

Plasma proteins as potential targets of abnormal Savda syndrome in asthma patients treated with unique Uighur prescription

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Abstract. The therapeutic effect of Uighur prescription on abnormal Savda in asthma patients was evaluated using plasma proteomics in order to elucidate the biological mechanism and identify potential therapeutic targets of abnormal Savda. In the present study, 40 asthma patients with abnormal Savda including abnormal Savda Munziq and Savda Mushil were enrolled and treated with Uighur prescription. The effect of Uighur prescription on protein expression and potential targets was investigated by isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics and bioinformatics analysis. Expression of candidate proteins was verified by an enzyme-linked immunosorbent assay. Following treatment with the Uighur prescription, 22 proteins were differentially expressed in the plasma of patients with asthma and abnormal Savda. The majority of these proteins were localized in intermediate filaments and the cytoskeleton and acted as antioxidant enzymes and binding proteins. Furthermore, they participated in the defense and inflammatory response, and the response to oxidative stress and wound healing. Peroxiredoxin 2 and carboxypeptidase B2 expression was significantly upregulated, whereas S100A7 was considerably downregulated in the whole plasma of patients (all $P < 0.05$) in accordance with the iTRAQ proteomics data. Uighur prescription of abnormal Savda may affect the whole regulatory network of protein expression that is altered following the development of abnormal Savda in patients with asthma.

Introduction

Asthma is a chronic disease of airway inflammation that presents with varying and recurrent symptoms, including cough, chest tightness and shortness of breath (1). Progress has been made in understanding the pathogenesis, diagnosis and treatment of asthma (2), and novel drugs, formulations and dosing instruments have been applied to treat asthma (3,4). However, asthma still presents a considerable threat to health, due to its high morbidity and mortality rates (5). Therefore, the development of novel drugs and complementary therapies, possibly in the form of traditional medicine, such as traditional Uighur medicine, is urgently required.

Uighur medicine is a well-established branch of medicine comprised of unique types of medicines, including munziq and mushil of abnormal savda. It is currently practiced by physicians and clinicians from the Xinjiang Uighur autonomous region in China (6,7). Uighur medicine shares an origin with Greco-Arab medicine and describes the incidence of illnesses associated with abnormal Hilits (representing syndromes), which are caused by an imbalance amongst four normal Hilits (representing humors), known as Safra, Kan, Phlegm and Savda (6,8). Quantitative or qualitative changes in any Hilit and the resulting disturbance of dynamic homeostasis of these Hilits may result in the development of corresponding symptoms, including increased quantity of urine, facial edema and weak pulse. Among these, abnormal Savda is the dominant syndrome in disease progression and often develops in conjunction with other abnormal Hilits (8). In traditional Uighur medicine, Savda is the major syndrome responsible for almost all complex diseases, including asthma, type II diabetes mellitus, cancer, and various cardiovascular and neurodegenerative illnesses (8). Previous studies have demonstrated that abnormal Savda is associated with relatively consistent biological changes in complex diseases that are manifested holistically within a population (9-11). Abnormal Savda may be treated with a unique Uighur prescription, comprised of ten herbal ingredients mixed in specific proportions. These are *C. dichotoma* (10.6%), *A. italic* (10.6%), *G. Uralensis* (7.1%), *A. capillus-veneris* (4.9%), *E. humifusa* (4.9%), *Z. jujuba* (4.9%), *L. angustifolia* (4.9%), *F. vulgare* (4.9%), *M. officinalis*

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(4.9%) and *A. pseudoalhagi* (42.3%) (7). A number of studies have demonstrated that Uighur prescription may mitigate oxidative stress associated with abnormal Savda, possibly by protecting cells from mitochondrial oxidative damage (12). Uighur medicine may also modulate abnormal changes in the neuroendocrine-immunity network and prevent carcinogenesis in murine models (13). Furthermore, flavonoids isolated from abnormal Savda Munziq are capable of inducing cell-cycle arrest and apoptosis of tumor cells (14). The results of these studies indicate that the biological basis of Savda may change as abnormal Savda develops, but is restored following Uighur prescription treatment.

In the disease state, protein expression is dynamically altered in a spatiotemporal manner and modulated by post-translational processing and chemical modifications. Along with the application of emerging proteomics techniques, serum/plasma has become a biomedium for the study of disease etiology, diagnostic biomarkers and drug targets of asthma in contemporary medicine (15). In particular, proteomics analysis has improved knowledge on the allergens of asthma and the effects of clinical therapy, and has even guided personalized therapy (16). In addition, abnormal changes in the whole regulatory network of protein expression are associated with the overall pathological state of patients with asthma (13). This holistic concept of understanding disease etiology through understanding of biological systems is shared by the theories of traditional Uighur medicine.

The present study assessed the effect of Uighur prescription of abnormal Savda on the regulatory network of relevant plasma proteins in asthma patients using proteomics. It also identified differentially expressed proteins that are potentially targeted by Uighur prescription. The aim of the present study was to provide evidence for the role of Uighur prescription in treating abnormal Savda at the proteomics level and to contribute to the scientific interpretation and application of Uighur medicine.

Materials and methods

Uighur prescription. Abnormal Savda Munziq is a traditional Uyghur medicinal herbal preparation that consists of the following (10,12,13): 15 g *Cordia dichotoma* fruit and *Ziziphus jujube* fruit, 7 g *Anchusa italic* plant, *Dracocephalum moldavica* L., *Lavandula angustifolia*, *Adiantum capillus-veneris*, *Foeniculum vulgare* Mill, *Euphorbia humifusa* Willd, 10 g *Glycyrrhiza uralensis* Fisch and 15 g *Alhagi pseudalhagi*. Abnormal Savda Mushil preparations contain: 45 g *Alhagi pseudalhagi* and *Fructus Cassiae fistulae*, 15 g *Pogonatherum crinitum*, *Terminalia chebula* Retz., and *Tenninlia chebula*, 12 g *Rosa rugosa*, 6 g *Polypodium vulgare*, *Glycyrrhiza Uralensis* Fisch. and *Foeniculum vulgare* Mill., 10 g *Lavandula angustifolia*, *Adiantum capillus-veneris*, *Euphorbia humifusa* Willd, *Anchusa italic*, *Iberis pectilata* L., *Viola tianschanica* Maxim, *Nymphaea* L. and *Amygdalus communis* L., 18 g *Raisin*, 30 g *Cordia dichotoma* fruit and *Qizil Guliqent* and 21 g *Cassia angustifolia*. All ten herbs were originally grown in Xinjiang, China and collected by the Chi Kang Barbour Pharmaceutical Co. (Xinjiang, China) by professional herbal growers.

Patient data. In total, 40 patients diagnosed with bronchial asthma between August 2013 and January 2015 were selected

for the present study according to the diagnostic criteria of the Guide for Prevention and Treatment of Bronchial Asthma established by the Chinese Medical Association in 2013 (17) and the Global Initiative for Asthma in 2010 (revised in 2012) (18). The present study consisted of 23 males and 17 females, with a mean age of (36.93±12.14) years old (age range, 20 and 60 years old).

Abnormal Hilits, including abnormal Kan, Phlegm, Safra or Savda, were identified by an independent diagnosis of two experienced physicians specialized in Uighur medicine. The classification outcome as abnormal Savda was further defined according to the established criteria of Uighur medicine (6,7) and using the assessment of symptom scores for abnormal Savda, including slow pulse (<60 beats/min), bleary eyes, dark purple lips, blue tongue, cool skin temperature (<36.6°C), dreams (or nightmares), night sweating, turbid urine and dry stools lasting a duration >1 month.

All enrolled individuals were orally administered with an Uighur prescription of abnormal Savda (patent no. ZL 02130082.8, China), including abnormal Savda Munziq extract powder at a dose of 3-6 g twice daily for 10-15 day, followed by Savda Mushil powder at a dose of 3-6 g twice daily for 3-5 days based upon the specific status of the asthma patients, as described previously (7). The entire clinical therapy involved completion of ≥3 courses of treatment, with each course consisting of treatment with abnormal Savda Munziq at a dose of 3-6 g for 10-15 days, followed by a 3-5 day therapy with abnormal Savda Mushil at a dose of 3-6 g.

The study design was approved and monitored by the Ethics Committee of the Xinjiang Medical University (Urumqi, China). All procedures of the study were in accordance with the Helsinki Declaration. Informed consent was obtained from all patients, and patient data were analyzed anonymously throughout the experiment.

Sample collection. Blood samples (2 ml) were obtained from 40 asthma patients by venipuncture into evacuated blood collection tubes containing EDTA-K2 anticoagulant (Promega Corporation, Madison, WI, USA). Plasma samples were separated by centrifugation at 3,000 x g for 10 min at 4°C and preserved at -80°C. Blood samples were collected twice: Prior to treatment (at baseline level) and once following final treatment with abnormal Savda Mushil.

Protein extraction and proteomic analysis. Proteomics analysis was performed using 8-plex isobaric tags for relative and absolute quantitation (iTRAQ, Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Plasma samples were collected twice; once at baseline and once following treatment, and were randomly and equally divided into four subgroups (n=4 in each group). Up to five samples from different individuals in each subgroup were mixed in equal volumes to form a pooled sample for further testing. The resulting samples were enriched for low-abundance proteins by depletion of medium- and high-abundance proteins using a pre-packed 1-ml affinity liquid chromatography (LC) column provided with a ProteoMiner™ low abundance protein enrichment kit (catalogue no. 56-2588-44; Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. Following enrichment, precipitated proteins were

dissolved in 300 μ l lysis buffer consisting of 6 M urea, 4% 3-cyclohexylamino propanesulfonic acid 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulfonate hydrate, 2 mM EDTA (all from Promega Corporation) and 1 mM phenyl-methane sulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Following reduction, 10 mM dithiothreitol and 100 mM NH_4HCO_3 (Sigma-Aldrich; Merck KGaA) were added at 56°C for 45 min. Subsequently, alkylation was performed with 55 mM iodoacetic acid (Promega Corporation) and 100 mM NH_4HCO_3 for 45 min. Samples were then precipitated in 80% acetone at -20°C overnight and dissolved in 0.8 M urea and 500 mM tetraethylammonium bromide (TEAB; pH 8.5). Each protein sample (30 μ g) was digested by supplementing sequencing-grade modified trypsin (Promega Corporation) at an enzyme/substrate ratio of 1:30 (w/w) in dissociation buffer (0.1% in TEAB; Promega Corporation) at 37°C for 24 h. The tryptic peptides were then labeled with 8-plex iTRAQ reagents (AB Sciex, Foster City, CA, USA) according to the manufacturer's instructions (iTRAQ113, 114, 115 and 116 for samples of the baseline, and 117, 118, 119 and 121 for samples following treatment). The reaction solvents were removed by speed vacuum at 3,000 x g and the labeled peptides were dissolved in 20 mM NH_4FA (pH 10.0; Sigma-Aldrich; Merck KGaA) for subsequent analysis.

Peptide fractionation by strong cation exchange chromatography and C18 column reversed-phase (RP) chromatography. High-resolution strong cationic exchange (SCX) chromatography was performed to remove redundant iTRAQ reagents and any interfering substances that could affect mass spectrometry (MS) analysis. Labeled peptides were loaded onto an SCX column (Luna SCX, 4.6x250 mm; Phenomenex Inc., Torrance, CA, USA), and eluted by a stepwise linear elution program as follows: 0-10 min equilibration in Buffer A at pH 3.0 including 25% acetonitrile (ACN), 20 mM KCl and 10 mM KH_2PO_4 . A 10-15 min fast elution was also completed using 0-5% Buffer B prepared with 25% ACN, 1 M KCl and 10 mM KH_2PO_4 , and a pH 3.0, 15-50 min linear elution with 5-30% Buffer B, and 50-55 min washing elution with 30-80% Buffer B. For desalting and further fractionation, peptide fractions were loaded onto an RP column (Luna C18, 4.6 mm inner diameter x250 mm length, Phenomenex, Inc.), and eluted by a step linear elution program as follows: 0-10 min equilibration in 100% solution A (2% ACN and 20 mM NH_4FA , pH 10.0), 10-15 min fast elution with 0-12% solution B (80% ACN and 20 mM NH_4FA , pH 10.0), 15-50 min linear elution with 12-56% solution B, and 50-55 min washing elution with 56-80% solution B. All procedures were performed using a prominence high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) with a flow rate of 1.0 ml/min and the peptides were monitored at 214 nm. In addition, the fractions containing the peptides were collected at a rate of 1 tube/min during a linear elution period.

Peptide analysis by nano-liquid chromatography coupled with Q-exactive MS. Peptide fractions were loaded onto a nano-RP column (5 μ m Hypersil C18 phenomenex Luna SCX 100A; 75 μ m x 100 mm; Thermo Fisher Scientific, Inc.) mounted in a Prominence Nano HPLC system (Shimadzu Corporation). The peptides were then eluted with an ACN gradient from

5-40% containing 0.1% formic acid (Sigma-Aldrich; Merck KGaA) for 65 min at 400 nl/min. Elutes were then transferred to a Thermo Scientific™ Q-exactive™ mass spectrometer (Thermo Fisher Scientific, Inc.), which was run in positive ion mode and in a data-dependent manner with a full MS scan of 350 to 6,000 m/z, 70,000 resolution, 320°C, 400 nl/min flow rate under a nubuliser pressure of 1,800 V, MS/MS scan with minimum signal threshold 17,500 and isolation at 2 kDa. In order to evaluate the performance of MS on iTRAQ-labeled samples, two MS/MS acquisition modes-higher collision energy dissociation and collision induce dissociation-were employed. In order to optimize the MS/MS acquisition efficiency of higher collision energy dissociation, normalized collision energy was systemically examined from 25 to 70%.

Database search and quantitative data analysis. Raw MS/MS data were converted into Mascot generic format (MGF) using Proteome Discoverer 1.3 (Thermo Fisher Scientific Inc.). Exported MGF files were searched using Mascot 2.3 (Matrix Science, Inc., Boston, MA, USA) against the Uniprot Human 2009-12 database (www.uniprot.org) with a precursor mass tolerance set at 15 ppm and product ion tolerance of 0.02 kDa. An automatic decoy database search was performed. Carbamidomethylation of cysteines was set as a fixed modification (C), and oxidation of methionines (M), Gln to pyro-Glu (N-term Q) and 8-plex iTRAQ modifications of N-term, K and Y were considered variable modifications. A maximum of one miscleavage was acceptable.

A protein with at ≥ 1 unique peptide and a false discovery rate < 0.01 qualified for further quantification analysis. Moreover, the fold change in protein abundance was defined as the median ratio of all significantly matched spectra with tag signals. Based on Proteome Discoverer 1.3 software analysis (Thermo Fisher Scientific, Inc.), the coefficient of variation distribution of all quantified proteins and quantitative results derived from duplicated injections were compared in parallel. The differential expression of all proteins was presented as a fold change in iTRAQ ratios. In addition, the upregulation of a protein was indicated by an increase of ≥ 1.2 -fold, and down-regulation by a decrease of ≤ 0.83 -fold (1.0/1.2).

Bioinformatics analysis by MetaCore™ software. Differentially expressed proteins were further characterized using a the MetaCore™ 6.18 software package (<http://thomsonreuters.com/metacore>, Thomson Reuters Co., New York, NY, USA) and an online database (<https://portal.genego.com>; Thomson Reuters Co.) in order to understand the underlying signaling pathways and protein-protein interaction networks, and to evaluate candidate proteins as potential biomarkers.

Enzyme-linked immunosorbent assay (ELISA). The plasma level of candidate proteins was verified for plasma samples collected at baseline and following ELISA using commercially available reagents (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions. ELISA reagents were used for the following candidate proteins: Peroxiredoxin 2 (PRDX2; catalogue no. SEF757Hu), protein S100A7 (catalogue no. SEC035Hu), transforming growth factor- β 1 (TGF- β 1; catalogue no. SEA124Hu), myeloperoxidase (MPO; catalogue no. SEA601Hu), carboxypeptidase B2 (CPB2; catalogue

no. SEA615Hu) and keratin type II cytoskeletal 6A (KRT6A; catalogue no. SED234Hu). The final data were confirmed by three independent measurements of each protein.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 statistical software for windows (SPSS, Inc., Chicago, IL USA). All P-values were two-sided, and $P < 0.05$ was used to indicate a statistically significant difference. The data of protein expression derived from the ELISA experiment at baseline and following treatment were statistically compared using a paired samples t-test.

Results

Identification of proteins differentially expressed by Nano-LC coupled with Q-Exactive MS. Therapeutic evaluation models for the treatment of asthma patients with abnormal Savda receiving unique Uighur prescription were established using proteomics analysis. Following preparation of pooled samples at baseline and following treatment by depletion of high-abundance proteins, enzymatic digestion and iTRAQ labeling, resulting peptides were simultaneously analyzed by nano-LC coupled with Q-Exactive MS, leading to the output of peptide spectra with 95% confidence intervals representing the relative and absolute quantitation of each sample. To analyze the effect of Uighur treatment on proteomic profiles, peptide spectra data of pooled samples corresponding to biological replicates at baseline and following treatment were collectively analyzed using a functional module of Proteome Discoverer software. Analysis of peptide spectra set a fold change ≥ 1.2 or ≤ 0.83 (1.0/1.2) as the cutoffs for quantitative differences, and 22 proteins were identified as differentially expressed in response to the corresponding treatment (Table I). Among these, 16 proteins were upregulated whereas 6 were downregulated.

Bioinformatics analysis of differentially expressed proteins by MetaCore™ software and ontology database. The role of the 22 identified proteins during disease development was further evaluated by bioinformatics analysis using MetaCore™ 6.16 software and an online database (<http://www.genego.com>). Gene Ontology analysis revealed that the majority of these proteins were localized in intermediate and keratin filaments, as well as the cytoskeleton. These proteins acted as peroxidases, oxidoreductases, acceptors of peroxides or carbohydrate binding proteins, and participated in the process of cytoskeleton remodeling, development, impaired lipoxin A4 signaling and stimulating transforming growth factor beta (TGF β) signaling. Regarding disease pathology, these proteins were largely involved in the defense and inflammatory response and the response to oxidative stress and wound healing (Fig. 1). Biomarker Assessment analysis based on the Disease Ontology database identified myeloperoxidase (MPO) and TGF β 1 as potential biomarkers for asthma and chronic obstructive pulmonary disease (COPD), keratin type II cytoskeletal 6A (KRT6A) as a biomarker for asthma and retinol-binding protein 4 (RBP4) for COPD (Fig. 1). The results of the present study suggested that these proteins, which were potentially targeted by the prescription for abnormal Savda, were most likely associated with dysregulation of

overall protein interaction and signaling network during the development and progression of asthma.

As presented in Table I, 6 out of 22 proteins, namely PRDX2, CBP2, MPO, TGF β 1, S100 A7 and KRT6A, may be molecular targets of Uighur prescription for abnormal Savda. Thus, these proteins served as pivotal candidate biomarkers for abnormal Savda-type asthma. A potential interaction and regulation network is associated with these proteins regarding cellular signaling and gene expression (Fig. 2), which was distributed in the extracellular space, membrane and cytoplasm. These proteins interacted with diverse effectors, including endopeptidases, matrix metalloproteinases and phosphatases, and were primarily regulated by transcription factors of distinct downstream signaling pathways.

Verification of changes in candidate proteins by ELISA. To verify the data from the proteomics and bioinformatics analysis, the plasma levels of the 6 candidate proteins were determined using whole blood (plasma) samples collected at baseline (before treatment) and after treatment by ELISA (Table II). The analysis demonstrated a significant upregulation of PRDX2 and CPB2, and downregulation of S100A7 and KRT6A in the plasma of patients in response to treatment (all $P < 0.05$). However, no difference was found for levels of MPO and TGF β 1 ($P > 0.05$). Due to the consistency between the results of iTRAQ proteomics for PRDX2, CPB2 and S100A7 (Table I) and the ELISA data (Table II), these proteins may be the potential targets of Uighur prescription for abnormal Savda. However, the ELISA results for the expression of KRT6A were inconsistent with those of iTRAQ proteomics, which indicated upregulation in response to the corresponding treatment.

Discussion

Recent proteomics studies of asthma have made significant progress in the identification of biomarkers and drug targets for asthma (10,12,13). Proteomics analysis of bronchial epithelium of asthma patients who were prescribed with budesonide (glucocorticoids) identified a number of differentially expressed proteins, including fibronectin 1, secretoglobin 1, KRT6A, interleukin enhancer-binding factor 3, dihydropyrimidinase like 5, cofilin 1, enolase 1 and vimentin as potential drug targets (16). Plasma-based proteomics revealed that heat-shock protein 70 and eotaxin were upregulated whereas vitamin D binding protein 3 was downregulated in patients diagnosed with asthma. These alterations were reversed following treatment with glucocorticoids (19). In a mouse model of acute-phase asthma, serum-based proteomics identified that immunomodulatory proteins were differentially expressed following glucocorticoid therapy (20). In addition, proteomics analysis determined that expression of 78 kDa glucose-regulated protein (GRP78) was upregulated in the lungs of mice with acute-phase asthma, and bronchial perfusion of anti-GRP78 small interfering RNA may modulate the inflammatory response induced by eosinocytes and bronchial hyper-responsiveness (21). Furthermore, increased expression of glycoprotein 39 and intercellular adhesion molecule 1 was detected in bronchoalveolar lavage fluid and lung tissues from asthma models of mice and monkeys, which was reversed

Table I. Identification of differentially expressed proteins in the plasma of patients with asthma and with an abnormal Savda in response to the treatment with the unique Uighur prescription.

Nos.	Protein information	Rec. Symbol	Uniprot ID	MW (kDa)	Calc. pI	Peptide detection		
						Peptide score	Peptide coverage	Unique peptides
1	Keratin, type II cytoskeletal 6A	KRT6A	P02538	60	8	1,803.12	55.32	3
2	Calmodulin-like protein 3	CALL3	P27482	16.9	4.42	37.41	8.05	1
3	Keratin, type II cytoskeletal 1	K2C1	P04264	66	8.12	1,883.75	53.73	34
4	Keratin, type II cytoskeletal 72	K2C72	Q14CN4	55.8	6.89	177.81	7.24	1
5	Keratin, type I cytoskeletal 27	K1C27	Q7Z3Y8	49.8	5.05	44.26	6.54	1
6	Annexin A1	ANXA1	P04083	38.7	7.02	65.14	8.96	2
7	InaD-like protein	INADL	Q8NI35	196.2	4.94	18.24	0.33	1
8	Keratin, type I cytoskeletal 10	KRT10	P13645	58.8	5.21	1,061.74	44.18	21
9	Thioredoxin	THIO	P10599	11.7	4.92	56.05	8.57	1
10	Carboxypeptidase B2	CPB2	Q96IY4	48.4	7.71	54.12	3.55	2
11	Serum amyloid A-2 protein	SAA2	P0DJ19	13.5	9.14	161.27	31.97	1
12	Peroxiredoxin-2	PRDX2	P32119	21.9	5.97	48.54	9.09	2
13	Myeloperoxidase	MPO	P05164	83.8	8.97	30.51	1.61	1
14	Keratin, type II cytoskeletal 2 epidermal	KRT2	P35908	65.4	8	718.83	28.01	10
15	Zinc finger and BTB domain-containing protein 18	ZBT18	Q99592	58.3	5.69	28.1	1.34	1
16	Transforming growth factor beta-1	TGFB1	P01137	44.3	8.53	35.31	7.69	2
17	Thrombospondin-4	TSP4	P35443	105.8	4.68	142.57	6.35	4
18	Protein S100-A7	S100A7	P31151	11.5	6.77	50.31	10.89	1
19	Retinol-binding protein 4	RBP4	P02753	23	6.07	123.03	9.95	2
20	Platelet factor 4 variant	PF4VL	P10720	11.5	9.1	193.28	48.08	2
21	Lysozyme C	LYSC	P61626	16.5	9.16	115.72	18.24	3
22	Protein CASC1	CASC1	Q6TDU7	83.1	5.29	24.76	1.40	1

*For a candidate protein, the fold-change (ratio) of >1.2 (base 2 logarithm values) or <0.80 (-1.2) in the plasma content was considered and reported as upregulated or downregulated following treatment with prescriptions, respectively. Rec. Symbol, recommended symbol; Calc.PI, theoretically calculated isoelectric point.

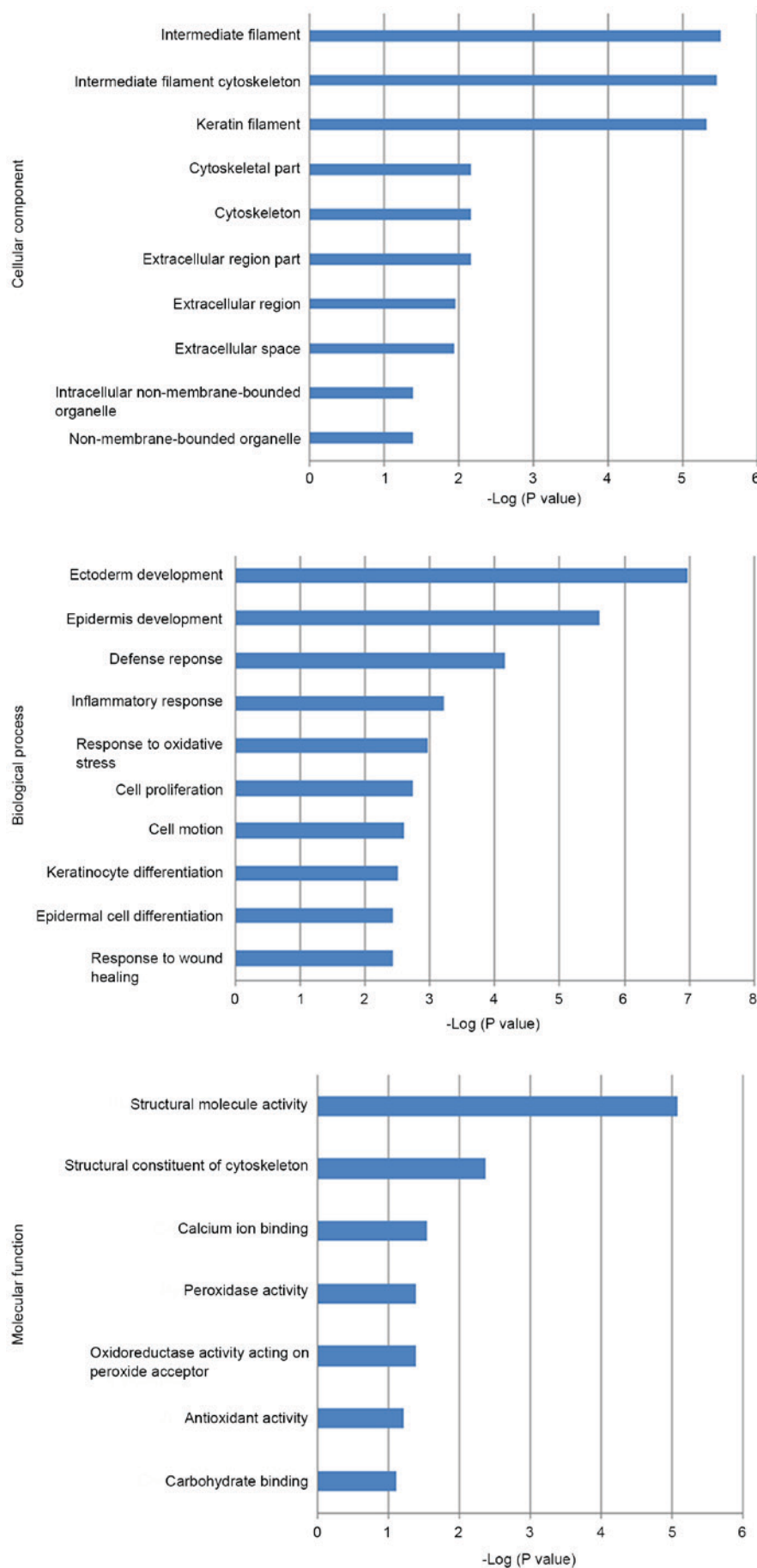


Figure 1. Gene ontology of annotation of the differentially expressed proteins. This analysis included assessment of the function of 22 proteins as described in Table I. The components for biological processes, cellular components, and molecular function of differentially expressed proteins are presented according to the gene ontology database (<http://www.genego.com/>; version 6.16).

Table II. ELISA verification of candidate protein expression in response to the treatment of abnormal Savda in asthma patients with Uighur prescription.

Protein	Plasma protein level (ng/ml) ^a		Paired t-test P-value
	Prior to treatment (mean ± SD)	Following treatment (mean ± SD)	
PRDX2	379.170±40.978	419.180±48.579	<0.001 ^b
CBP2	1,693.570±114.878	1,783.030±194.455	0.030 ^b
MPO	253.900±46.917	273.606±46.263	0.065
TGFβ1	0.410±0.314	0.445±0.306	0.531
S100A7	1.747±0.115	1.686±0.111	0.026 ^b
KRT6A	0.592±0.128	0.557±0.131	0.027 ^b

^aSamples from 40 patients; ^bP<0.05 indicating a statistically significant difference. CBP2, carboxypeptidase B2; MPO, myeloperoxidase; TGFβ1, transforming growth factor β1; S100A7, protein S100 A7; KRT6A, keratin type II cytoskeletal 6A; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

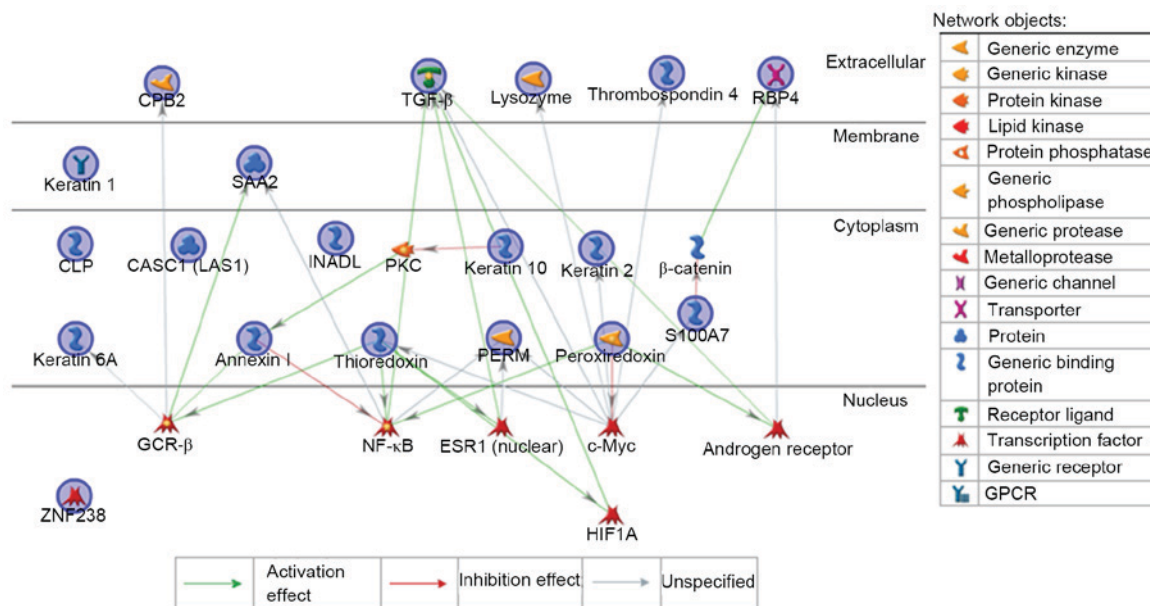


Figure 2. Network profile of protein interactions associated with eight candidate proteins at the cellular and genetic levels by MetaCore. This network was generated using the shortest path algorithm to map interaction among different proteins. The arrowheads indicated the direction of the interaction. The color of the lines between nodes indicated activating (green), inhibiting (red) and unspecified (black) interactions.

following glucocorticoid treatment (22). Although different drugs may have distinct molecular targets, the progression or clinical therapy of asthma may be causally associated with overall changes in the expression of proteins throughout the whole body, which may be integrated into the blood plasma. Therefore, it is necessary to investigate the entire regulatory network of protein expression associated with abnormal Savda, which is potentially targeted by Uighur prescription of abnormal Savda through a holistic concept that is shared by both systems biology and Uighur medicine.

In the present study, a therapeutic evaluation model was established and was used to evaluate abnormal Savda in patients with asthma receiving treatment with unique Uighur prescription. In total, proteomics identified 22 differentially expressed proteins in response to the corresponding treatment. Bioinformatics analysis demonstrated that a majority

of these proteins were localized in intermediate filaments and the cytoskeleton, indicating that these proteins may act as antioxidant enzymes and binding proteins, and be crucial in the defense and inflammatory response, and the response to oxidative stress or wound healing. A database search identified that MPO, TGFβ1, KRT6A and RBP4 acted as biomarkers for asthma or COPD, as reported previously (19). There was a discrepancy between the iTRAQ and ELISA data collected in the present study, therefore the association of these four proteins with the effect of the prescription was not fully confirmed in the current study. The majority of participants in the current study presented with no severe side effects or adverse events following treatment with Uighur prescription. A total of 4 patients suffered from a slight degree of diarrhea, which may be controlled. Our group plans on analyzing adverse events that occur following

administration of Munziq and Mushil of abnormal Savda in patients with asthma. It has been demonstrated that abnormal Savda asthma can induce metabolic disorder, and disrupt gluconeogenesis and host immunity (23). However, abnormal Savda Munziq is capable of counteracting abnormal metabolism and is important in upregulating gluconeogenesis and immune disorders (24). The present study aimed to identify a novel medication target of applying the Munziq and Mushil in treating abnormal Savda asthma from the perspective of protein genomics.

The consistency between the iTRAQ and ELISA data for PRDX2, CPB2 and S100A7 indicated that the expression of these proteins may be associated with abnormal Savda in asthma patients and that these proteins may be targeted by Uighur prescription. Of these biomarkers, PRDX2 is one of the non-selenium-dependent peroxidases with anti-oxidative activities and serves a potential role in the modulation of oxidative stress-related signaling and disease processes (25). PRDX2 may participate in a variety of cellular signaling pathways, and may reduce hyper-responsiveness during allergic airway inflammation by eliminating cellular reactive oxygen species (26). CPB2 is a zinc-containing carboxypeptidase that is involved in regulating the coagulation-fibrinolysis balance in a variety of diseases, including cancer and cardiovascular disease (27). It has been demonstrated that CPB2 regulates thrombin-mediated tissue inflammation and other inflammatory responses by inactivating a variety of active inflammatory mediators, including bradykinin, C3a, C5a and thrombin-cleaved osteopontin (28). S100A7/psoriasin is expressed in the airway epithelium, lung epithelial cells and macrophages (29). S100A7 is induced by oxidative stress and is involved in a variety of inflammatory diseases (30). The expression of S100A7/psoriasin is increased in the nasal lavage fluid of allergic patients and the S100A7 gene polymorphism is associated with allergic rhinitis (31). In addition, S100A7 may be important in the development of breast cancer by increasing inflammatory cell infiltration, stimulating the pro-inflammatory response, and promoting oxidative stress and angiogenesis (32,33). These observations are in accordance with the results of the present study, which suggest that treatment of abnormal Savda in asthma patients with Uighur prescription upregulates the expression of PRDX2 and CPB2 and thus the anti-oxidative and anti-inflammatory response capacity of the body, whereas it downregulates the expression of S100A7, which may reduce oxidative stress and inflammatory responses.

In conclusion, the results of the current study indicate that the therapeutic effect of Uighur prescription for abnormal Savda in patients with asthma is achieved by predominantly targeting the entire regulatory network of protein expression, particularly of those proteins involved in responses to inflammation and oxidative stress. Further *in vitro* and *in vivo* studies are required to identify the underlying mechanism of the therapeutic effect of the prescription for abnormal Savda during asthma progression, which may be revealed by verifying alternative identified proteins.

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