conditions. The LC eluent was subjected to positive ion nanoflow electrospray analysis using a Qstar XL MS/MS system (Applied Biosystems: Thermo Fisher Scientific, Inc.) in an information-dependent acquisition (IDA) mode. In IDA mode, a TOFMS survey scan was acquired (m/z 400-1,800), with up to six most intense multiply charged ions in the survey scan sequentially subjected to product ion analysis. Product ion spectra were accumulated for 2 sec in the mass range m/z 100-2,000 with a modified Enhance All mode Q2 transition setting favoring low mass ions, so that the reporting iTRAQ ion (113, 114, and 115 m/z) intensities were enhanced for quantitation.

Data analysis. All LC MS/MS data were acquired by Analyst QS 3.1 (Applied Biosystems: Thermo Fisher Scientific, Inc.). MS/MS data were analyzed using ProteinPilot v3.0 (Applied Biosystems: Thermo Fisher Scientific, Inc.) which uses the Paragon Algorithm to perform database searching. The search results were further processed by the Pro Group Algorithm to remove redundant hits and comparative quantitation so that the minimal set of justifiable identified proteins could be found. The protein database used for all searches was Swiss-Prot human (downloaded on Sept. 15, 2016). Loading error was normalized by bias correction calculated using ProteinPilot software. All reported data were based on 95% confidence for protein identification as determined by ProteinPilot (Prot score ≥1.3). The relative protein quantitation was calculated as an average ratio. The confidence level of the altered expression of proteins was calculated by ProteinPilot as P-value, which allows the results to be evaluated based on the confidence level of expression change, not just by the magnitude of the change.

ELISA. Blood samples taken from the patients and normal control, were grouped and numbered (5 ml elbow vein blood, low temperature centrifugation, at the speed of 1,500 x g for 15 min at 4˚C, supernatant sub-packaging, -80˚C in storage) and then recorded. Specific detection was performed according to iTRAQ technology and ELISA kit.

Preparation of the reagents, samples and standards: 100 μl of sample were added, standard or blank to each well, and incubated for 90 min at 37˚C. A total of 100 μl of 1X biotinylated detection anti-human SAA (EA8001-1; AssayPro, St. Charles, MO, USA), CD14 (227920; Biomol GmbH, Hamburg, Germany), CFL1 (LS-F20976; LifeSpan BioSciences, Inc., Seattle, WA, USA) and TPM4 (EKC35896; Biomatik Corp., Cambridge, ON, Canada) polyclonal antibodies (1:300) were aspirated and added, and then incubated for 1 h at 37˚C. Then aspirated and washed 3 times. 1X HRP conjugate (100 μl) was

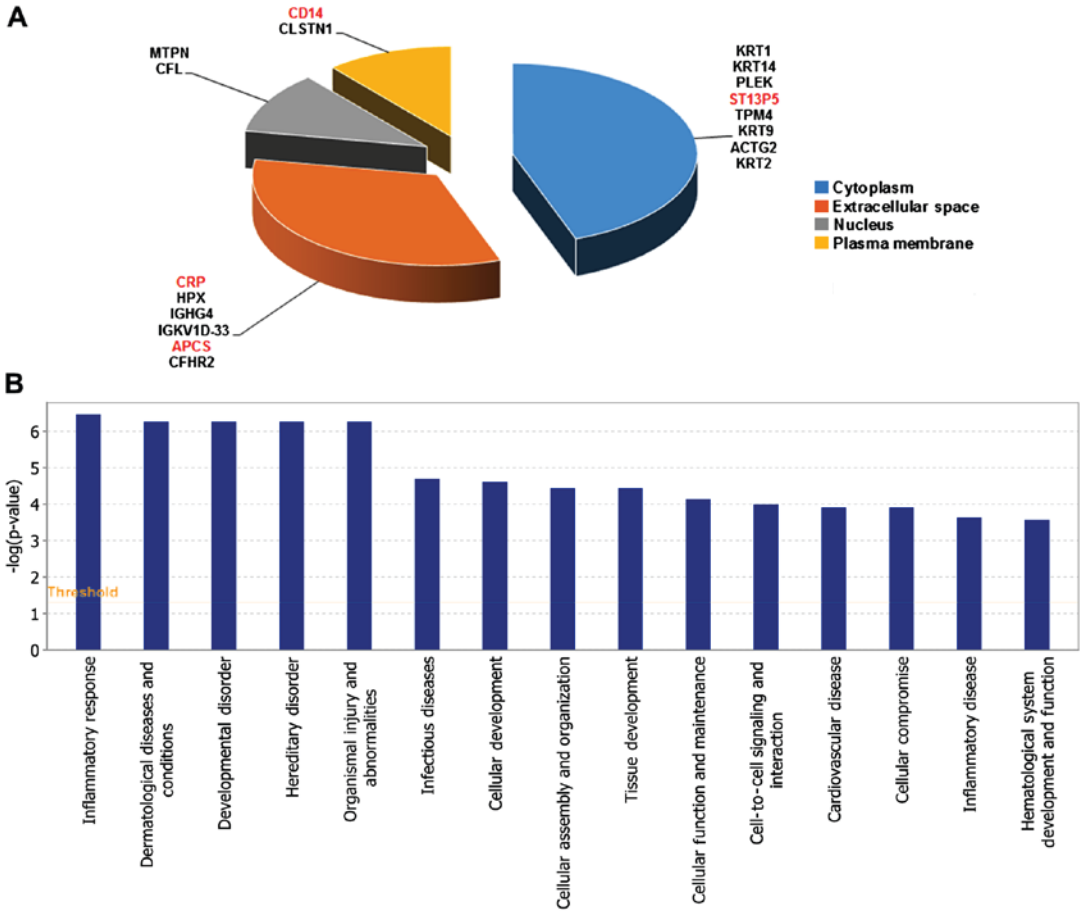

Figure 1. (A and B) The subcellular location and functional distribution of differentially expressed proteins of group A and B. Group A: wheal duration <2 h, 10 cases; group B: wheal duration 12-24 h, 10 cases. Red, upregulated; black, downregulated. CRP, C-reactive protein.

added and incubated for 30 min at 37˚C. Aspirated and washed 5 times. TMB substrate solution (90 μl) was added and incubated for 15 min at 37˚C. Finally, 50 μl of stop solution were added and reading was performed immediately at 450 nm.

Serum measurement: ELISA assays for IGFBP2 and LCAT (Cloud-Clone Corp., Wuhan, China), SHBG (R&D Systems Europe, Ltd., Abingdon, UK), GRP78 (Enzo Life Sciences, Inc., Exeter, UK) and calprotectin (BioLegend, Inc., San Diego, CA, USA) were performed in duplicate using commercial kits following the manufacturer's instructions. The mean coefficients of variance for duplicate analysis for each assay were as follows: IGFBP2, 8.1%; LCAT, 8.4%; SHBG, 7.4%; GRP78, 3.1%; and calprotectin, 4.5%.

Statistical analysis. All reported data were based on 95% confidence for protein identification as determined by ProteinPilot (Prot score ≥1.3). The relative protein quantitation was calculated as an average ratio. The confidence level of the altered expression of proteins was calculated by ProteinPilot as P-value, which allows the results to be evaluated based on the confidence level of expression change, not just by the magnitude of the change. $P<0.05$ was considered as a statistically significant difference.

Results

Proteomic and biological functional analysis in serum. There were in total 370 proteins identified in sera of groups A, B and D using iTRAQ labeling combined with 2D-LC-MS/MS. There were 18 significant differentially expressed proteins between group B and A. Among them, 4 proteins were upregulated and 14 were downregulated (Table I). The cell location and functional distribution of the 18 differentially expressed proteins are displayed in Fig. 1A and B, respectively. There were 20 significantly different proteins in group D compared with group B, 5 proteins were upregulated and 15 were downregulated (Table II). Fig. 2A and B, shows the 20 differentially expressed proteins in cell location and functional distribution. These differentially expressed proteins were classified in cell components by GO. They were both mainly distributed in cytoplasm, outer matrix components, plasma membrane and the nucleus.

There were 31 significantly different proteins between group A and D, 12 proteins were upregulated and 19 were downregulated (Table III). These 31 differentially expressed proteins were classified in cell components by GO, and were mainly distributed in extracellular matrix components, cytoplasm, plasma membrane and nucleus (Fig. 3A). For IPA bio function, the first three ranks are cell-to-cell signaling and interaction, hematological system development and function and inflammatory response (Fig. 3B).

The differentially expressed proteins identified among these three groups were also compared. SAA, CD14, CFL1 and TPM4 proteins had significant change. CRP, as a non-specific inflammatory marker, has been studied and

Table II. Differentially expressed proteins of group B and D.

Accession no.	Entry name	Protein names	Ratio B/D
P0DJI8	SAA1_HUMAN	SAA-1 protein	3.118620690
P67936	TPM4_HUMAN	Tropomyosin α-4 chain	0.225594016
Q9Y490	TLN1_HUMAN	Talin-1	0.425138687
P02741	CRP_HUMAN	CRP	4.628658177
P35527	K1C9_HUMAN	CK-9	0.377987984
P13645	K1C10_HUMAN	CK-10	0.424446977
P04264	K2C1_HUMAN	CK-1	0.393632417
Q92954	PRG4_HUMAN	Proteoglycan 4	1.678792288
P08567	PLEK_HUMAN	Pleckstrin	0.180288202
Q15746	MYLK_HUMAN	Myosin light chain kinase	1.783790265
P81605	DCD_HUMAN	Dermcidin	2.342048293
P35908	K22E_HUMAN	CK-2e	0.226904558
P31629	ZEP2_HUMAN	Transcription factor HIVEP2	0.666550825
P01137	TGFB1_HUMAN	Transforming growth factor β-1	0.629120347
Q9HBI1	PARVB_HUMAN	β-parvin (affixin)	0.182152123
P23528	COF1_HUMAN	Cofilin-1	0.226166454
P02533	K1C14_HUMAN	CK-14	0.431996357
O00194	RB27B_HUMAN	Ras-related protein Rab-27B	0.405605322
P68363	TBA1B_HUMAN	Tubulin α-1B chain	0.609584125
O60234	GMFG_HUMAN	Glia maturation factor γ	0.447163743

Group B: wheal duration 12-24 h, 10 cases; group D: healthy control group, 10 cases. SAA, serum amyloid A; CRP, C-reactive protein; CK, cytokeratin.

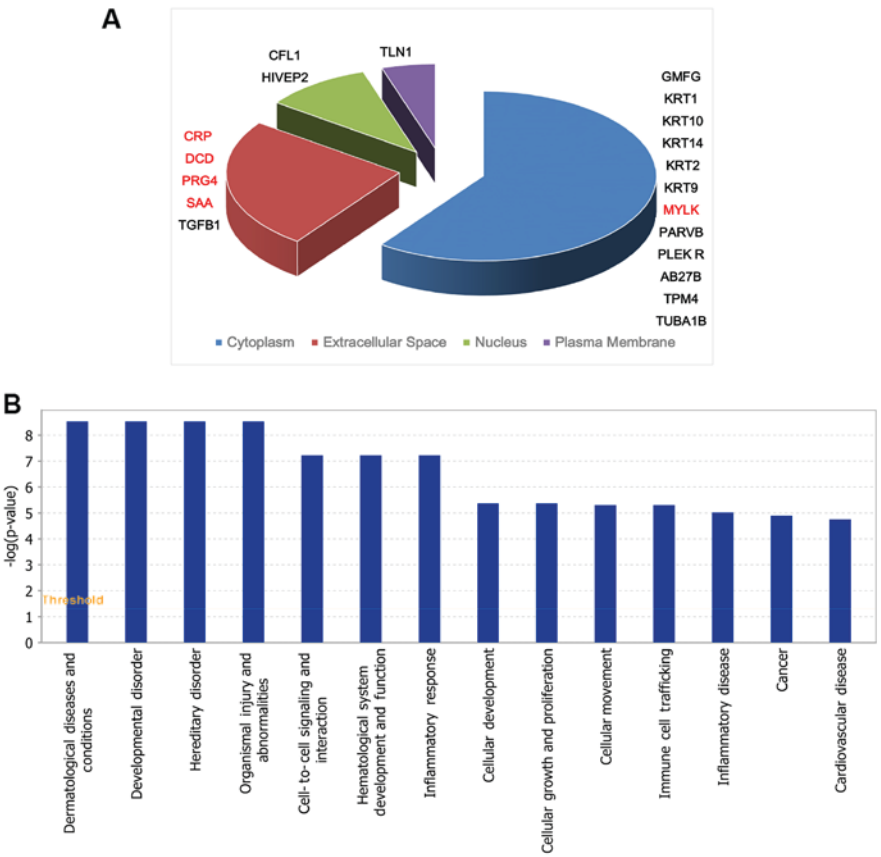

Figure 2. (A and B) The subcellular location and functional distribution of differentially expressed proteins between groups B and D. Group B: wheal duration 12-24 h, 10 cases; group D: healthy control group, 10 cases. Red, upregulated; black, downregulated. CRP, C-reactive protein.

Table III. Differentially expressed proteins of group A and D.

Accession no.	Entry name	Protein names	Ratio A/D
P01008	ANT3_HUMAN	ATIII	0.522774597
P0DJI8	SAA1_HUMAN	SAA-1 protein	2.838068966
Q96RU2	UBP28_HUMAN	Ubiquitin carboxyl-terminal hydrolase 28	0.662010526
P67936	TPM4_HUMAN	Tropomyosin-4	0.406512327
Q9Y490	TLN1_HUMAN	Talin-1	0.595211961
P02741	CRP_HUMAN	CRP	1.709707346
Q15485	FCN2_HUMAN	Ficolin-2	0.553688195
P13645	K1C10_HUMAN	CK-10	0.554630198
O43866	CD5L_HUMAN	CD5 antigen-like	0.662852444
P02753	RET4_HUMAN	Retinol-binding protein 4	1.523612657
Q92954	PRG4_HUMAN	Proteoglycan 4	1.818843216
P08567	PLEK_HUMAN	Pleckstrin	0.297942514
Q15746	MYLK_HUMAN	MLCK, smooth muscle	1.598593238
P02675	FIBB_HUMAN	Fibrinogen β chain	0.648407879
P51587	BRCA2_HUMAN	Breast cancer type 2 susceptibility protein	0.413992962
P55056	APOC4_HUMAN	Apolipoprotein C-IV	2.046185998
P81605	DCD_HUMAN	Dermcidin	1.578517902
P35749	MYH11_HUMAN	Myosin-11	1.744439599
P35908	K22E_HUMAN	CK-2e	0.46258381
P31629	ZEP2_HUMAN	Transcription factor HIVEP2	0.579206487
P01705	LV223_HUMAN	Immunoglobulin λ variable 2-23	1.68477803
Q9HBI1	PARVB_HUMAN	β-parvin (affixin)	0.273112113
P01019	ANGT_HUMAN	Angiotensinogen (serpin A8)	0.644745326
P00748	FA12_HUMAN	Coagulation factor XII	1.885902876
P02790	HEMO_HUMAN	Hemopexin (β-1B-glycoprotein)	1.76584849
P02533	K1C14_HUMAN	CK-14	0.650596923
P08571	CD14_HUMAN	Monocyte differentiation antigen CD14	0.537576093
O94985	CSTN1_HUMAN	Calsyntenin-1	2.147996993
O00194	RB27B_HUMAN	Ras-related protein Rab-27B (C25KG)	0.480318932
O00602	FCN1_HUMAN	Ficolin-1	0.644163875
O60234	GMFG_HUMAN	Glia maturation factor γ (GMF-γ)	0.584795322

Group A: wheal duration <2 h, 10 cases; group D: healthy control group, 10 cases. ATIII, antithrombin-III; SAA, serum amyloid A; CRP, C-reactive protein; CK, cytokeratin; MLCK, myosin light chain kinase.

reported in literature. Further 4 proteins, SAA, TPM4, CFL1 and CD14, based on the biological and functional information have close relationship with dermatological diseases and therefore were chosen to be validated as potential disease biomarkers by ELISA.

ELISA. The expression level of SAA in the sera of group G (wheal duration of 12-24 h) was higher than that of healthy control; while there was no significant difference in the expression of SAA between group Y (wheal duration <2 h) and G, and between group Y and J (healthy group) (Fig. 4A) indicating that serum SAA levels are significantly elevated in CSU patients with longer duration of wheals. In other words, the severity of symptoms in CSU patients is directly proportional to the serum level of SAA.

The expression levels of CFL1 and TPM4 were decreased in group G compared to group Y and J (Fig. 4B and C). The expression levels of CFL1 and TPM4 were significantly decreased in the serum of CSU patients with longer wheal duration compared to shorter wheal duration and healthy control as shown by ELISA. The expression levels of CFL1 and TPM4 had no significant change in the serum of CSU patients with shorter wheal duration and healthy control.

The results showed that in CSU patients with wheal duration of 12-24 h the expression level of SAA increased (P=0.047767), but CFL1 and TPM4 levels decreased (P=0.049229 and P=0.0049, respectively) in comparison with healthy controls. Compared with the group of wheal duration of <2 h, the expression level of SAA in CSU patients with 12-24 h wheal duration had no significantly difference, while the expression level of CFL1 and TPM4 decreased (P=0.01684 and P=0.0186, respectively). However, there was no significant difference in sera levels of SAA, CFL1 and TPM4 between CSU patients with wheal duration of <2 h group and healthy

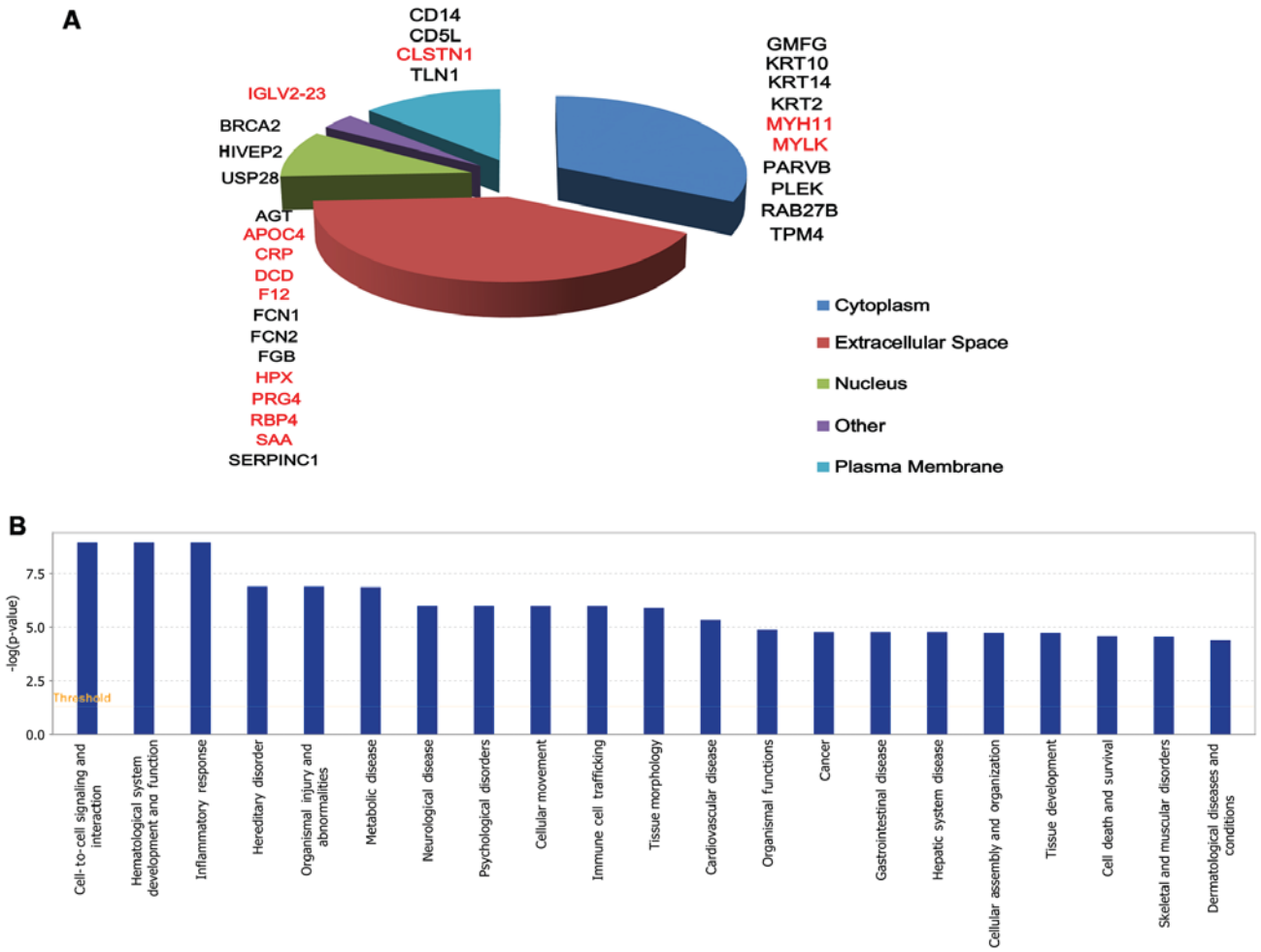

Figure 3. (A and B) The subcellular location and function distribution of differentially expressed proteins between group A and D. Group A: wheal duration <2 h, 10 cases; group D: healthy control group, 10 cases. Red, upregulated; black, downregulated. CRP, C-reactive protein.

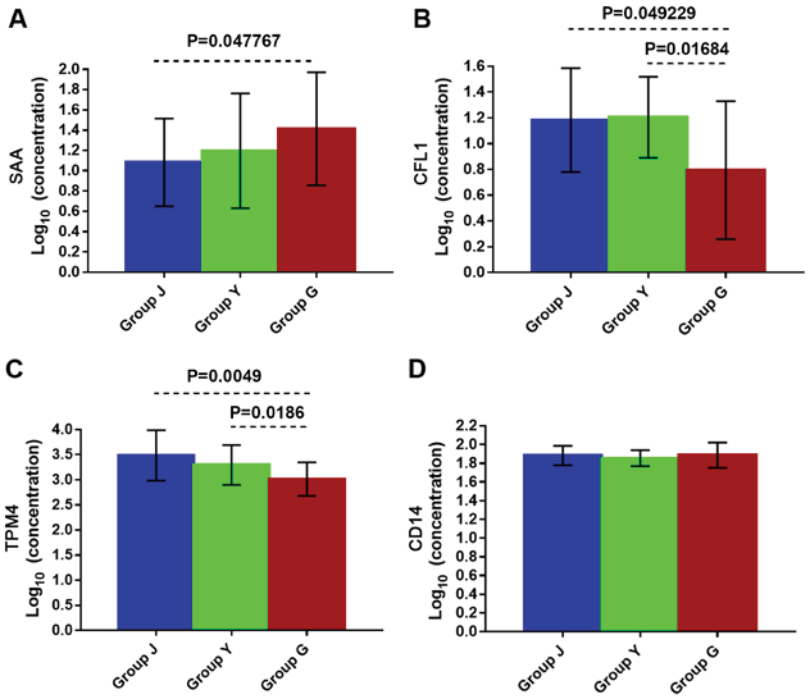

Figure 4. (A) The SAA, (B) the CFL1, (C) the TPM4, and (D) the CD14 levels in serum of CSU patients of different groups by ELISA. Group Y: wheal duration <2 h, 21 cases; group G: wheal duration 12-24 h, 21 cases; group J: healthy control group, 21 cases. Statistical significance, P<0.05. SAA, serum amyloid A; CSU, chronic spontaneous urticaria; ELISA, enzyme-linked immunosorbent assay.

control. There was no statistically significant change in the expression level of CD14 of the three groups (Fig. 4D).

Discussion

CSU is an inflammatory disease, characterized by acute phase response (APR) (23,24). The impact of APR on the body is a change in plasma protein concentration accompanied by a series of physiological and biochemical changes. Among them, the plasma protein concentration increased by >25%, is known as positive acute phase protein (APP), such as CRP, SAA and fibrinogen (FIB). CSU might be caused by an interactive combination of immune, genetic, and environmental factors, including infections (25-27). In addition, the altered function of the neuroendocrine-immune system has been recognized in CSU pathogenesis (28,29). Relevant research between coagulation and inflammation markers and the severity of acute exacerbation of the level of indicators and CU severity is also one of the hot spots in recent years; D-dimer and CRP levels are closely related to the activation of CU (11,30,31).

In the present study, the expression level of SAA in serum of CSU patients with wheal duration of 12-24 h increased significantly compared with the healthy control group as resulted by ELISA test. While there was no difference between healthy group and wheal duration of <2 h group. Serum amyloid A (SAA) is one of the most dominent positive acute-phase proteins, which increases sharply in serum due to inflammation, infection, neoplasia and tissue injury (32-34). The high expression level of SAA in the serum of CSU patients plays an important role in the long duration of the wheals. SAA is highly induced during the inflammatory response and is involved in the systemic regulation of the innate and adaptive immune responses. SAA possesses a number of cytokine-like properties reported in recent research. It can also stimulate the release of mature IL-1β from neutrophils, mast cells, macrophages and fibroblasts (33,35,36). IL-1β is a potent pro-inflammatory cytokine involved in many inflammatory diseases, including autoimmune and allergic diseases (37). The expression level of SAA was also significantly increased in allergic rhinitis and asthma patients (38).

Interestingly, in our experiment the expression levels of CFL1 and TPM4 were significantly downregulated in sera of CSU patients with longer duration of wheals compared to healthy and CSU patients with shorter duration of wheals. There was no difference between the serum in CSU patients with shorter duration of wheals and healthy groups. The role of CFL1 and TPM4 in the disease mechanism of CSU is still unclear. CFL1 is a key member of the actin-binding protein family that cleaves microfilaments and binds to actin monomers, to accelerate actin conversion rate *in vivo*.

Eosinophils are rich in actin, and the cytoskeleton is dynamic and very sensitive to changes in the cell's environment. This desirable property may benefit a motile cell such as the eosinophil to migrate into an inflammatory site. The dual function of cofilin, namely depolymerization and severing, serve as a key moducule controlling the actin dynamics. Thus, cofilin might be a possible candidate which connects early signal transduction events with the functional role of the cytoskeleton upon eosinophil activation (39). The number of eosinophils is increased in patients with CSU peripheral blood (40) and skin tissue (39). It has also been reported that the immunopathology of CSU is based on eosinophils and basophil-mediated hypersensitivity reactions characterized by secretion of Th0 cytokines (41). Therefore, CFL1 is abnormally expressed in CSU patients with longer periods of wheals and is probably due to eosinophil changes of function, which needs to be further confirmed.

Tropomyosins are coiled-coil dimmers, one of the major allergenic proteins of crustacean aquatic animals, that is located from head-to-tail polymers along actin filaments and regulate interactions of other proteins, including actin depolymerizing factor (ADF)/cofilins and myosins, with actin (42-45).

There are at least 40 tropomyosin subtypes in eukaryotes, and not all tropomyosins can compete with cofilin for binding to F-actin. The results of this experiment showed that TPM4 expression was consistent with that of CFL1 in the sera of CSU patients with longer duration of wheals, which seemed to be not competitive with F-actin binding. The role of TPM4 and CFL1 in the pathogenesis of CSU and the relationship between them need also to be further studied.

The pathogenesis of CSU remains unclear. As one of the major allergens of crustacean aquatic animals, TPM4 is abnormally expressed in the sera of CSU patients with longer period of wheals. The possible explanation is that the crustacean aquatic animal is one of the main causes in CSU patients with longer duration of the wheals and this result would be further observed in combination with clinical cases.

CD14, one of glycosylphosphatidylinositol-anchored proteins, plays multiple roles in microbial recognition and signaling. It assists to recognize the ligands of TLR1, 2, 3, 4, 6, 7, and 9, and it contributes in many ways to the trigger of the signaling pathways activated in response to LPS (46). Although CD14 protein was expressed in all serum of CSU patients, the expression of CD14 in serum of patients with wheal duration of <2 h was lower than that in healthy controls (ratio of A/D was ~0.54), and higher in sera of patients with long duration of wheals (ratio of B/A was ~1.66). However, the results of further validation did not confirm statistically significant differences between groups of patients with different duration of the wheals and with healthy individuals. CSU is a chronic inflammatory disease caused by the interaction of immune, genetic, environmental and infection factors. The results of ELISA validated that CD14 has no practical significance in the pathogenesis of CSU.

In conclusion, the results of serum identification of CSU patients with different durations of wheals using iTRAQ technique showed that there were different proteins in the serum of CSU patients with different duration. The level of SAA was positively associated with the duration of the wheals in sera of CSU patients, as shown by ELISA. The expression levels of CFL1 and TPM4 were different in sera of CSU patients with different duration of wheals; the expression levels were decreased in the serum of CSU patients with wheal duration of 12-24 h. The results suggest that there is an association between SAA, CFL1 and TPM4 and the duration of wheals in CSU patient, and therefore may be considered as new potential inflammatory biomarkers associated with CSU.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YC and SX conceived and designed this study. WK and HC analyzed the data and interpreted the results. YX acquired the data, performed the ELISA assays and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Pudong New Area People's Hospital (Shanghai, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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