ORIGINAL ARTICLE

Study of Urinary Protein Biomarkers in Hereditary Angioedema

Wu J[1,2*], Tang X[1,3*], Zhou N[4*], Wang X[4], Liu P[2], Zhang Z[2], Zhang S[2,5], Zhi Y[4]

[1]Institute of Clinical Medicine, National Infrastructure for Translational Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[2]State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science, Beijing, China
[3]State Key Laboratory of Common Mechanism Research for Major Diseases, Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China
[4]Department of Allergy and Clinical Immunology, National Clinical Research Center for Immunologic Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[5]Department of Cardiology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
*Jianqiang Wu, Xiaoyue Tang, and Nan Zhou contributed equally to this study.

J Investig Allergol Clin Immunol 2025; Vol. 35(5)
doi: 10.18176/jiaci.1032

Abstract

Background: Hereditary angioedema (HAE) is a rare and potentially life-threatening disease, and diagnosis is often missed or delayed. We aimed to identify noninvasive urinary protein biomarkers and to evaluate their potential roles in diagnosis and evaluation of disease severity.
Methods: Using data-independent acquisition (DIA)–based urinary proteomics, we identified proteins that were differentially expressed between patients with HAE and healthy control (HC) groups. Then, the parallel reaction monitoring (PRM)–targeted proteomics method was used to validate promising biomarker candidates in other HAE patients and HCs. Furthermore, enzyme-linked immunosorbent assay (ELISA) was conducted to verify levels of several key proteins in HAE, histamine-mediated angioedema, and HCs.
Results: Differential expression between HAE patients and HCs was observed in 269 of the 2562 urinary proteins identified. In the biofunction analysis, these differentially expressed proteins were significantly enriched in leukocyte migration, adhesion of immune cells, endothelial cell development, permeability of the vascular system, and death of immune cells. Moreover, a biomarker panel (C1 esterase inhibitor, pro-epidermal growth factor, and kininogen-1) was validated in 2 independent clinical cohorts with area under the curve values of 0.910 and 0.949 for a diagnosis of HAE. Additionally, the urinary clusterin level was found to be significantly correlated with HAE severity scores ($R=-0.758$, $P<.01$).
Conclusions: This study describes the first application of a DIA-PRM-ELISA workflow to identify noninvasive urine biomarkers of HAE. The results will contribute to our understanding of the pathogenesis of HAE and may also provide a potential alternative method for diagnosis and evaluation of disease severity.

Key words: Hereditary angioedema. Biomarkers. Urine. Proteomics. Pathogenesis.

Resumen

Antecedentes: El angioedema hereditario (AEH) es una enfermedad rara y potencialmente mortal. A menudo se pasa por alto y se retrasa su diagnóstico. Nuestro objetivo ha sido identificar biomarcadores proteicos urinarios no invasivos e intentar evaluar sus posibles funciones para el diagnóstico y la evaluación de la enfermedad.
Métodos: Utilizando la proteómica urinaria basada en la adquisición independiente de datos (AID), se identificaron proteínas expresadas diferencialmente entre los grupos de AEH y de controles sanos (CS). A continuación, se utilizó el método de proteómica dirigida de monitorización de reacción paralela (MRP) para validar los biomarcadores candidatos prometedores en otros pacientes con AEH y CS. Además, se realizaron ensayos inmunoenzimáticos (ELISA) para verificar niveles de varias proteínas clave en AEH, en angioedema histaminérgico y en CS.
Resultados: De las 2562 proteínas urinarias identificadas, 269 mostraron una expresión diferencial entre AEH y CS. En el análisis de biofunción, estas proteínas expresadas diferencialmente se encontraron enriquecidas significativamente asociadas a la migración de leucocitos, la adhesión de células inmunitarias, el desarrollo de células endoteliales, la permeabilidad del sistema vascular y la muerte celular de células inmunitarias. Además, en dos cohortes clínicas independientes se validó un panel de biomarcadores (inhibidor de C1 esterasa, factor de crecimiento proepidérmico y kininógeno-1) con valores de área bajo la curva (ABC) de 0,910 y 0,949 para el diagnóstico del AEH. Además, se observó que el nivel urinario de clusterina estaba significativamente correlacionado con las puntuaciones de gravedad de la enfermedad del AEH ($R = -0,758$, $p <0,01$).
Conclusiones: Este estudio describe la primera aplicación de un flujo de trabajo AID-MRP-ELISA para identificar biomarcadores urinarios no invasivos de AEH. Estos hallazgos contribuirán a comprender la patogénesis del AEH y también pueden proporcionar un método alternativo potencial para el diagnóstico y la evaluación de la gravedad de la enfermedad.

Palabras clave: Angioedema hereditario. Biomarcadores. Orina. Proteómica. Patogénesis.

Accepted Article

© 2024 Esmon Publicidad

Summary box

- **What do we know about this topic?**
  Hereditary angioedema (HAE) is a rare and potentially life-threatening disease. Its pathogenesis is not well understood. Moreover, noninvasive and disease-specific biomarkers are required for the early diagnosis and clinical management of HAE.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  We describe the first application of a DIA-PRM-ELISA workflow to identify and validate noninvasive urine biomarkers of HAE. This approach will contribute to our understanding of the pathogenesis of HAE and provide a potential alternative method for diagnosis and evaluation.

Accepted Article

Introduction

Hereditary angioedema (HAE) is a rare disease with a prevalence of 1/50 000 in the general population. It is characterized by recurrent and unpredictable episodes of subcutaneous and submucosal edema, usually affecting the face, extremities, trunk, genitalia, upper respiratory tract, and gastrointestinal tract [1]. The most dangerous clinical symptom of HAE is laryngeal edema, which has a 40% lifetime mortality rate [2,3]. Treatment of HAE has evolved in recent years and now focuses on 3 therapeutic strategies to prevent the onset, progression, and morbidity and mortality of edema, namely, on-demand acute attack treatment, short-term prophylaxis, and long-term prophylaxis [4-6]. It is easy to misdiagnose gastrointestinal edema as acute abdomen due to severe abdominal pain, resulting in unnecessary surgery [7]. However, the interval between the onset of symptoms and the final diagnosis of HAE has been reported to be approximately 10 years worldwide [8,9] and 12.6 years in China [10]. Long diagnostic delay and inappropriate treatment aggravate the humanistic and economic burden of HAE [11]. Early diagnosis and evaluation of HAE patients are, therefore, essential for improving their clinical management and quality of life.

Classic HAE can be subdivided into C1-esterase inhibitor (C1-INH) antigen deficiency (type 1 HAE, HAE-1) and C1-INH dysfunction (type 2 HAE, HAE-2), both of which are caused by mutations in the *SERPING1* gene [12]. HAE-1 accounts for most cases of HAE in China, with HAE-2 accounting for <5% of cases [13]. Currently, diagnosis of HAE-1/2 depends on the detection of the levels of antigenic C1-INH and complement C4 in serum and the activity of functional C1-INH in plasma. Given the absence or malfunction of C1-INH, the kallikrein-kinin system is overactivated in HAE, leading to uncontrolled generation of bradykinin. Bradykinin can bind to B2 receptors in endothelial cells and increase vascular permeability, leading to angioedema attacks [1]. HAE can also be caused by mutations in genes of factor XII (*F12*), angiopoietin-1 (*ANGPT1*), plasminogen (*PLG*), kininogen (*KNG1*), myoferlin (*MYOF*), and heparan sulfate-glucosamine 3-O-sulfotransferase 6 (*HS3ST6*) [14]. However, the underlying molecular mechanism of HAE remains unclear. To date, the study of biomarkers for the diagnosis and evaluation of HAE has focused on pathophysiologic pathways, including the complement, coagulation, and fibrinolysis systems and the vascular endothelium.

Proteomics has proven to be a powerful and popular tool in biomarker discovery and analysis of pathogenesis [15]. Mass spectrometry (MS)–based proteomics techniques enable high-throughput, accurate, and sensitive measurement of thousands of proteins in clinical samples. Urine is an ideal sample source for biomarker research because it can be collected noninvasively and in large volumes. More importantly, urine can be used to identify systematic changes in the body without regulation of homeostasis and can reflect mild and early pathological conditions [16,17]. With the rapid development of MS techniques, urinary proteomics has become a promising field in biomarker discovery [18]. In addition to its application in urological diseases [19], urinary proteomics is now also applied in cardiovascular diseases [20], brain diseases [21], and other diseases [22-24]. Although urinary biomarkers have been investigated in many diseases, no urinary biomarkers are currently available for diagnosis, monitoring, and evaluation of therapeutic efficacy in HAE [25]. Advances in high-throughput proteomics technology can enhance molecular characterization and identification of promising noninvasive biomarkers of HAE.

In this study, we first used data-independent acquisition (DIA)–based proteomics technology for urine proteomics profiling of HAE patients and healthy controls (HCs). Bioinformatics analysis was performed to annotate the biofunctions and pathways of the differentially expressed proteins of HAE. Then, the candidate biomarkers were validated in other HAE patients and HCs using parallel reaction monitoring (PRM)–targeted proteomics technology. Finally, enzyme-linked immunosorbent assay (ELISA) was performed to verify several critical proteins associated with HAE in an independent cohort, including HAE patients, patients with histaminergic angioedema (disease control [DC]), and HCs. This study identified several biomarkers in the urine of HAE patients and may provide a potential alternative method for diagnosis of HAE and evaluation of disease severity. In addition, our findings may improve our understanding of the pathogenesis of HAE.

Methods

HAE Patients and Controls

Consecutive HAE patients were recruited from Peking Union Medical College Hospital between 2021 and 2023.

 © 2024 Esmon Publicidad

The patients selected for the study were aged 18 to 75 years. The diagnosis of HAE was based on the clinical history and laboratory tests, as follows: (*1*) a history of recurrent skin angioedema without urticaria and/or recurrent attacks of abdominal pain and/or laryngeal edema; and (*2*) repeated confirmation of lower levels of C4 and C1-INH antigen in serum or lower levels of C1-INH function in plasma. Histaminergic angioedema is defined as recurrent angioedema with or without urticaria, which can be controlled by antihistamines, corticosteroids, epinephrine, and omalizumab. Unlike HAE mediated by bradykinin, histaminergic angioedema is mediated by mast cell mediators, including histamine [12]. All participants in this study provided their written informed consent, and the Ethics Committee of Peking Union Medical College Hospital approved the study (HS-2402).

Study Design

The discovery cohort comprised 20 adult HAE patients (15 HAE-1 and 5 HAE-2) and 29 healthy controls (HCs) recruited for DIA-based quantitative proteomics analysis. Validation cohort 1 comprised 24 HAE-1 patients and 18 HCs who were enrolled and validated using PRM-targeted proteomics. A further 60 HAE-1 patients, 30 DC patients, and 30 HCs were recruited in validation cohort 2 to verify key proteins identified above using ELISA. In validation cohort 2, a very small subset of HAE patients used medication irregularly. No medicine was used for over a week before urine sampling.

Urine Sample Preparation

A total of 5 mL of midstream first-morning urine sample was collected from each participant. Urine samples were from asymptomatic HAE patients (at least 2 weeks from their last episode). All participants had normal urine test results. The urine samples were centrifuged at 3000*g* for 20 minutes at 4°C to remove cell debris, and the supernatants were stored at –80°C for further proteomics analysis.

A filter-aided method was used to prepare the urine samples [26]. Briefly, an alkylation step was performed with 15 mM iodoacetamide at room temperature for 30 minutes after urine samples were reduced with 5 mM dithiothreitol at 100°C for 10 minutes. Subsequently, urinary proteins were precipitated with acetone and resuspended in 20 mM Tris buffer. In the next step, 100 µg of protein was loaded onto a 30-kD filter device and centrifuged at 14 000*g* at 18°C. The samples were washed 3 times with 20 mM Tris buffer and digested overnight with trypsin at 37°C. Two micrograms of each peptide sample was used for the proteomics analysis.

DIA Proteomics Analysis

An Orbitrap QE HF mass spectrometer coupled with an EASY-nLC 1200 UHPLC system (Thermo Fisher) was used for the proteomics analysis. The peptide samples were loaded onto a trap column and separated on an analytical column (75 µm × 500 mm, Kyoto Monotech). A 60-minute elution procedure was run with 5%-30% buffer B (0.1 formic acid in 99.9% acetonitrile). An iRT kit (Biognosys) was added to align retention times among the samples [27]. DIA mode was selected on the MS, and the parameters were as follows: 120 000 resolution and 350-1500 m/z range for the full scan, automatic gain control at 3e6, injection time <100 ms, and precursors with a +2 to +6 charge state. For quality control (QC), a mixed sample was inserted after every 10 samples during the DIA analysis.

PRM-Targeted Proteomics Validation

First, 6 technical replicates were applied in a pooled peptide sample. A spectrum library was built using Skyline software, and peptides were screened for PRM analysis. A total of 2 to 5 unique peptides per targeted protein were selected based on the following criteria: q value <1%, digested entirely by trypsin, 8-18 amino acid residues, and exclusion of the first 25 N-terminal amino acids. A retention time segment (RT) for each targeted peptide was set to ±2 minutes based on its expected RT from pooled samples. To determine the stability of the instrument signal, a mixed sample was used after every 10 samples. Additionally, an iRT standard was added to each sample, and chromatography retention times were evaluated during analysis to ensure data quality. In order to minimize system biases, different groups of samples were analyzed randomly.

Mass Spectrometry Data Processing

The DIA spectra library was generated from the raw MS data using Spectronaut software (Biognosys AG). Data were searched for in the UniProt human database with parent ion tolerances of 10 ppm and fragment ion mass tolerances of 0.05 Da. Trypsin digestion allowed up to 2 missed cleavage sites. Cysteine carbamidomethylation was defined as a fixed modification and methionine oxidation as a variable modification. Spectronaut Pulsar X was used to search the DIA raw files against the self-built spectra library. Using the iRT calibration strategy, an optimal extraction window for XIC was determined. A local regression was used to normalize the cross-run data [28], and data were filtered with a Q value of 0.01. Quantification of peptide intensity was based on the sum peak areas of the fragment ions in MS2.

Skyline software was used to process MS data in the PRM experiment. In Skyline, the transition settings were as follows: precursor charges +2 to +5; ion types b, y, and p; product ions from ion 3 to last ion −1; and ion match tolerance of 0.02 m/z. The 6 most intense product ions were selected, and the variable "min dotp" was set at 0.7. Progenesis QI for Proteomics software (Nonlinear Dynamics) was used to collect total ion current chromatograms for each sample. To correct for sample size and mass signal intensity errors, the mass spectra of each peptide were normalized with their associated total ion current chromatograms. The results for each peptide were quantitatively analyzed, and the differential proteins identified were compared with the DIA results.

Immunoassay Verification of Several Key Proteins

According to the manufacturer's instructions, ELISA kits were used to validate 3 urine proteins, namely, C1-IHN (Raybio, ELH-SERPING1), proepidermal growth factor (EGF) (Raybio, ELH-EGF), and KNG1 (AssayGenie, HUDL01639). Briefly, urine samples were diluted with different dilution

Accepted Article

© 2024 Esmon Publicidad

doi: 10.18176/jiaci.1032

factors. The standard and urine samples were added to ELISA plates for incubation. Following washing with wash buffer, the plates were incubated for 1 hour with an antibody cocktail. Each well was washed, and HRP-conjugated streptavidin was added to catalyze the reaction. Finally, the catalytic reaction was stopped by the addition of sulfuric acid, and the optical density was measured at 450 nm using a microplate reader (Thermo Scientific). The concentration of these biomarkers was normalized using the protein concentration of urine samples.

Bioinformatics and Statistical Analysis

For pathway enrichment analysis, the UniProt numbers of the differential proteins were uploaded to the ingenuity pathway analysis (IPA) software (Qiagen), and the biofunction categories and canonical pathways were enriched. A protein–protein interaction network was constructed using the STRING database (http://string-db.org/). Cytoscape software (version 3.9.1, https://cytoscape.org/) was used to construct and visualize the PPI network, and another Cytoscape plugin, cytoHubba, was used to identify the hub proteins. The statistical analysis was performed using SPSS 22.0 for Windows (IBM Corp.) and Prism (GraphPad Software). Receiver operating characteristic (ROC) analysis was performed using MedCalc software. The diagnostic model was established using the "Biomarker discovery" module on the MetaboAnalyst website (https://www.metaboanalyst.ca).

Ethics Approval

This study was approved by the Ethics Committee of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences (No. HS-2402). All participants provided their written informed consent.

Results

Clinical Characteristics of Participants

The design of this study is shown in Figure 1. The participants were divided into 3 cohorts. In the discovery cohort, 20 drug-naive HAE patients and 29 HCs were included for differential proteome analysis. In validation cohort 1, 24 newly diagnosed and drug-naive HAE-1 and 18 HCs were recruited for PRM-targeted validation. In validation cohort 2, 60 HAE-1 patients, 30 DC patients, and 30 HCs were recruited for the ELISA analysis (Table). HAE, HCs, and DCs were matched by age and sex. Among patients with HAE, 89.4%, 62.5%, and 63.5% reported previous skin, gastrointestinal, and laryngeal edema, respectively.

DIA Proteomics Analysis

In the biomarker discovery phase, the DIA-based quantitative proteomics method was used to analyze urine samples from 20 HAE patients and 29 HCs. QC samples were used to assess the stability of the proteomics results. A satisfactory correlation was found between the quantified protein intensities of the QC samples (R2 values >0.90, Figure 2A). There were 2562 protein groups identified in urine samples, and 1716 proteins quantified with at least 2 unique peptides were used for further analysis.

A total of 269 urine proteins were differentially expressed between HAE patients and HCs (ratio >1.5, *P*<.05). A total of 203 proteins were significantly upregulated, and 66 were significantly downregulated in HAE patients (Figure 2B). The orthogonal partial least squares discriminant analysis and heatmap results also showed that there was a significantly different urinary protein expression pattern between HAE patients and HCs (Figure 2C-D). Compared to HAE-2 patients

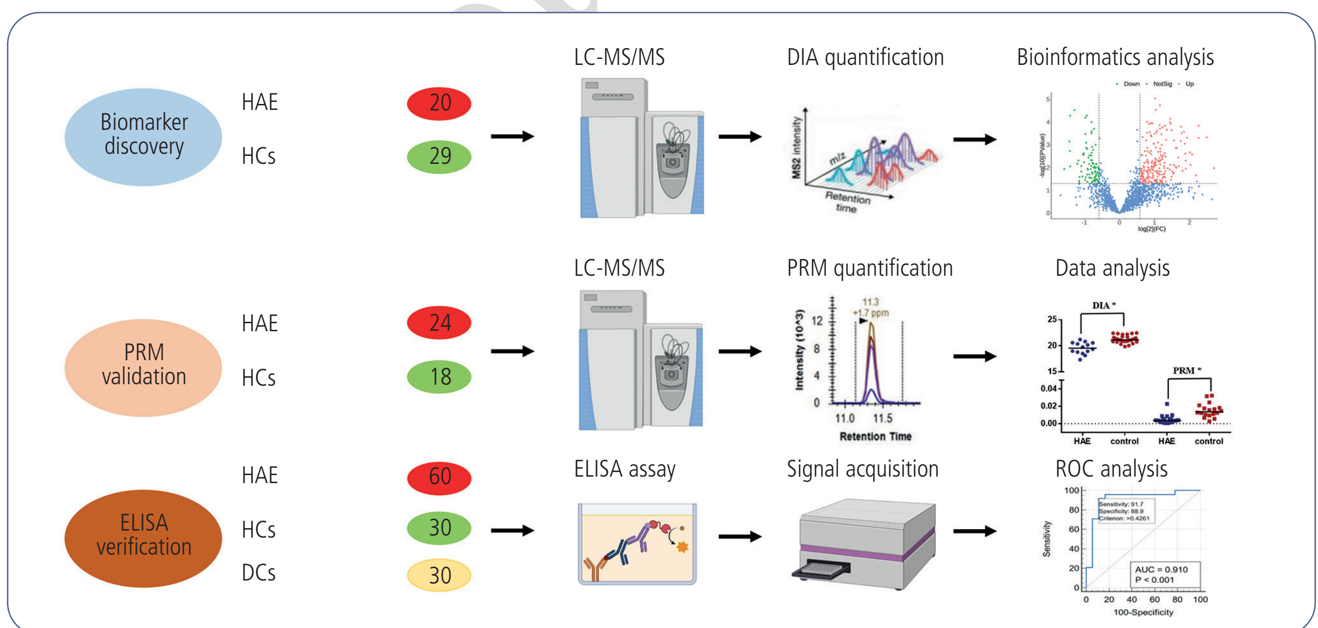

Figure 1. Study workflow. HAE indicates hereditary angioedema; HCs, healthy controls; DCs, disease controls; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; DIA, data-independent acquisition; PRM, parallel reaction monitoring; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic curve analysis; AUC, area under the curve.

© 2024 Esmon Publicidad

Table. Clinical Characteristics of the Study Participants.[a]

Variable	Discovery cohort			Validation cohort 1			Validation cohort 2			
	HAE	HCs	*P* value	HAE	HCs	*P* value	HAE	DCs	HCs	*P* value
No.	20	29	NA	24	18	NA	60	30	30	NA
Sex, M/F	6/14	9/20	.938	9/15	5/13	.508	21/39	11/19	12/18	.898
Mean (SD) age, y	43.25 (12.98)	43.00 (12.74)	.915	42.79 (11.30)	41.83 (7.89)	.760	39.85 (13.74)	38.03 (13.71)	39.61 (11.41)	.822
Skin edema	17	0	NA	18	0	NA	58	30	0	NA
Gastrointestinal edema	12	0	NA	9	0	NA	44	0	0	NA
Laryngeal edema	14	0	NA	15	0	NA	37	0	0	NA

Abbreviations: DCs, disease controls, histaminergic angioedema; HAE, hereditary angioedema; HCs, healthy controls.
[a]Groups were compared using analysis of variance, the *t* test, or the χ^2 test.

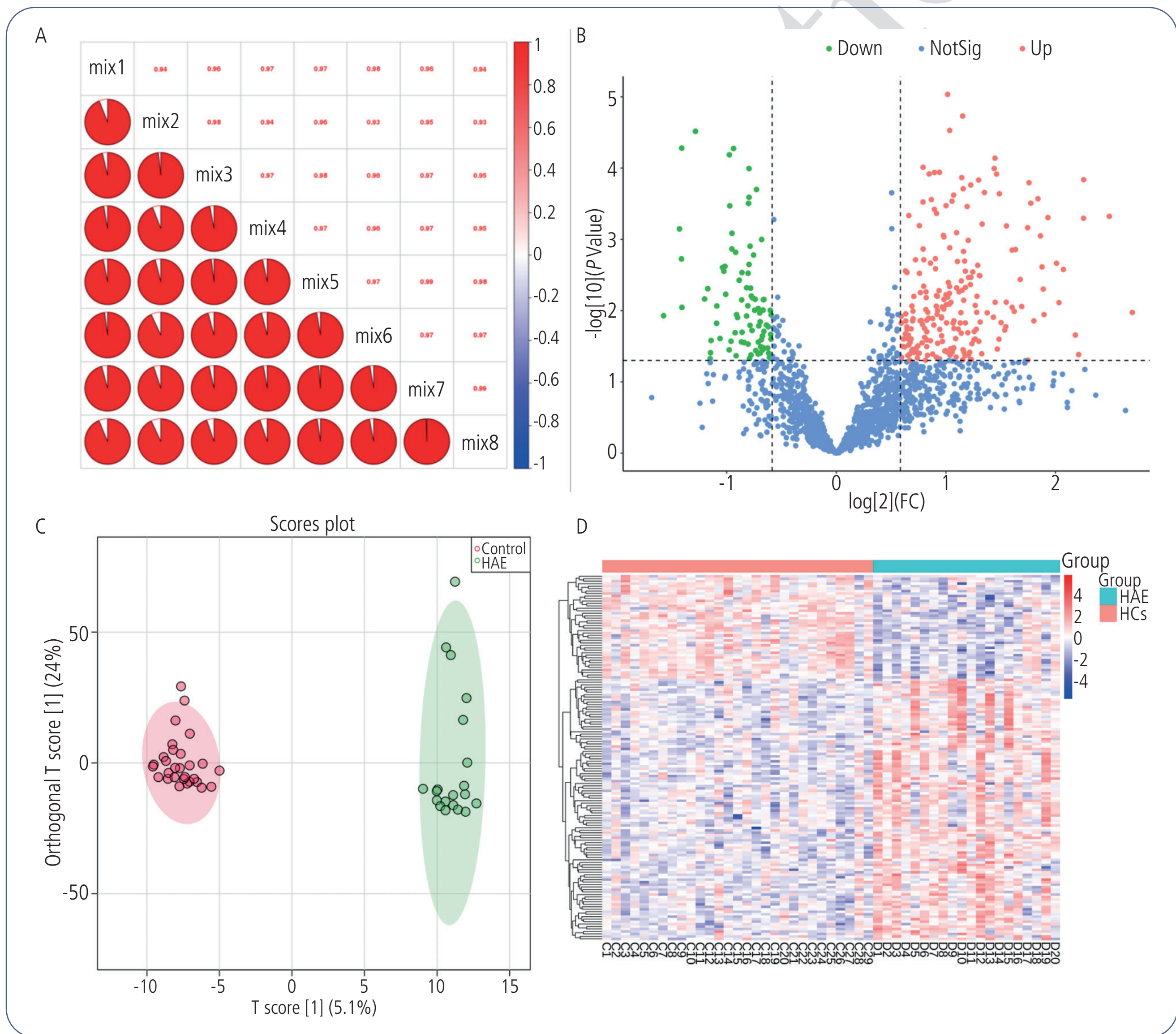

Figure 2. Urine proteomics profiling of HAE patients. A, Correlation analysis of quality control samples in DIA proteomics quantification. B-D, The volcano plot, orthogonal partial least squares discriminant analysis, and heatmap analysis of urine samples between HAE and HCs. HAE indicates hereditary angioedema; HCs, healthy controls.

© 2024 Esmon Publicidad

doi: 10.18176/jiaci.1032

and HCs, urinary C1-INH expression was significantly downregulated in HAE-1 patients.

Bioinformatics Analysis of Differential Proteins Between HAE patients and HCs

The 269 differentially expressed proteins were further analyzed using IPA in order to identify canonical pathways and biofunctions in the pathogenesis of HAE. The results showed that IL-15 signaling, B-cell receptor signaling, phagosome maturation, phospholipase C signaling, the coagulation system, acute phase response signaling, communication between innate and adaptive immune cells, the complement system, leukocyte extravasation signaling, and actin cytoskeleton signaling were significantly enriched in HAE (Figure 3A). The disease and biofunction analysis showed that the differentially expressed proteins were significantly enriched in movement of leukocytes, adhesion of immune cells, immune-mediated inflammatory disease, cell-cell contact, endothelial cell development, organization of cytoskeleton, permeability of the vascular system, vascular lesions, death of immune cells, and HAE (Figure 3B). According to these results, urinary proteomics can reflect changes in pathophysiology associated with HAE.

A coexpression network was generated using STRING and Cytoscape based on differential proteins in HAE. The most significant cluster consisted of 10 hub proteins, including clusterin (CLU), C1-INH, CD59 glycoprotein, kininogen-1 (KNG1), complement C4-A, EGF, ras-related C3 botulinum toxin substrate 1 (RAC1), angiotensin-converting enzyme, aminopeptidase N (ANPEP), and glutamyl aminopeptidase (Figure 3C). Furthermore, a machine learning–based biomarker diagnostic model was constructed using hub proteins. A biomarker panel consisting of C1-INH, EGF, and KNG1 had good diagnostic performance with an area under the curve (AUC) value of 0.956 (Figure 3D).

We also investigated the association between urinary proteome and disease severity in order to demonstrate the clinical utility of the in-depth proteomics analysis. Severity of HAE was assessed using a scoring system based on age at onset, location of edema, clinical manifestations, and need for long-term prophylaxis [29]. Based on our study, 2 urinary proteins (RAC1 and junctional adhesion molecule C) exhibited positive correlations with disease severity. Meanwhile, 6 urine proteins exhibited negative correlations with severity of HAE, including CLU, EGF, ANPEP, glutaminyl-peptide cyclotransferase, ß-1,4-galactosyltransferase 1, and mannosyl-

Article

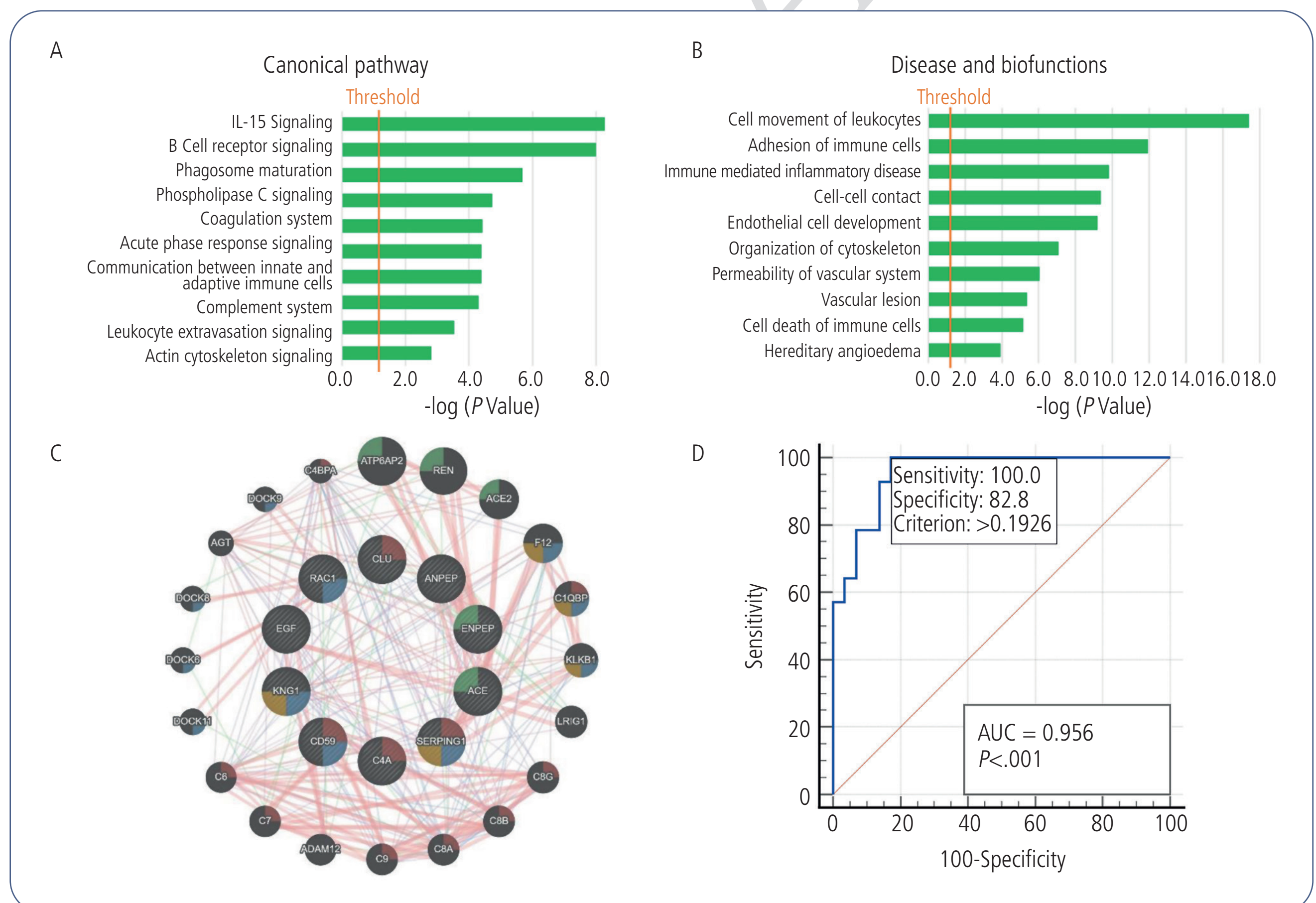

Figure 3. Bioinformatics analyses of expressed proteins differentially between HAE patients and healthy controls. A-B, Canonical pathway and disease/biofunction annotation of differential proteins in ingenuity pathway analysis. C, Coexpression network analysis of differential proteins of HAE. D, Receiver operating characteristic curve analysis result of a biomarker panel (EGF + C1-INH + KNG1). IPA indicates ingenuity pathway analysis; ROC, receiver operating characteristic; AUC, area under the curve.

doi: 10.18176/jiaci.1032
© 2024 Esmon Publicidad

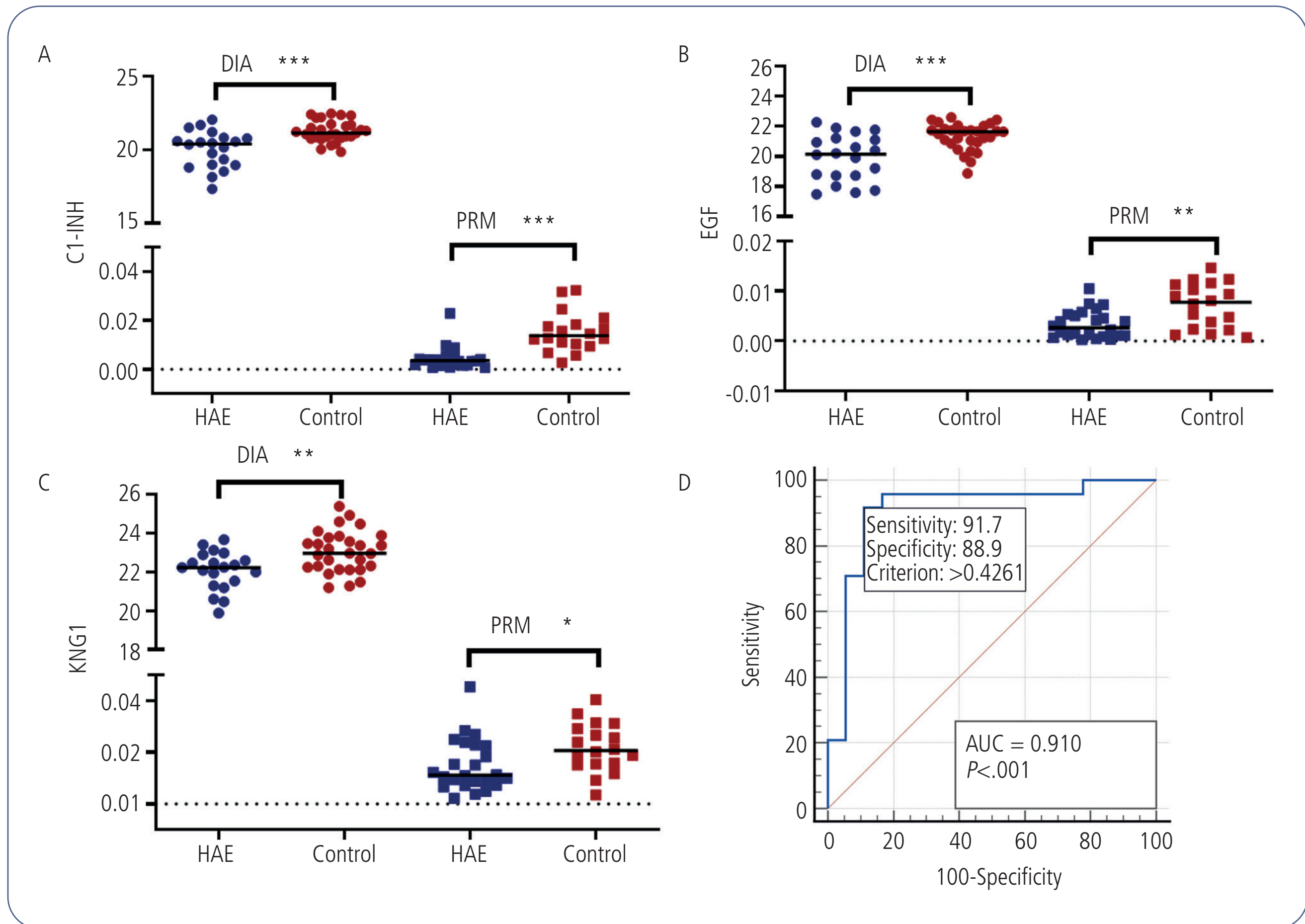

Figure 4. Targeted proteomics validation of promising biomarkers in another cohort. A-C, Protein abundance of C1-INH, EGF, and KNG1 in urine samples using DIA- and PRM-based quantitative proteomics. D, ROC analysis of the biomarker panel (C1IN + EGF + KNG1) for diagnosis of HAE. DIA indicates data-independent acquisition; PRM, parallel reaction monitoring; C1-IHN, C1-esterase inhibitor; EGF, proepidermal growth factor; KNG, kininogen; AUC, area under the curve; HAE hereditary angioedema; ROC, receiver operating characteristic.

oligosaccharide 1,2-α-mannosidase IA. Moreover, CLU was the most significant urine protein associated with severity of HAE (R=–0.758, P<.01), suggesting urinary CLU level may predict the severity of HAE.

PRM Validation of Promising Biomarkers of HAE

Biomarker candidates identified from the DIA experiment were further validated in validation cohort 1, which comprised 24 HAE patients and 18 HCs. QC samples were analyzed after every 10 samples during targeted proteomic analysis to ensure system stability and limit systemic bias. A Pearson correlation coefficient >0.95 was found in the correlation map for the QC samples, demonstrating good stability of the PRM-targeted proteomics analysis platform.

Based on targeted quantitative proteomics, a total of 10 urinary protein biomarkers exhibited trends similar to those quantified in the DIA experiment, including C1-INH (ratio=0.32, P<.001), EGF (ratio=0.50, P<.003), KNG1 (ratio=0.68, P=.029), malate dehydrogenase (ratio=0.49, P<.001), kallikrein-1 (ratio=0.48, P<.001), a disintegrin and metalloproteinase with thrombospondin motifs 1 (ratio=2.06, P=.054), napsin-A (ratio=0.51, P=.039), profilin-1 (ratio=1.57, P=.061), immunoglobulin heavy constant γ 4 (ratio =0.55, P =0.016), and heat shock protein ß-1 (ratio=2.07, P=.205). Furthermore, ROC curve analysis was performed on the biomarker panel (C1-INH, EGF, and KNG1) to evaluate its diagnostic performance for HAE. This biomarker panel performed well, with a sensitivity of 91.7% and specificity of 88.9%, resulting in an AUC of 0.910 (Figure 4).

Immunoassay Verification of 3 Critical HAE-Associated Proteins

Given that several key proteins associated with HAE were significantly changed in the DIA proteomics analysis and validated by targeted quantitative proteomics, we further verified 3 urinary proteins of the biomarker panel (C1-INH, EGF, and KNG1) in validation cohort 2 using ELISA to confirm that they could be used in clinical practice. The urinary expression levels of C1-INH, EGF, and KNG1 in the HAE group were significantly downregulated compared with those in the DC and HC groups (P<.05). ROC curve

© 2024 Esmon Publicidad

doi: 10.18176/jiaci.1032

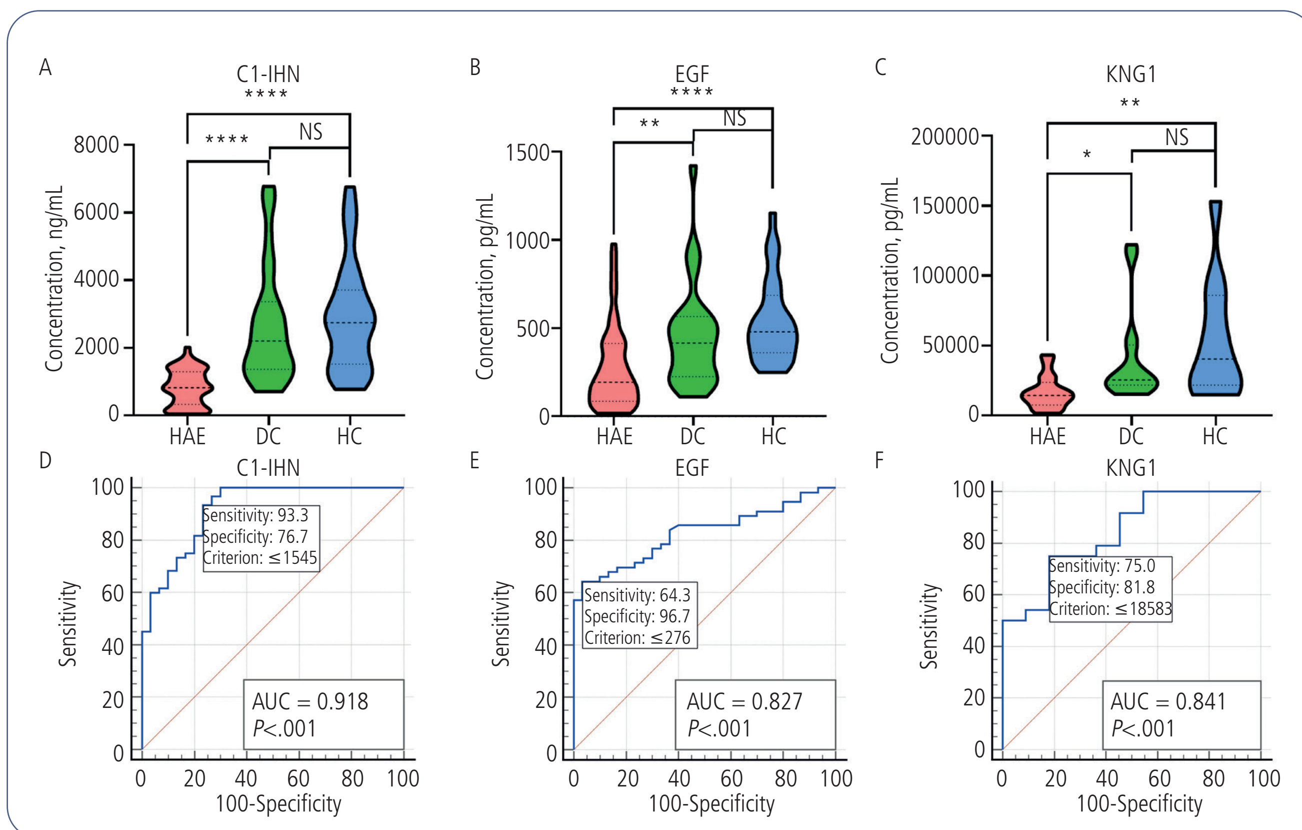

Figure 5. ELISA verification of C1-INH, EGF, and KNG1 in validation cohort 2. A-C, Comparison of concentrations of C1-INH, EGF, and KNG1 between HAE patients, histaminergic angioedema patients (DCs), and healthy controls (HCs). D-F, ROC analysis of C1-INH, EGF and KNG1 between HAE patients and healthy controls. HAE indicates hereditary angioedema; ROC, receiver operating characteristic curve; C1-IHN, C1-esterase inhibitor; EGF, proepidermal growth factor; KNG, kininogen; AUC, area under the curve.

analysis showed that the AUCs of C1-INH, EGF, and KNG1 were 0.918, 0.827, and 0.841, respectively (Figure 5). When the 3 proteins were combined, this biomarker panel achieved a higher diagnostic efficacy, with an AUC value of 0.949 (sensitivity, 100; specificity, 76; *P*<.001).

Discussion

This study describes the first application of a DIA-PRM-ELISA workflow to identify and validate noninvasive and HAE-specific biomarkers in urine. Some biological biofunctions and signaling pathways associated with the pathogenesis of HAE were significantly changed, and several promising biomarkers of HAE were further validated in 2 independent cohorts using targeted quantitative proteomics and ELISA. This is the first application of proteomics analysis in urine samples from patients with HAE. The results may improve our understanding of the pathogenesis of HAE and provide a potential noninvasive method for diagnosing and evaluating HAE.

The pathogenic mechanism underlying HAE is not fully understood. Previous studies have shown that the kallikrein-kinin, complement, coagulation, and fibrinolytic systems are involved in pathogenesis [30]. In addition, activation of the contact system would result in bradykinin production, which could activate the receptors on vascular endothelial cells to promote increased vascular permeability and angioedema [1,31]. In the present study, the proteomics results of HAE also suggested that some signaling pathways were significantly changed, including IL-15 signaling, B-cell receptor signaling, phagosome maturation, phospholipase C signaling, the coagulation system, acute phase response signaling, communication between innate and adaptive immune cells, the complement system, leukocyte extravasation signaling, and actin cytoskeleton signaling. Recently, B-cell receptor signaling, IL-15 signaling, phagosome maturation, and acute phase response signaling were reported to be associated with acute HAE attacks [32]. Phospholipase C signaling can mediate vasodilation and formation of edema [33]. Recent evidence has also shown that innate and adaptive immune responses are involved in the pathophysiology of HAE and that immune cells are a source of vasoactive mediators [34]. Our results indicate an increased immune-inflammatory load in HAE patients, albeit during the symptom-free periods. In addition, leukocyte extravasation signaling involves leukocyte rolling and docking on the endothelial surface of vessels, leading to contraction of the actin cytoskeleton in endothelial cells. Actin cytoskeleton signaling plays a vital role in endothelial permeability and formation of edema [35,36]. Our findings demonstrate that

 © 2024 Esmon Publicidad

urinary proteomics could reflect pathophysiological changes in HAE and that urine proteins may serve as promising biomarkers for diagnosis and evaluation of HAE. In addition, we observed that the expression of *F12*, *PLG*, and *KNG1* was significantly changed in urine samples from HAE patients (Figure S1). As rare variants in these 3 genes may trigger HAE with normal C1-INH (HAE-nC1-INH) [37], abnormal levels of these proteins in the urine of HAE-1/2 patients are interesting but need further investigation.

We designed a machine learning–based diagnostic model to assess HAE. The hub proteins in the coexpression network analysis of differential proteins were used to construct the biomarker panel for diagnosis of HAE. Our results showed that the combination of 3 urinary biomarkers (C1-INH, EGF, KNG1) performed well for HAE. Furthermore, this biomarker panel has been validated in 2 independent clinical cohorts using targeted quantitative proteomics and ELISA, demonstrating its good prospects for translation to and application in clinical practice, where serum C1-INH levels and plasma C1-INH activity can be used for diagnosis of HAE type 1 and type 2. However, it is unknown whether urinary C1-INH can be used to diagnose HAE. Urine is formed through selective glomerular filtration and tubular reabsorption of blood in the kidney. Control of the homeostasis mechanism may enable protein changes to be more sensitively detected in urine than in blood [38]. Thus, there is no direct correlation between urinary proteins and blood proteins. In this study, we report for the first time that urinary C1-INH can be used in the diagnosis of HAE. EGF has rarely been reported in HAE. Further research is needed to investigate the role of EGF in the pathogenesis of HAE. KNG1, also a protease inhibitor, is involved in the blood coagulation process. Mutations in the *KNG1* gene can cause a rare type of HAE with a normal C1-INH level [37,39]. Furthermore, urinary CLU expression was significantly decreased in HAE patients and was negatively correlated with severity of HAE. The urinary CLU measured is the soluble form of CLU, which is generated by shedding the membrane-bound form or vesicle release. The role of CLU in the pathogenesis of HAE has not been reported. Previous studies showed that extracellular CLU is a multifunctional cell protector that can sequester harmful agents and help remove them [40]. We speculate that the decrease in urinary CLU level may result from its increased consumption. Urinary CLU may be a potential biomarker of the severity of HAE, and further research is needed to validate its performance in more samples.

HAE is a potentially life-threatening disease, although it is easily misdiagnosed owing to its rarity and nonspecific clinical manifestations. In this study, the DIA-PRM-ELISA workflow was used to identify and validate urine biomarkers. These HAE-specific biomarkers also have the potential to be an alternative screening method for HAE. Urine can be collected noninvasively and is more stable and less complex than other biofluids, and in prospective studies, urine samples can be collected from the same individual repeatedly.

Our study is subject to a series of limitations. As HAE is a rare disease, most of the HAE patients in this study were HAE-1. In the future, more HAE-2 and HAE-nC1-INH patients will be recruited for further validation. In addition, the urine samples used in this study were collected during symptom-free periods. In future research, urine samples during HAE attacks can be used to analyze disease heterogeneity, predict response to treatment, and monitor HAE attacks.

Conclusion

This study describes the first application of a DIA-PRM-ELISA workflow to identify and validate noninvasive and HAE-specific biomarkers in urine. The results revealed that urinary proteomics can reflect pathophysiological changes in HAE and that urine proteins have the potential to serve as noninvasive biomarkers for diagnosing and evaluating HAE. The role of these biomarkers in the pathogenesis of HAE and their possible application as an alternative method for screening and evaluation of the activity of HAE require further research and validation.

Funding

This study was supported by Beijing Natural Science Foundation (No. L222082, 5244054), the National Natural Science Foundation of China (No. 82271815), the National Key Research and Development Program of China (No. 2016YFC0901501), the National High Level Hospital Clinical Research Funding (2022-PUMCH-B-090), and the CAMS Innovation Fund for Medical Sciences (CIFMS, No.2021-I2M-1-003; No.2022-I2M-C&T-B-004).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Data Availability

The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium via the iProX partner repository with data set identifier PXD045435. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Busse PJ, Christiansen SC. Hereditary Angioedema. N Engl J Med. 2020;382(12):1136-48.
2. Xu YY, Zhi YX, Liu RL, Craig T, Zhang HY. Upper airway edema in 43 patients with hereditary angioedema. Ann Allergy Asthma Immunol. 2014;112(6):539-44 e1.
3. Bork K, Hardt J, Witzke G. Fatal laryngeal attacks and mortality in hereditary angioedema due to C1-INH deficiency. J Allergy Clin Immunol. 2012;130(3):692-7.
4. Branco Ferreira M, Baeza ML, Spinola Santos A, Prieto-Garcia A, Leal R, Alvarez J, et al. Evolution of Guidelines for the Management of Hereditary Angioedema due to C1 Inhibitor Deficiency. J Investig Allergol Clin Immunol. 2023;33(5):332-62.
5. Entrala A, Loli-Ausejo D, Losantos I, Perez T, Cabanas R, Caballero T. Real-life experience of subcutaneous (SC) plasma derived C1-inhibitor (pdC1INH) as long-term prophylaxis

© 2024 Esmon Publicidad

doi: 10.18176/jiaci.1032

Accepted Article

(LTP) in HAE-C1INH. J Investig Allergol Clin Immunol. 2024;34(4):261-3.

6. Baynova K, Cimbollek S, Quiralte J, Garcia R, Lucena JM, Lasa Luaces E, et al. Subcutaneous C1 Inhibitor for Long-term Prophylaxis of Hereditary Angioedema: A Real-life Experience. J Investig Allergol Clin Immunol. 2024;34(3):198-9.
7. Cao Y, Liu S, Zhi Y. Recurrent and acute abdominal pain as the main clinical manifestation in patients with hereditary angioedema. Allergy Asthma Proc. 2021;42(2):131-5.
8. Zanichelli A, Magerl M, Longhurst H, Fabien V, Maurer M. Hereditary angioedema with C1 inhibitor deficiency: delay in diagnosis in Europe. Allergy Asthma Clin Immunol. 2013;9(1):29.
9. Busse PJ, Christiansen SC, Riedl MA, Banerji A, Bernstein JA, Castaldo AJ, et al. US HAEA Medical Advisory Board 2020 Guidelines for the Management of Hereditary Angioedema. J Allergy Clin Immunol Pract. 2021;9(1):132-50 e3.
10. Xu YY, Jiang Y, Zhi YX, Yin J, Wang LL, Wen LP, et al. Clinical features of hereditary angioedema in Chinese patients: new findings and differences from other populations. Eur J Dermatol. 2013;23(4):500-4.
11. Liu S, Wang X, Xu Y, Xu Q, Zhi Y. Risk factors for diagnostic delay in Chinese patients with hereditary angioedema. Allergy Asthma Proc. 2019;40(5):343-9.
12. Maurer M, Magerl M, Betschel S, Aberer W, Ansotegui IJ, Aygoren-Pursun E, et al. The international WAO/EAACI guideline for the management of hereditary angioedema-The 2021 revision and update. Allergy. 2022;77(7):1961-90.
13. Liu S, Xu Y, Liu Y, Zhi Y. Hereditary angioedema: a Chinese perspective. Eur J Dermatol. 2019;29(1):14-20.
14. Miyata T, Horiuchi T. Biochemistry, molecular genetics, and clinical aspects of hereditary angioedema with and without C1 inhibitor deficiency. Allergol Int. 2023;72(3):375-84.
15. Wu J, Wang W, Chen Z, Xu F, Zheng Y. Proteomics applications in biomarker discovery and pathogenesis for abdominal aortic aneurysm. Expert Rev Proteomics. 2021;18(4):305-14.
16. Gao Y. Urine-an untapped goldmine for biomarker discovery? Sci China Life Sci. 2013;56(12):1145-6.
17. Wu J, Gao Y. Physiological conditions can be reflected in human urine proteome and metabolome. Expert Rev Proteomics. 2015;12(6):623-36.
18. Zhao M, Li M, Yang Y, Guo Z, Sun Y, Shao C, et al. A comprehensive analysis and annotation of human normal urinary proteome. Sci Rep. 2017;7(1):3024.
19. Tofte N, Lindhardt M, Adamova K, Bakker SJL, Beige J, Beulens JWJ, et al. Early detection of diabetic kidney disease by urinary proteomics and subsequent intervention with spironolactone to delay progression (PRIORITY): a prospective observational study and embedded randomised placebo-controlled trial. Lancet Diabetes Endocrinol. 2020;8(4):301-12.
20. Wang W, Wu J, Liu P, Tang X, Pang H, Xie T, et al. Urinary Proteomics Identifying Novel Biomarkers for the Diagnosis and Phenotyping of Carotid Artery Stenosis. Front Mol Biosci. 2021;8:714706.
21. Wu J, Zhang J, Wei J, Zhao Y, Gao Y. Urinary biomarker discovery in gliomas using mass spectrometry-based clinical proteomics. Chin Neurosurg J. 2020;6:11.
22. Tian W, Zhang N, Jin R, Feng Y, Wang S, Gao S, et al. Immune suppression in the early stage of COVID-19 disease. Nat Commun. 2020;11(1):5859.
23. Virreira Winter S, Karayel O, Strauss MT, Padmanabhan S, Surface M, Merchant K, et al. Urinary proteome profiling for stratifying patients with familial Parkinson's disease. EMBO Mol Med. 2021;13(3):e13257.
24. Sun Y, Guo Z, Liu X, Yang L, Jing Z, Cai M, et al. Noninvasive urinary protein signatures associated with colorectal cancer diagnosis and metastasis. Nat Commun. 2022;13(1):2757.
25. Porebski G, Kwitniewski M, Reshef A. Biomarkers in Hereditary Angioedema. Clin Rev Allergy Immunol. 2021; 60(3):404-15.
26. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359-62.
27. Escher C, Reiter L, MacLean B, Ossola R, Herzog F, Chilton J, et al. Using iRT, a normalized retention time for more targeted measurement of peptides. Proteomics. 2012;12(8):1111-21.
28. Zhang B, Chambers MC, Tabb DL. Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. J Proteome Res. 2007;6(9):3549-57.
29. Bygum A, Fagerberg CR, Ponard D, Monnier N, Lunardi J, Drouet C. Mutational spectrum and phenotypes in Danish families with hereditary angioedema because of C1 inhibitor deficiency. Allergy. 2011;66(1):76-84.
30. Grover SP, Kawano T, Wan J, Tanratana P, Polai Z, Shim YJ, et al. C1 inhibitor deficiency enhances contact pathway-mediated activation of coagulation and venous thrombosis. Blood. 2023;141(19):2390-401.
31. Zuraw BL, Christiansen SC. HAE Pathophysiology and Underlying Mechanisms. Clin Rev Allergy Immunol. 2016;51(2):216-29.
32. Singh U, Bernstein JA. Determining biomarkers for evaluation and diagnosis of hereditary angioedema. Clin Transl Allergy. 2022;12(10):e12202.
33. Marceau F, Bachelard H, Bouthillier J, Fortin JP, Morissette G, Bawolak MT, et al. Bradykinin receptors: Agonists, antagonists, expression, signaling, and adaptation to sustained stimulation. Int Immunopharmacol. 2020;82:106305.
34. Ferrara AL, Cristinziano L, Petraroli A, Bova M, Gigliotti MC, Marcella S, et al. Roles of Immune Cells in Hereditary Angioedema. Clin Rev Allergy Immunol. 2021;60(3):369-82.
35. Debreczeni ML, Nemeth Z, Kajdacsi E, Schwaner E, Mako V, Masszi A, et al. MASP-1 Increases Endothelial Permeability. Front Immunol. 2019;10:991.
36. Wu MA, Bova M, Berra S, Senter R, Parolin D, Caccia S, et al. The central role of endothelium in hereditary angioedema due to C1 inhibitor deficiency. Int Immunopharmacol. 2020;82:106304.
37. Bork K, Wulff K, Rossmann H, Steinmuller-Magin L, Braenne I, Witzke G, et al. Hereditary angioedema cosegregating with a novel kininogen 1 gene mutation changing the N-terminal cleavage site of bradykinin. Allergy. 2019;74(12):2479-81.
38. Li M, Zhao M, Gao Y. Changes of proteins induced by anticoagulants can be more sensitively detected in urine than in plasma. Sci China Life Sci. 2014;57(7):649-56.

Accepted Article

© 2024 Esmon Publicidad

39. Adenaeuer A, Barco S, Trinchero A, Krutmann S, Nazir HF, Ambaglio C, et al. Severe high-molecular-weight kininogen deficiency: clinical characteristics, deficiency-causing KNG1 variants, and estimated prevalence. J Thromb Haemost. 2023;21(2):237-54.
40. Klock G, Baiersdorfer M, Koch-Brandt C. Chapter 7: Cell protective functions of secretory Clusterin (sCLU). Adv Cancer Res. 2009;104:115-38.

Manuscript received March 30, 2024; accepted for publication August 26, 2024.

Yuxiang Zhi

Department of Allergy and Clinical Immunology
National Clinical Research Center for Immunologic Diseases
Peking Union Medical College Hospital
E-mail: yuxiang_zhi@126.com

Shuyang Zhang

State Key Laboratory of Complex Severe and Rare Diseases
Peking Union Medical College Hospital
E-mail: shuyangzhang103@nrdrs.org

Accepted Article

© 2024 Esmon Publicidad

doi: 10.18176/jiaci.1032