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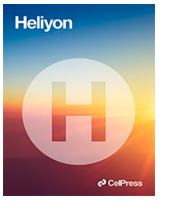

Analysis of urine differential proteins in patients with allergic rhinitis

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ABSTRACT

Background: Allergic rhinitis (AR) is one of the most common clinical allergic diseases. Early diagnosis and medical intervention will benefit patients with allergic rhinitis. In this study, we focused on changes in urine proteomics in AR patients to investigate their potential clinical utility in AR diagnosis and evaluation.

Material and methods: TMT-labeled mass spectrometry-based proteomics was carried out to identify differentially expressed proteins (DEPs) in urine between allergic rhinitis patients and normal control groups. The molecular biological role of DEPs was investigated by Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and protein-protein interaction (PPI) network analysis.

Results: Enrichment analysis showed that the differentially expressed proteins were mainly related to cell-cell adhesion, complement and coagulation cascades, peptidase activity regulation, MAP kinase activity, etc. Compared with the NC group, HLA-DRB1, WFDC12, and DEFA4, among the top ten up-regulated proteins in the urine of the AR group, were related to the biological process of the humoral immune response. Among the top 10 down-regulated proteins, GUSB, SQSTM1, and KIT are related to protein domain-specific binding in terms of molecular function.

Conclusions: We found differential protein changes between AR patients and normal subjects may be related to the pathophysiological changes of AR, which provides the possibility for further exploration of urinary proteomics biomarkers in the future.

1. Introduction

Allergic rhinitis (AR) is one of the most common clinical allergic diseases. Although the global incidence of AR varies by region, due to the impact of global industrialization, the incidence has gradually increased in recent years [1,2]. The incidence of AR can reach up to 40%, and more than 500 million patients worldwide have been affected by allergic rhinitis [3]. Patients with AR often have nasal symptoms such as nasal itching, sneezing, and nasal congestion. AR can also lead to eye and lower respiratory symptoms. In addition, AR is also one of the risk factors for asthma [4]. Studies have shown that AR affects the patient's quality of life, psychological state, and even the growth and development of children [5]. Early diagnosis and medical intervention will benefit patients with allergic rhinitis.

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The diagnosis of allergic rhinitis mainly relies on the patient's medical history, characteristic symptoms and signs, and the presence of allergen-specific IgE. However, patients' symptoms and signs are often changeable and lack specificity [6]. An immediate hypersensitivity skin test may cause a systemic allergy [7]. Serological IgE immunoassay is relatively expensive, and sensitivity varies widely [8]. This requires finding new biomarkers that are easy to detect and have particular diagnostic specificity and sensitivity.

Urine proteomics refers to the systematic analysis of protein composition and biological functions in urine at an overall level using proteomics techniques [9]. Urine is easily collected in large quantities and repeatedly through noninvasive procedures and is a good resource for clinical proteomics research. The protein composition of urine is relatively stable compared to other biological fluids, such as plasma or serum, that are prone to proteolytic degradation during and after sampling [9]. Although the total amount of protein in normal urine is much smaller than in plasma, thousands of proteins have been found in urine. Because the protein components in urine include proteins from the urinary system itself and proteins that are secreted into the blood from other organs and enter the proximal tubule through the filtration barrier and are not entirely recovered [10], urine protein can be used to study the pathological process of systemic and kidney diseases, and to discover new biomarkers [11]. With the development of technology, urine proteomics has become one of the most prevalent research areas for biomarker discovery [12].

In our previous studies, we found differences between the urine protein of AR patients and the normal population [13]. This study will analyze the bioinformatics characteristics of the above-mentioned differential proteins to investigate the potential clinical utility of differential proteins in the urine of AR patients.

2. Materials and methods

2.1. Study population

From March 2020 to June 2020, a total of 20 patients with allergic rhinitis, according to the diagnostic and treatment principle for allergic rhinitis and a recommended scheme [14], were included in the allergic rhinitis group (AR group). The exclusion criteria for participants were as follows: (1) comorbidities of asthma, eczema, or any other allergic diseases; (2) pregnant women; (3) chronic diseases such as chronic kidney disease, hypertension, diabetes, rheumatism, and hepatitis; (4) history of somatic or psychiatric abnormalities in the medical records; (5) taking antihistamines, glucocorticoids and other drugs within one week; and (6) concomitant medication history during the preceding two weeks. All participants were permanent residents of the area and had lived there for more than six months.

As described in our previous studies [13], the patients in the AR group were divided into the AR1 group with the total serum IgE $>$ 125 IU/ml and the AR 2 group with the total IgE $\leq$ 125 IU/ml. Ten healthy subjects without allergic diseases were enrolled in the NC group. As our previous studies showed, there was no significant difference in age, gender, and CRP levels among the three groups [13].

This study was approved by the ethics committee of Beijing Shijitan Hospital, and the participants all gave their informed consent following the provisions of the Helsinki Declaration.

2.2. Sample collection

Blood samples were collected from all subjects after an overnight fast following a standard protocol to minimize preanalytical bias. The volunteers' midstream urine samples were put into dry, clean containers and taken in the morning. Immediately after collection, urine samples were centrifuged at 4000 r/min for 5 min. Then, we collected the supernatant and divide it into equal parts, and freeze it at −80 °C. Patients with medicine usage (corticosteroids, antibiotics, et al.) 2 weeks before the urine sample collection should be excluded. No urine samples exhibited hematuresis, and urinary albumin/creatinine ratios (A/Cr) were less than 30 mg/g. Ten urine samples in each group were mixed and analyzed three times.

2.3. Total IgE measurements

Serum total IgE was measured using a protein analyzer (Siemens BNII, Siemens, Germany).

2.3.1. Urinary proteomics

2.3.1.1. Protein extraction and labeling. The samples were diluted until the final concentration falls within the range of the standard curve. The Bradford method was used to measure the concentration of the extracted protein.

100 μg protein of each sample was placed in a centrifuge tube. Adjust final volume to 100 μl with solution buffer. The pellet was resuspended in a centrifuge tube with 5 μl 200 mM reducing agent buffer, and incubated at 55 °C for 1 h. Reduced proteins were alkylated for 40 min at room temperature in the dark by adding IAA to a final concentration of 50 mM. A solution of iodoacetamide (5 μL 375 mM) was added to the sample and placed for incubation in the dark at room temperature for 30 min. The sample was transferred to an ultrafiltration tube add 200 μL 100 mM dissolution buffer. After centrifugation, the upper phenol layer was transferred to a new tube. This procedure was repeated four times, and 100 mM dissolution buffer was used to bring the volume up to 100 μL. Finally, proteins were then digested with 2.5 μL of 1 μg/μL trypsin per sample for 14 h at 37 °C.

After thawing the TMT (Tandem mass tag) reagent at room temperature, add 41 μL absolute ethanol into 0.8 mg TMT reagent per tube, centrifuge after shake for 5 min. 41 μL TMT labeling reagent was added into 100 μg (100 μL/sample) of digested peptides and

reacted for 1 h at 25 °C. Quenching Reagent (8 μL 5%) was added and incubated for 15 min to stop the reaction. The mixed labeled samples were centrifuged to the bottom of the tube after vortex oscillation. The labeled mixed samples were stored after lyophilization.

2.3.1.2. Peptide identification. The peptide fractions were added in 20 μL buffer A (0.1% FA, 2% ACN) and centrifuged at 12,000 rpm for 10 min. 10 μL of supernatant was injected into the nano UPLC-MS/MS system composed of an EASY nLC 1000 HPLC system (Thermo Scientific) and an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). The samples were added to an Acclaim PepMap100C18 column and separation was started. The mass spectrometer operates in positive ion mode, and the full MS scans were performed with 120,000 resolutions in the range of 300–1500 *m/z*. The 20 most abundant multiply charged ions were chosen for high-energy collision dissociation fragmentation following a full MS scan. Human protein database UniProt_HUMAN (2019.4.20) was used in this experiment. The MS/MS data were processed by proteome discoverer 1.4.

2.3.1.3. Protein analysis. After mass spectrometry analysis, results were retrieved from the database, and relevant proteins were separated. Differentially expressed proteins (DEPs) were identified based on fold changes (FC) calculated by comparing AR1 and NC, as well as AR2 and NC groups, and p-values were obtained by t-tests. The protein was identified using the retrieval parameter settings shown in Table 1. Proteins that were significantly differentially expressed had FC > 1.5, FC $< 2/3$, and a p-value of 0.05.

2.3.1.4. Functional enrichment and pathway analysis, and protein-protein interaction (PPI) network construction. We analyzed the functions and signaling pathways of DEPs using Metascape (http://metascape.org). Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched based on Metascape for biological process, cellular component, and molecular function categories. Only terms with a P-value < 0.01, a minimum count of 3, and an enrichment factor >1.5 were considered significant. The most statistically significant term in the cluster is selected as the term representing the cluster. A subset of enriched items is selected and presented as a network graph to identify further relationships between items, where items with a similarity >0.3 are connected. Protein-protein interaction enrichment analysis was performed using the BioGrid, InWeb_IM, and OmniPath databases. In addition, the Molecular Complex Detection (MCODE) algorithm was applied to identify significantly connected protein networks.

2.3.1.5. Statistical analysis. All data are presented as the mean ± standard deviation (SD). The student's t-test was used to evaluate differences among the NC group, AR1 group, and AR2 group. The Kruskal-Wallis test was used to compare nonnormal distributions among groups. The Bonferroni correction test was used to compare the two groups. A *P* value less than 0.05 was considered statistically significant (two-tailed). Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, USA).

3. Results

3.1. Clinical data and laboratory test characteristics

There were no significant differences among the three groups regarding smoking or not and the total number of neutrophils and basophils. There was no significant difference in the total white blood cell count between the AR2 and NC groups. The total number of eosinophils in the AR2 group was significantly higher than that in the NC group, but there was no significant difference compared with the AR1 group. The total number of white blood cells of patients in the AR1 group was significantly higher than those in the control group and the AR2 group. The specific IgE level of patients in the AR1 group was significantly higher than that in the AR2 group (median 13.0 vs. 1.1 IU/mL, $p = 0.030$) (Table 2).

3.2. Functional enrichment analysis of proteins that are expressed differently in the urine of people with allergic rhinitis

To explore the possible functions of DEPs found in our previous study, we performed GO and KEGG functional enrichment analyses in MetaScape (P-value < 0.05). The top 20 GO enrichment items were divided into three functional groups: biological process group,

Table 1
Parameter settings of the identification procedure.

Parameter	Value
Enzyme	Trypsin
Static modification	C carboxyamidomethylation (57.021Da)
Dynamic modification	Static modification, Dynamic modifications, Carboxyamidomethylation of Cys residues; Oxidation modification of Met residues
Species	Homo sapiens
Precursor ion mass tolerance	±15 ppm
Fragment ion mass tolerance	±0.02 Da
Max missed cleavages	2

Table 2
Comparison of clinical data and laboratory test results among groups.

Variables	NC group (n = 10)	AR1 group (n = 10)	AR2 group (n = 10)	p
Active smoking, n (%)	3 (30.0)	4 (40.0)	2 (20.0)	0.621
WBC, $\times10^9$/L	4.93 (0.84)	6.68 (1.26)	5.54 (1.31)	0.011
Neutrophil count, $\times10^9$/L	2.40 (2.10,3.10)	3.68 (3.09,4.20)	2.69 (2.49,4.05)	0.126
Basophil, $\times10^9$/L	0.01 (0.01,0.03)	0.02 (0.02,0.04)	0.02 (0.01,0.03)	0.378
Eosinophil, $\times10^9$/L	0.05 (0.04,0.07)	0.23 (0.18,0.58)	0.14 (0.07,0.30)	<0.001
sIgE, IU/mL	N/A	13.00 (5.90,90.80)	1.11 (0.01,5.20)	0.030

molecular function group, and cellular component group.

Compared with normal people, the up-regulated proteins in the urine of AR patients mainly participate in the cellular components of collagen-containing extracellular matrix, vesicle lumen lytic vacuole, etc. The participating biological processes mainly include cell-cell adhesion, response to wounding, regulation of peptidase activity, the biological process involved in symbiotic interaction, endocytosis, etc., the molecular functions of the protein are mainly cell adhesion molecule binding, calcium ion binding, and glycosaminoglycan binding. (Fig. 1, Table 3). The top 20 KEGG pathways of urinary DEPs for AR patients are shown in Fig. 2 and Table 4. KEGG pathway analysis showed that Complement and coagulation cascades, endocytosis, Lysosome, ECM-receptor interaction, cell adhesion molecules, and other signaling pathways are related to AR.

The cellular components involved in the down-regulated protein in the urine of AR patients are mainly lytic vacuoles, and the biological processes involved mainly include positive regulation of MAP kinase activity, response to inorganic substances, and adaptive immune response. The molecular function of the protein is mainly manifested as protease binding. (Fig. 3, Table 5). KEGG analysis failed to find significantly enriched signaling pathways.

To better understand the pathophysiological relationship between DEPs and AR, we performed a functional enrichment analysis of the protein interaction network and MCODE components. As shown in Fig. 4, the biological functions of upregulated proteins in AR

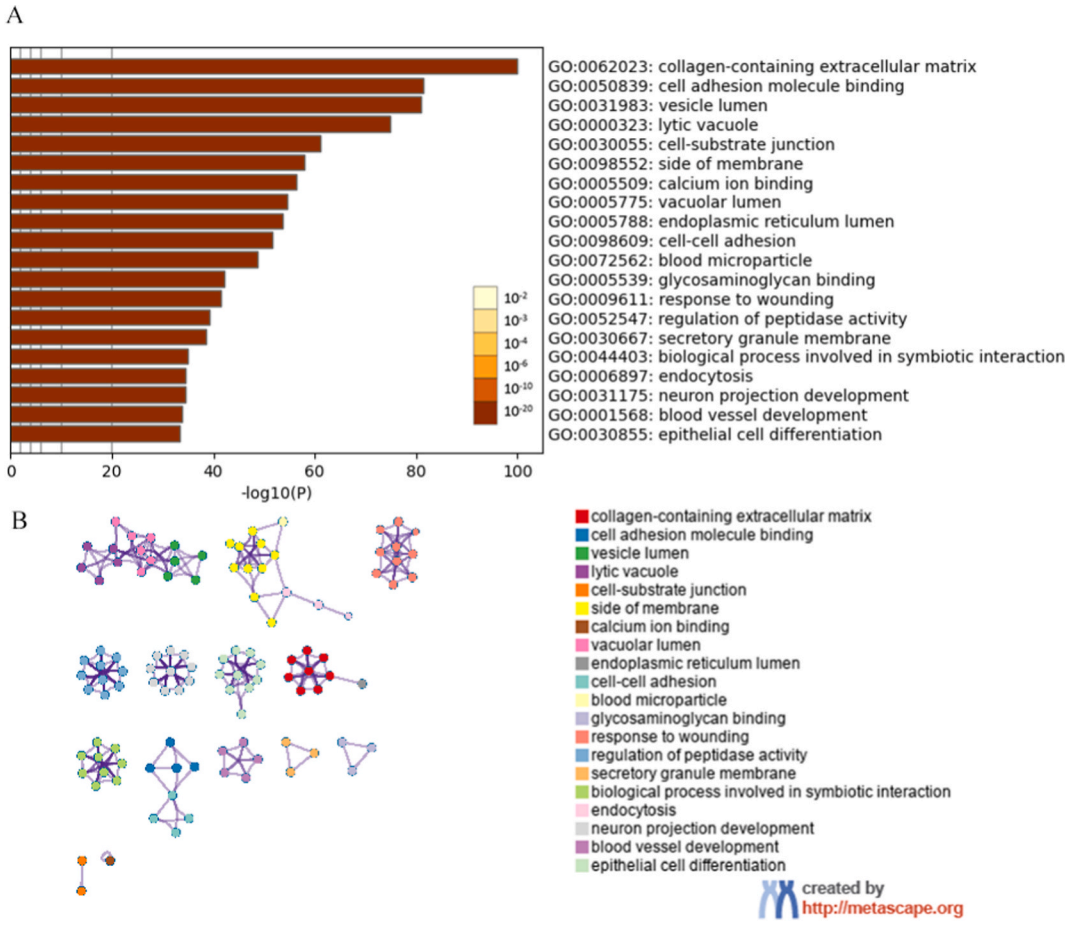


Fig. 1. GO enrichment analysis of urinary upregulated proteins in AR patients A: Enrichment analysis heat map; B: Network of GO enriched terms colored by cluster ID, where nodes that share the same cluster-ID are typically close to each other.

Table 3
The GO function enrichment analysis of up-expressed DEPs in AR.

GO	Category	Description	Count	%	Log10(P)	Log10(q)
GO:0062023	GO Cellular Components	Collagen-containing extracellular matrix	170	11.39	−100	−96.12
GO:0031983	GO Cellular Components	Vesicle lumen	129	8.65	−80.87	−77.22
GO:0000323	GO Cellular Components	Lytic vacuole	182	12.2	−74.86	−71.46
GO:0030055	GO Cellular Components	Cell-substrate junction	126	8.45	−61.06	−57.71
GO:0098552	GO Cellular Components	Side of membrane	154	10.32	−57.9	−54.63
GO:0005775	GO Cellular Components	Vacuolar lumen	79	5.29	−54.67	−51.46
GO:0005788	GO Cellular Components	Endoplasmic reticulum lumen	101	6.77	−53.63	−50.45
GO:0072562	GO Cellular Components	Blood microparticle	68	4.56	−48.68	−45.61
GO:0030667	GO Cellular Components	Secretory granule membrane	85	5.7	−38.5	−35.59
GO:0098609	GO Biological Processes	Cell-cell adhesion	129	8.65	−51.63	−48.48
GO:0009611	GO Biological Processes	Response to wounding	105	7.04	−41.55	−38.59
GO:0052547	GO Biological Processes	Regulation of peptidase activity	104	6.97	−39.26	−36.32
GO:0044403	GO Biological Processes	Biological process involved in symbiotic interaction	74	4.96	−34.94	−32.12
GO:0006897	GO Biological Processes	Endocytosis	103	6.9	−34.55	−31.76
GO:0031175	GO Biological Processes	Neuron projection development	119	7.98	−34.45	−31.68
GO:0001568	GO Biological Processes	Blood vessel development	102	6.84	−33.75	−31.02
GO:0030855	GO Biological Processes	Epithelial cell differentiation	112	7.51	−33.43	−30.73
GO:0050839	GO Molecular Functions	Cell adhesion molecule binding	165	11.06	−81.37	−77.61
GO:0005509	GO Molecular Functions	Calcium ion binding	155	10.39	−56.47	−53.23
GO:0005539	GO Molecular Functions	Glycosaminoglycan binding	78	5.23	−42.03	−39.06

patients are mainly related to cell adhesion molecule binding. As shown in Fig. 5, the down-regulated proteins in the urine of AR patients were primarily associated with the regulation of MAP kinase activity.

3.3. Functional analysis of the top differentially expressed protein between the AR and NC groups

Among the up-regulated proteins in AR1 vs. NC and AR2 vs. NC, the top ten with the most significant changes are protein HLA-DRB1, WFDC12, JAML, CREB3L4, TGM4, SPINT1, C8orf58, DEFA4, PDZK1IP1, and SUPT6H (Table 6). The top ten with the most significant changes for the down-regulated proteins are LAIR1, STXBP2, SLC10A3, VIPR1, SDC1, IFT122, GUSB, SQSTM1, LYPD1, and KIT (Table 7).

Enrichment analysis showed that HLA-DRB1, WFDC12, and DEFA4, among the top ten proteins in the up-regulation range, were related to the biological process of the humoral immune response. Among the top 10 down-regulated proteins, STXBP2, SDC1, GUSB, and SQSTM1 are related to the lytic vacuole in terms of cellular components, and GUSB, SQSTM1, and KIT are related to protein domain-specific binding in terms of molecular function (Fig. 6).

4. Discussion

Urine proteome detection technology can provide helpful information for disease diagnosis and monitoring. Based on our previously identified differentially expressed proteins from the urine of AR patients [13], functional enrichment analysis in the present study showed that these proteins might be potential biomarkers associated with AR pathogenesis.

The functional enrichment and protein interaction network analysis in this study found that, compared with normal people, the up-regulated proteins in the urine of AR patients mainly participate in the biological processes involved in cell-cell adhesion, and the signaling pathways are related primarily to complement and coagulation cascades. The adhesion and activation of inflammatory and immune cells play an essential role in the immune response to allergic rhinitis [15,16]. Studies have found that various cell adhesion-related molecules in nasal mucus and serum are associated with the severity of AR disease and have biomarker value [17,18], however, there are no protein biomarkers related to cell adhesion in the urine of AR patients. reports. Complement is a serum protein that mediates immune and inflammatory responses and is activated by antigen-antibody complexes [19]. The coagulation cascade is a process in which calcium-dependent zymogen-serine proteases are coordinated to transform into thrombin. Animal studies have found that the coagulation cascade is involved in the pathogenesis of allergic rhinitis [20]. Thwaites et al. found that allergens can induce complement activation in nasal mucus and biphasic activation of the coagulation cascade system [21]. RNA-seq analysis for allergic rhinitis by Li et al. found functional enrichment of complement and coagulation cascades in differentially expressed genes in nasal fibroblasts [22]. The findings of this study suggest that changes in urinary proteomics in patients with allergic rhinitis may be consistent with molecular biology in blood and nasal mucus.

The functional enrichment and protein interaction network analyses in this study confirmed that the down-regulated proteins in the urine of AR patients are mostly involved in the biological process of regulating MAP kinase activity in a positive way. The mitogen-activated protein (MAP) kinase signaling pathway plays a crucial role in many aspects of immune-mediated inflammatory responses [23], and phosphorylation of MAPK can lead to the production of inflammatory mediators and promote allergic inflammatory reactions [24]. Studies have shown that mitogen-activated protein kinase (MAPK) is involved in allergic rhinitis activation [25]. MAPK is an essential target for suppressing allergic inflammation [26]. This study found that the proteins related to the activation of MAPK were downregulated in the urine of AR patients, which was speculated to be associated with the negative feedback of the body's

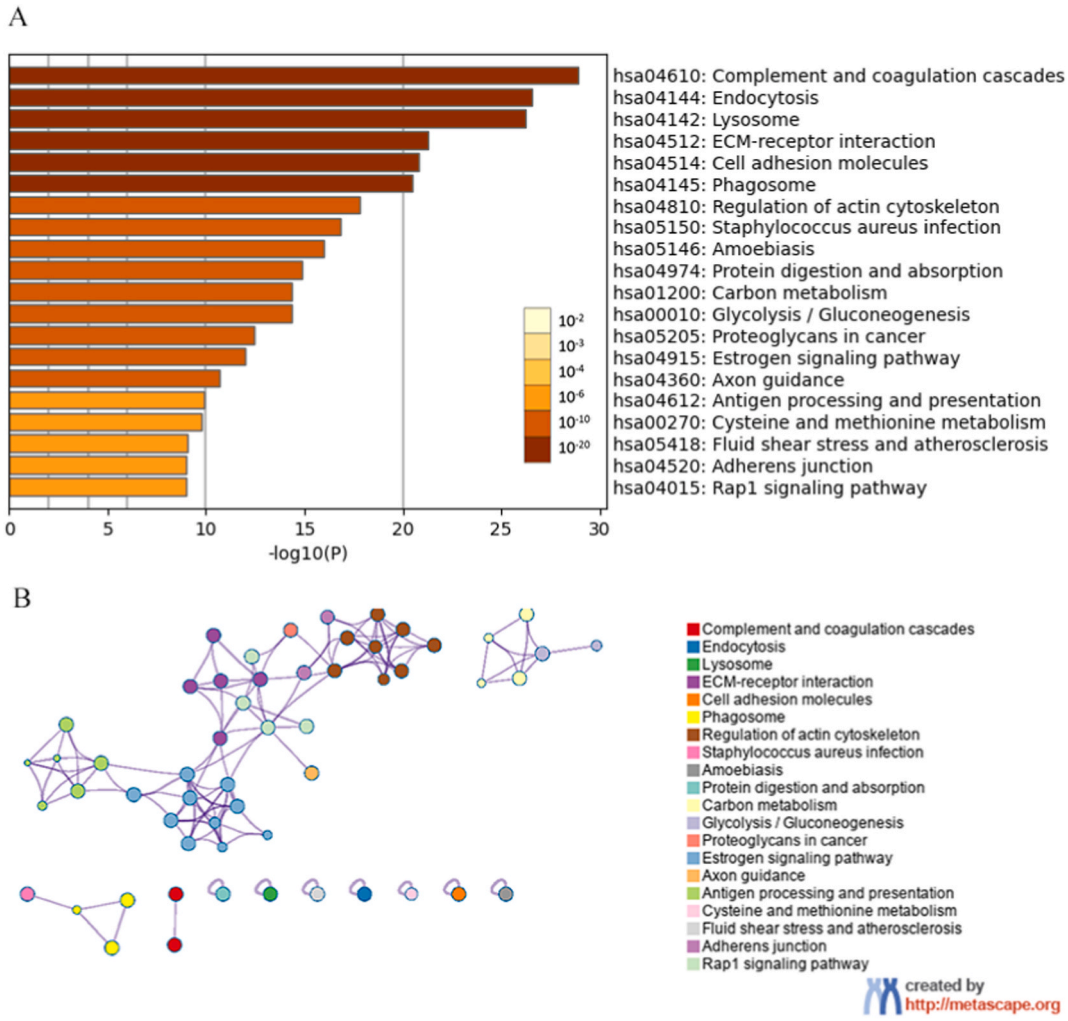


Fig. 2. KEGG pathway analysis of urinary proteins upregulated in AR patients. A: Enrichment analysis heat map; B: Network of KEGG enriched pathways colored by cluster ID, where nodes that share the same cluster-ID are typically close to each other.

immune regulation.

We then identified and analyzed the top ten differentially expressed proteins in urine that were up-regulated and down-regulated. Among the up-regulated proteins, the most differentially expressed protein was the HLA class II histocompatibility antigen, DRB1-10 beta chain (HLA-DRB1), a beta chain of an antigen-presenting major histocompatibility complex class II (MHCII) molecule. Together with the chain, they form a functional protein complex known as the HLA-DR antigen-binding heterodimer, which presents foreign peptides to the immune system to initiate an immunological response [27,28]. Regarding biomarker applications, Klimenta et al. found that patients with HLA-DRB1 gene mutations had an increased risk of developing rheumatoid arthritis [29]. HLA-DRB1 gene polymorphisms are associated with the efficacy of allergen-specific immunotherapy in Chinese AR patients [30]. Zhang et al. conducted gene expression profiling analysis in nasal epithelial cells and found that HLA-DRB1 is a hub gene for differentially expressed genes. However, unlike the results of this study, the study found that the expression of HLA-DRB1 was down-regulated [31]. In the future, more studies with a large sample population will help to make sense of this paradox.

The most differentially down-regulated protein was leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1). LAIR-1 is widely expressed on most immune cells and is a targeted receptor suppressing immune responses. It plays a vital role in avoiding excessive activation of the immune system and maintaining immune homeostasis [32]. Currently, there is no research on using LAIR-1 as a biomarker in allergic diseases. This study suggests that the expression of LAIR-1 in urine is significantly down-regulated, which may be related to the imbalance of immune function in patients with allergic rhinitis. The clinical diagnosis and predictive value of LAIR-1 should be further studied.

This study has certain limitations. As an exploratory study, although we found differential expression of urinary proteins at the level of urine proteomics, there may be inconsistent changes in the expression of other body fluids or tissues. In addition, whether the differential proteins found by the highly sensitive TMT marker quantitative proteomics detection can be found in the corresponding

Table 4
The KEGG function enrichment analysis of up-expressed DEPs in AR.

GO	Category	Description	Count	%	Log10(P)	Log10(q)
hsa04610	KEGG Pathway	Complement and coagulation cascades	40	2.68	−28.93	−26.39
hsa04144	KEGG Pathway	Endocytosis	63	4.22	−26.55	−24.31
hsa04142	KEGG Pathway	Lysosome	46	3.08	−26.23	−24.17
hsa04512	KEGG Pathway	ECM-receptor interaction	34	2.28	−21.25	−19.31
hsa04514	KEGG Pathway	Cell adhesion molecules	44	2.95	−20.79	−18.95
hsa04145	KEGG Pathway	Phagosome	43	2.88	−20.5	−18.74
hsa04810	KEGG Pathway	Regulation of actin cytoskeleton	48	3.22	−17.83	−16.29
hsa05150	KEGG Pathway	Staphylococcus aureus infection	31	2.08	−16.8	−15.3
hsa05146	KEGG Pathway	Amoebiasis	31	2.08	−15.95	−14.49
hsa04974	KEGG Pathway	Protein digestion and absorption	30	2.01	−14.9	−13.5
hsa01200	KEGG Pathway	Carbon metabolism	31	2.08	−14.33	−12.99
hsa00010	KEGG Pathway	Glycolysis/Gluconeogenesis	24	1.61	−14.32	−12.99
hsa05205	KEGG Pathway	Proteoglycans in cancer	39	2.61	−12.43	−11.12
hsa04915	KEGG Pathway	Estrogen signaling pathway	31	2.08	−12.01	−10.72
hsa04360	KEGG Pathway	Axon guidance	34	2.28	−10.7	−9.46
hsa04612	KEGG Pathway	Antigen processing and presentation	21	1.41	−9.92	−8.7
hsa00270	KEGG Pathway	Cysteine and methionine metabolism	17	1.14	−9.76	−8.57
hsa05418	KEGG Pathway	Fluid shear stress and atherosclerosis	27	1.81	−9.03	−7.87
hsa04520	KEGG Pathway	Adherens junction	19	1.27	−8.99	−7.85
hsa04015	KEGG Pathway	Rap1 signaling pathway	34	2.28	−8.97	−7.85

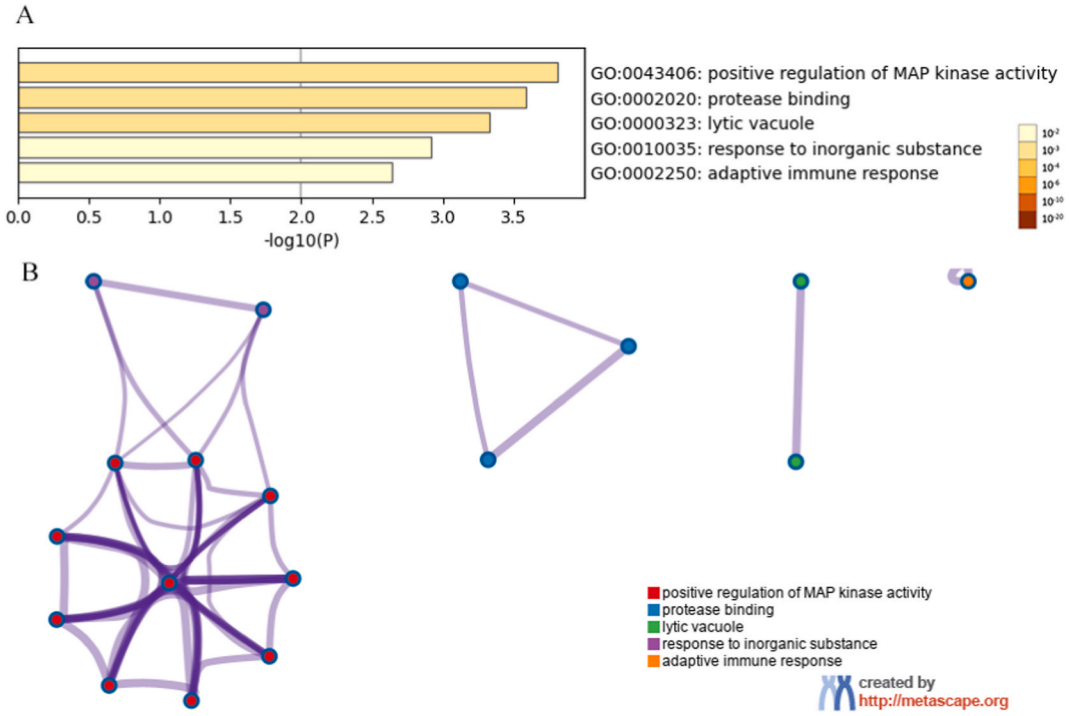
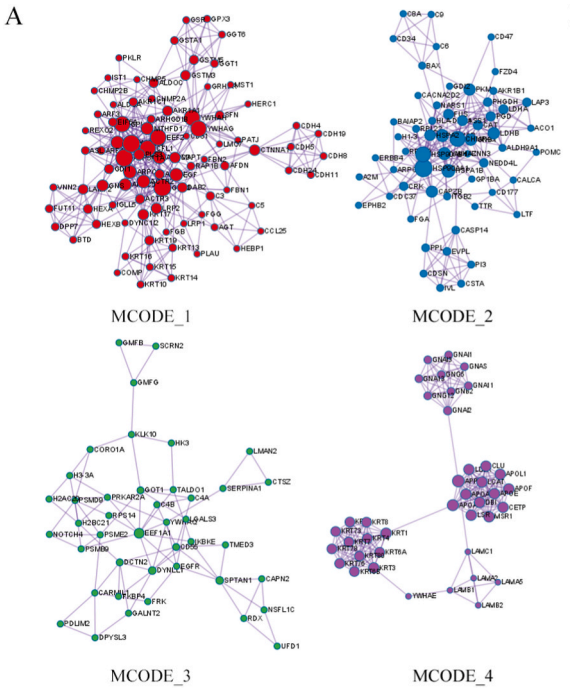


Fig. 3. GO enrichment analysis of urinary down-regulated proteins in AR patients. A: Enrichment analysis heat map; B: Network of GO enriched terms colored by cluster ID, where nodes that share the same cluster-ID are typically close to each other.

Table 5
The GO function enrichment analysis of down-expressed DEPs in AR.

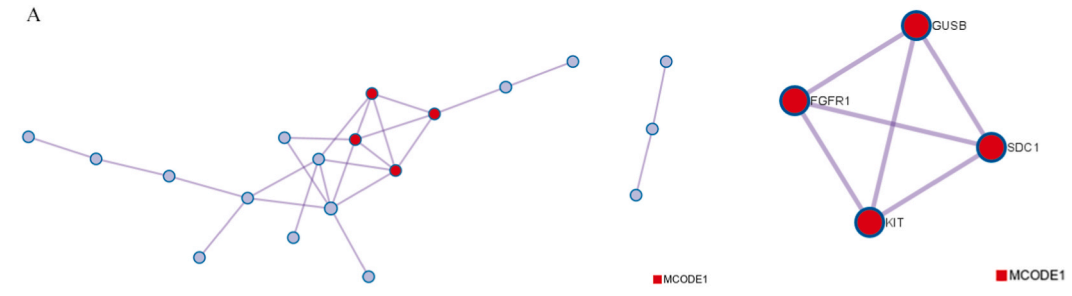
GO	Category	Description	Count	%	Log10(P)	Log10(q)
GO:0000323	GO Cellular Components	Lytic vacuole	5	18.52	−3.33	0.00
GO:0043406	GO Biological Processes	Positive regulation of MAP kinase activity	3	11.11	−3.81	0.00
GO:0010035	GO Biological Processes	Response to inorganic substance	4	14.81	−2.91	0.00
GO:0002250	GO Biological Processes	Adaptive immune response	4	14.81	−2.64	0.00
GO:0002020	GO Molecular Functions	Protease binding	3	11.11	−3.59	0.00



B

Color	MCODE	GO	Description	Log10(P)
	MCODE_1	GO:0050839	cell adhesion molecule binding	-15.7
	MCODE_1	GO:0034774	secretory granule lumen	-13.4
	MCODE_1	GO:0060205	cytoplasmic vesicle lumen	-13.4
	MCODE_2	GO:0050839	cell adhesion molecule binding	-12.8
	MCODE_2	GO:0001533	cornified envelope	-10.6
	MCODE_2	GO:0045296	cadherin binding	-10.4
	MCODE_3	GO:0101002	ficolin-1-rich granule	-9.8
	MCODE_3	GO:0030864	cortical actin cytoskeleton	-8.9
	MCODE_3	GO:0030863	cortical cytoskeleton	-8.1
	MCODE_4	GO:0030280	structural constituent of skin epidermis	-26.1
	MCODE_4	GO:0032994	protein-lipid complex	-25.7
	MCODE_4	GO:0034358	plasma lipoprotein particle	-23.5

Fig. 4. A protein-protein interaction (PPI) network of urinary up-regulated proteins and functional enrichment analysis of the four most important MCODE components. A: Modules selected from PPI network using MCODE. B: Functional enrichment analysis of MCODE components.



B

GO	Description	Log10(P)
GO:0043406	positive regulation of MAP kinase activity	-4.3
GO:0043405	regulation of MAP kinase activity	-3.7
GO:0009612	response to mechanical stimulus	-3.5

Fig. 5. Protein-protein interaction (PPI) of down-regulated proteins in the urine of AR patients. A: PPI network and most important MCODE components; B: independent functional enrichment analysis of MCODE components.

differential changes in routine laboratory inspection methods also needs further verification. The sample size of this study was small, and ROC analysis could not be performed on the diagnostic and predictive ability of differentially expressed proteins. Given the above limitations, we will verify the expression level, function, and predictive diagnostic value of potential urinary protein biomarkers in a larger sample population in the future based on the results of this exploratory study.

In conclusion, we found 1517 up-regulated and 27 down-regulated proteins in AR patients compared with normal control through urine proteomics research. Bioinformatics analysis showed that these differentially expressed proteins might be related to the pathophysiological changes of AR, which provides the possibility for further exploration of urinary proteomics biomarkers in the future.

Table 6
The top 10 proteins in the AR and NC groups in terms of up-regulation.

Protein Name	Description	Ratio.AR1_NC	pValue	Ratio.AR2_NC	pValue
HLA-DRB1	HLA class II histocompatibility antigen, DRB1-10 beta chain	24.93939	$p < 0.001$	28.06061	$p < 0.001$
WFDC12	WAP four-disulfide core domain protein 12	24.33333	$p < 0.001$	26.38164	$p < 0.001$
JAML	Junctional adhesion molecule-like	20.94054	$p < 0.001$	17.08649	$p < 0.001$
CREB3L4	Cyclic AMP-responsive element-binding protein 3-like protein 4	15.68857	$p < 0.001$	21.52857	$p < 0.001$
TGM4	Protein-glutamine gamma-glutamyltransferase 4	15.32889	$p < 0.001$	17.84444	$p < 0.001$
SPINT1	Kunitz-type protease inhibitor 1 (Fragment)	13.24542	$p < 0.001$	20.44689	$p < 0.001$
C8orf58	Uncharacterized protein C8orf58 (Fragment)	13.09058	$p < 0.001$	15.04348	$p < 0.001$
DEFA4	Neutrophil defensin 4	12.30435	$p < 0.001$	26.53044	$p < 0.001$
PDZK1IP1	PDZK1-interacting protein 1	11.71028	$p < 0.001$	16.18692	$p < 0.001$
SUPT6H	Transcription elongation factor SPT6	11.06349	$p < 0.001$	29.54762	$p < 0.001$

Table 7
The top 10 proteins in the down-regulated proteins between the AR and NC groups.

Gene Name	Description	Ratio.AR1_NC	pValue	Ratio.AR2_NC	pValue
LAIR1	Leukocyte-associated immunoglobulin-like receptor 1 (Fragment)	0.082131	$p < 0.001$	0.060436	$p < 0.001$
STXBP2	Syntaxin-binding protein 2 (Fragment)	0.152518	$p < 0.001$	0.22446	$p < 0.001$
SLC10A3	P3 protein	0.261772	$p < 0.001$	0.543291	$p < 0.001$
VIPR1	Vasoactive intestinal polypeptide receptor 1	0.328012	$p < 0.001$	0.340371	$p < 0.001$
SDC1	Syndecan-1	0.339803	$p < 0.001$	0.454819	$p < 0.001$
IFT122	Intraflagellar transport protein 122 homolog (Fragment)	0.411765	$p < 0.001$	0.423762	$p < 0.001$
GUSB	Beta-glucuronidase	0.421182	$p < 0.001$	0.39647	$p < 0.001$
SQSTM1	Sequestosome-1	0.469168	$p < 0.001$	0.28789	$p = 0.001$
LYPD1	Ly6/PLAUR domain-containing protein 1	0.477229	$p < 0.001$	0.540025	0.00011
KIT	Mast/stem cell growth factor receptor Kit	0.489996	$p = 0.003$	0.583503	$p = 0.004$

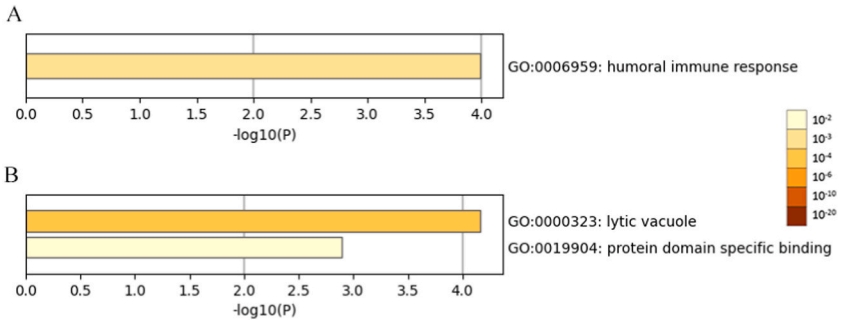


Fig. 6. Enrichment analysis of the top 10 urine differentially expressed proteins in the up-regulated and down-regulated ranges. A: GO heat map of the top 10 differentially expressed proteins that are upregulated; B: GO heatmap of down-regulated top 10 differentially expressed proteins.

Ethics statement

This study was approved by the ethics committee of Beijing Shijitan Hospital (2019 No. 74), and the participants all gave their informed consent following the provisions of the Helsinki Declaration.

Consent for publication

All the authors consent to the publication of the manuscript.

Author contribution statement

Na Liu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jitu Wang: Analyzed and interpreted the data; Wrote the paper.
Xueyan Wang: Contributed reagents, materials, analysis tools or data.
Man Zhang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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