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Original Research

Identification of Potential Predictive and Diagnostic Urinary Biomarkers for Acute Rejection in Renal Transplant Recipients: A Proteomics Study

Mohsen Nafar [1], Shiva Samavat [2], Nooshin Dalili [2], Shiva Kalantari [3], Leonard Foster [4], Kyung-Mee Moon [4], Somaye-Sadat Heidari [2, *]

1. Urology and Nephrology Research Center (UNRC), Shahid Labbafinejad Hospital, Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran; E-Mail: nafar@sbmu.ac.ir
2. Chronic Kidney Disease Research Center (CKDRC), Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran; E-Mails: shsamavat@gmail.com; dr.nooshindalili@gmail.com; somayesadat_heidary@yahoo.com
3. Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, USA; E-Mail: shivakalantari_81@yahoo.com
4. Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada; E-Mails: foster@msl.ubc.ca; kyungmee@mail.ubc.ca

* **Correspondence:** Somaye-Sadat Heidari; E-Mail: somayesadat_heidary@yahoo.com

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Abstract

Acute rejection (AR) is one of the main predictors of long-term survival of allograft. The development of noninvasive diagnostic biomarkers of AR is an unmet need for the timely detection. This study aimed to identify novel detective biomarkers of AR by analyzing the urine proteome profile of transplant patients. Forty-two transplant patients including 30 biopsy-proven AR patients (including antibody and T-cell mediated rejection) and 12 transplant patients with stable renal function (control group) were enrolled. Label-free quantification (LFQ) proteomics technique was performed on urine samples. Multivariate statistical analysis was applied for biomarker identification. The ELISA method validated EGF (epidermal growth

factor) from the top 10 candidate biomarkers in an independent cohort. Gene ontology and possible pathways were also analyzed. LFQ analysis revealed 453 identified proteins differentially expressed between groups that mainly participated in complement and coagulation pathways and proteolysis. Ten proteins with the highest AUCs (Area under the ROC Curve) were identified as candidate diagnostic biomarkers. Candidate biomarkers were mainly associated with extracellular matrix (ECM) degradation and epithelial-to-mesenchymal transition (EMT). Reduction of urinary EGF measured by ELISA in an independent group confirmed proteomics results. We introduced a unique set of diagnostic urinary biomarkers for AR. Interactions of biomarkers and validation of EGF among biomarker panels revealed that ECM remodeling and EMT might be the consequence of immunological processes in AR. If validated as a panel, the mentioned biomarkers might shed light on the pathogenesis of chronic injury after AR and point out the potential treatment strategies.

Keywords
Acute rejection; kidney; biomarker; proteomics; urine

1. Introduction

Kidney transplantation (KT) is the best renal replacement therapy in patients with end-stage renal disease (ESRD), improving both patient's quality of life and survival [1]. Despite advances in immunosuppressive therapies, allograft rejection and reduced graft survival has been a great challenge [2]. Acute rejection (AR) is one of the main reasons for allograft dysfunction and AR episodes may reduce graft function and long-term survival [3, 4]. Late rejections beyond the third post-transplantation month, antibody-mediated rejection, and treatment-resistant rejections are associated with poor allograft outcomes [5]. Thus, timely and accurate detection of transplant rejection for initiation of effective treatment increases the chance of allograft survival [6].

Currently, serum creatinine and urine protein assessment are strategies for monitoring allograft function, and although alterations in these parameters raise suspicion for rejection, they are not specific for rejection [7, 8]. Additionally, physicians might miss the subclinical rejections while waiting for changes to occur in serum creatinine. Needle biopsy, the gold standard for accurate diagnosis of rejection, is an invasive method with possible complications and sampling errors. Furthermore, the biopsy protocol for diagnosis of subclinical rejection is not applicable serially as it is a costly and undesirable method [9]. Therefore, a reliable biomarker for early and precise acute or subclinical rejection diagnosis is necessary.

Proteomics technology and identifying biomarkers of acute rejection in biological fluids are growing fields. Several biomarkers in biological fluids have been discovered in AR patients such as cytokines [10, 11], extracellular matrix proteins [12, 13] and acute phase proteins [14, 15]. So far, there has been little agreement on a unique biomarker [14, 16-18].

Alterations in the urine proteome might resemble changes in renal tissue. As an easily obtainable and noninvasive sample, urine is an ideal specimen in search of reliable noninvasive biomarkers using proteomics technology [19, 20].

This study sets out to identify variations in the expression of urine proteins in biopsy-proven

acute rejection and introduce potential biomarkers for early detection of AR by applying Label-Free Quantification (LFQ) proteomics.

2. Patients and Methods

2.1 Patients

All recipients aged between sixteen and sixty-seven years old who received their first kidney transplant in Labbafinejad Hospital (Tehran-Iran) were included in this case-control study. All patients were on triple immunosuppressive regimens (tacrolimus, mycophenolate mofetil and prednisone).

Patients with more than a 25% increase in serum creatinine above the baseline for three sequential measurements after transplantation were subjected to renal biopsy after ruling out factors such as obstruction and urinary tract infection. Two independent pathologists analyzed biopsy samples and reported according to Banff 2017 (the latest diagnostic criteria for allograft rejection) [21]. Biopsy-proven acute rejection patients were enrolled (case group). The control group was allograft recipients with clinically stable graft function and no fluctuation of serum creatinine more than 0.3 mg/dl in the last three months. There was no proteinuria and no rejection episode for at least 6 months. Allograft recipients with multiple organ transplantation, preformed donor-specific antibodies (DSAs), graft loss during the first three months, hepatitis, infection, and pregnancy were excluded from the study.

This study design was performed by the 2013 revision of the Declaration of Helsinki (1967) and approved by the Ethics Committee of Shahid Beheshti University of Medical Science (Tehran-Iran). Written informed consent was provided and obtained from all participants in the research.

2.2 Urine Sampling and Preparation

Second-morning urine samples were collected from patients on the day of indicated kidney biopsy procedure and from stable patients after diagnosis of stable allograft function according to inclusion criteria. Protease inhibitors and sodium azide (100 µl/10 ml urine) were added to each sample to protect samples from protease and bacterial activity. Amicon Ultra-15 Centrifugal Filter Units with a 3 kDa cutoff (Millipore, Billerica, MA, USA) were used to separate, desalt and concentrate the proteome part of urine samples according to the manufacturer's instruction. Samples were stored at −80°C for further evaluation.

2.3 Protein Fractionation, Digestion and Gel Cleaning-Up

A bicinchoninic acid protein assay kit (Thermo Scientific, USA) was used to measure the protein concentration. To obtain an appropriate separation of proteins, equal amounts of each protein sample (50 µg) were loaded on 10% SDS-PAGE gels and stained with blue silver after electrophoresis. Each lane of samples was separated into three parts based on the 70 kDa band (albumin likely) including: fraction 1(low abundant proteins band), fraction 2 (thick albumin band) and fraction 3 (other proteins). Protein fractions were digested into peptides with trypsin (Promega, USA) overnight at 37°C in 50 mM ammonium bicarbonate after reduction and alkylation with 0.5 M dithiothreitol and iodoacetamide for an hour.

Peptides were desalted on C18-StageTips columns (3 M Empore, USA), conditioned with

methanol and equilibrated by adding 0.5% trifluoroacetic acid. Samples were eluted with 80% acetonitrile in 0.1% formic acid and concentrated in a vacuum concentrator (Eppendorf, Germany).

2.4 Liquid Chromatography-Tandem Mass Spectrometry Analysis (LC-MS/MS)

Peptide separation and identification were performed using an EASY-nLC 1000 HPLC (Thermo Fisher, USA) coupled to Quadrupole Time-of-Flight Mass Spectrometry Impact II (Bruker Daltonics, Germany). Chromatographic columns included: trap column packed with 5 µm Aqua C-18 beads (Phenomenex Co., CA-USA) and analytical column packed with 1.9 µm-diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch GmbH, Ammerbuch-Entringen-Germany) maintained at 50°C with an in-house heater. The Nano electrospray ionization source was set at 1700 V capillary voltage, 0.20 bar nanoBooster pressure. Samples were resuspended in 5% acetonitrile and 0.5% formic acid and loaded on the trap column at 850 bar and the analysis was performed at 0.25 µl/min flow rate.

2.5 Data Analysis and Protein Identification

Data from mass spectrometry were analyzed using MaxQuant software (version 1.5.3.30). The search criteria were as follows: specificity to tryptic digestion up to 2 missed cleavage, carbamidomethylated cysteines as fixed modification and oxidized methionines and acetylated protein N-termini as variable modifications, a mass tolerance of 0.006 Da for precursor ion and 40 ppm for the fragment. Results were filtered with a false discovery rate (FDR) of 1% for both peptides and proteins.

2.6 Data Processing

Multivariate statistical analysis was applied for quantitative data of mass spectrometry using SIMCA software (SIMCA 15, Umetrics, Umea, Sweden). Before bioinformatics analysis, peak intensity values were converted to logarithmic values to normalize data. Unsupervised principal component analysis (PCA) was performed to visualize the variation of the data matrix and get an overview of data to detect clusters and pick up outliers. To identify and classify differential proteins between case and control groups, orthogonal projection to latent structure discriminant analysis (OPLS-DA) was performed and proteins with different abundance between groups were detected. Cross-validation scores and Cross-Validation ANOVA were calculated to estimate the most accurate model in the studied groups. The estimated model was acceptable if it was in the confidence interval of 95%. The specificity and sensitivity of the model were calculated with receiver operating characteristic (ROC) analysis. Variable Importance in the Projection (VIP) was used to identify proteins' influence on the model for further analysis.

2.7 Biomarker Identification

Proteins with the highest score of VIP in OPLS-DA were considered for identifying possible biomarkers. Proteins were sorted based on the most Importance in the model, and fold change for each variable (protein) was calculated as the ratio of medians of LFQ peak intensity in each group. The first five differentially expressed proteins were considered top candidate biomarkers. ROC analysis was used to determine specificity and sensitivity and, candidate biomarkers were sorted based on the highest area under the curve (AUC).

Candidate biomarkers with AUC values greater than 0.8 were selected and combined to identify a diagnostic panel. Logistic regression was used to calculate predicted probability and after ROC analysis, proteins with AUC values greater than 0.9 were considered for inclusion in a diagnostic panel.

2.8 ELISA Assay for Epidermal Growth Factor (EGF)

EGF was validated in an independent cohort of patients with AR (n = 28) and stable graft function (n = 18). ELISA kit (RAB0149 Sigma-Aldrich, USA) was used to measure the concentration of urinary EGF. Second-morning urine samples of AR and stable patients were collected respectively on the day of biopsy before the procedure and after ensuring the graft stability considering criteria introduced in the LFQ step. Assay was performed in duplicate for each urine sample according to the manufacturer's directions and, EGF values were normalized with urine creatinine.

2.9 Pathway Analysis and Functional Evaluation

Gene ontology of identified proteins and probable pathways in acute rejection were searched in Database for Annotation, Visualization and, Integrated Discovery (DAVID: http://www.david.niaid.nih.gov), Kyoto Encyclopedia of Genes and Genomes (KEGG: https://www.genome.jp) and BioCarta (http://www.biocarta.com). The relationship between candidate biomarkers was analyzed using online STRING network analysis (https://string-db.org).

3. Results

3.1 Demographic and Para-Clinical Results

Forty-two kidney transplant recipients (69% male), aged between 16 to 67 years old (average: 39 years), were enrolled in this study. Thirty patients with biopsy-proven acute rejection were considered the case group (AR) and 12 with stable graft function as the control group (stable). Lab data analysis showed that the estimated glomerular filtration rate (eGFR) and hemoglobin levels were significantly lower in the acute rejection group compared with the control group while urine protein was higher in the acute rejection group (Table 1). No significant differences were observed in the lipid profile between groups.

Table 1 Demographic and clinical characteristics of patients in studied groups.

Parameters	**Groups**			
	AR* (n = 30)	stable (n = 12)	All (n = 42)	P-value **
Recipient gender (male %)	63	83	57	
Donor gender (male %)	77	67	71	
Donor type (%)				
Deceased donor	15 (50)	5 (42)	20 (48)	
Living donor	15 (50)	7 (58)	22 (52)	
HLA mismatches (n)	27	10	37	
First transplantation	26	12	38	
Age at transplant	37.5 ± 15	38.4 ± 14.3	37.8 ± 14.6	

Age at biopsy in AR group (year)	39.7 ± 15	40.5 ± 9.8	
Biopsy <1 year after transplant (%)	73	5	
Lab data			
eGFR (mL/min/1.73 m2)	32 ± 13.8	79 ± 15.3	0.000
Urine protein (mg/ml)	10.4 ± 15.2	4.7 ± 7.7	0.002
Creatinine (mg/dl)	2.6 ± 1.5	1.1 ± 0.2	0.001
Hb (g/dl)	10.5 ± 1.6	13.9 ± 1	0.000
HcT (%)	32.5 ± 5.3	41.8 ± 2.8	0.000
Chol (mg/dl)	156.4 ± 50.2	196.6 ± 100.2	0.122
TG (mg/dl)	170.3 ± 136.7	128.6 ± 57.8	0.228
LDL-C (mg/dl)	81.5 ± 18.2	105.5 ± 38.7	0.205
HDL-C (mg/dl)	42.3 ± 9.4	44.1 ± 12.7	0.759
VLDL-C (mg/dl)	34 ± 27.3	25.7 ± 11.5	0.230

*. AR: acute rejection

**. Significance level was set as <0.05.

AR group included acute antibody-mediated rejection (AMR; n = 22) and cellular rejection (TCMR; n = 8) based on Banff 2017 classification.

3.2 Proteomics Analysis

Mass spectrometry analysis detected 1020 proteins in urine samples. Further analysis showed that 453 proteins were differentially expressed between groups. Among them, the expression of 210 proteins was upregulated and 240 proteins were downregulated in the AR group. Three proteins were just detected in nearly one-third of AR urine samples including: Asialoglycoprotein receptor 2 (in 10 AR patients), Corticotropin-releasing factor-binding protein (in 11 AR patients) and Chitinase-3-like protein 1(in 11 AR patients).

Heat map analysis was performed based on fold changes of peak intensities in LFQ to present quantitative proteomic data and visualize proteins with differential expression in AR patients (Supplementary 1). Among upregulated proteins, fibrinogen beta chain, serum amyloid A-1, fatty-acid binding protein 4, complement C1q subcomponent, microfibrillar associated protein 5, cystatin-B, complement factor D, plasminogen and apolipoprotein B-100 showed the greatest changes in AR patients. Roundabout homolog 4, epidermal growth factor, cadherin-2, cubilin and matrix–remodeling–associated protein 8 had the lowest expression in AR patients.

3.3 Multivariate Statistical Analysis of Proteome

Differentially expressed proteins between studied groups were subjected to PCA. The score plot of PCA showed no clusters or outliers in this analysis (Figure 1). These results imply that differences between urine protein profiles of patients are so small that supervised analysis might reveal them.

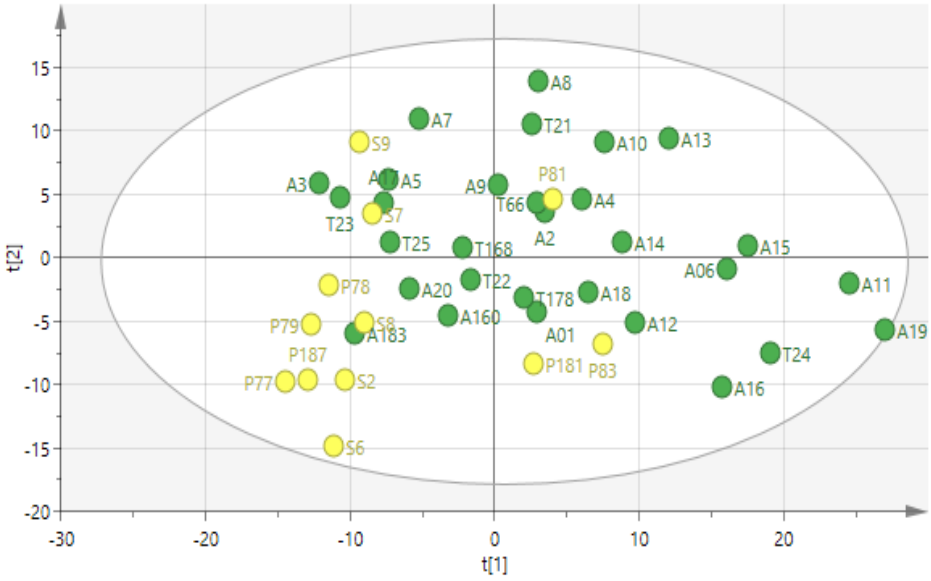

Figure 1 PCA score plot for all studied patients without grouping. No cluster and no outlier are observed in scoring. Green circles are representative of acute rejection patients and yellow circles are stable groups.

OPLS-DA was performed to identify differential proteins between groups. Determining two study groups, the OPLS-DA scores plot revealed clusters between groups along the predictive components of Q2 = 0.82 and R2 = 0.97 (Figure 2). CV score for modeling accuracy was significant (P-value = 0.05) and ROC analysis showed that the modeling had the highest sensitivity and specificity.

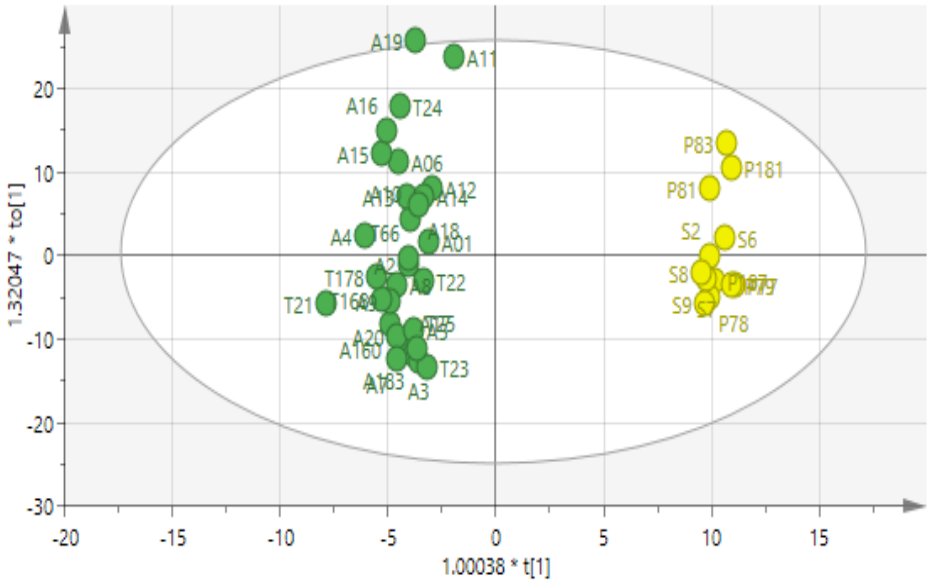

Figure 2 OPLS_DA score plot for 453 differentially expressed proteins in studied group based on grouping population study into acute rejection and stable groups. Separated clusters of groups are shown in this plot as a proper discrimination of proteins. Green circles are representative of acute rejection patients and yellow circles are stable groups.

3.4 Identification of Candidate Biomarkers

Differential proteins between groups were determined and plotted after modeling with OPLS-DA (Figure 3). The VIP list was achieved from OPLS-DA modeling and proteins with VIP scores greater than one were considered important proteins in groups' differentiation. Finally, 135 proteins significantly contributed to discriminate studied groups due to VIP scores. The fold change of each protein in the two groups was obtained by calculating the ratio of the median peak intensity in the AR group to the stable group. We considered five downregulated proteins besides five upregulated proteins to make an appropriate interpretation of the biomarker list. Possible candidate biomarkers were selected among the most up and down-regulated proteins with the highest AUCs and VIP scores (Table 2). It should be noted that modeling was made for subgroups of AR patients (AMR and TCMR) to exclude any common protein from identified candidate biomarkers. However, OPLS-DA could not make a model to differentiate subgroups of AR which might be due to the small sample size of subgroups. Therefore, all identified candidate biomarkers were considered specific for discriminating AR from stable groups (supplementary 2).

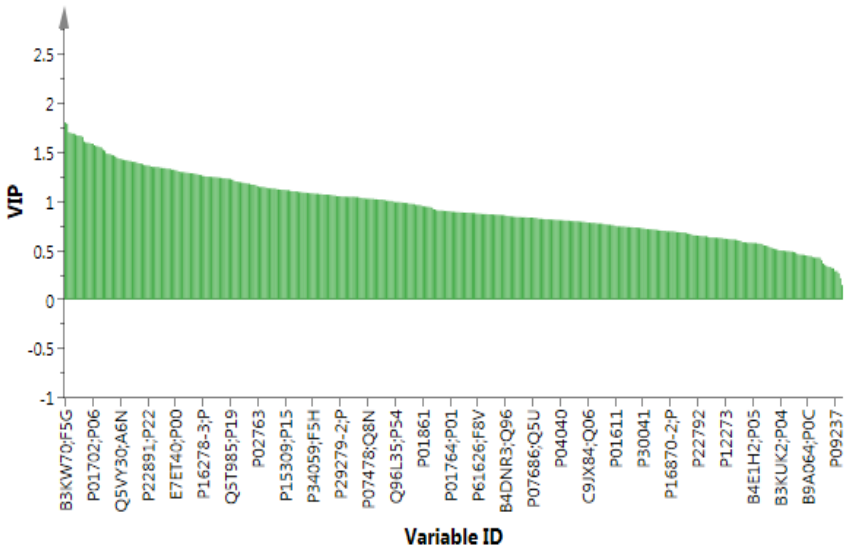

Figure 3 Protein importance plot of predictive model for acute rejection and stable groups. The X and Y axes show protein ID and protein importance respectively. The highest importance for differential proteins is 1.8.

Table 2 Urinary protein candidate biomarkers with the highest AUCs and VIP scores*.

Protein entry	Gene names	Protein names	Up/Down regulation	AUC**	Fold change#	P-value †
B3KW70	MFAP5	Microfibrillar-associated protein 5	up	0.9	6	0.06
P01133	EGF	Pro-epidermal growth factor	down	0.9	8	0.000
P15090	FABP4	Fatty acid-binding protein	up	0.8	7	0.03

P02746	C1QB	Complement C1q subcomponent subunit B	up	0.8	6	0.06
Q8WZ75	ROBO4	Roundabout homolog 4	down	0.8	9	0.001
P19022	CDH2	Cadherin-2	down	0.8	7	0.002
O60494	CUBN	Cubilin	down	0.8	6	0.000
P02675	FGB	Fibrinogen beta chain	up	0.7	63	0.007
P0DJI8	SAA1	Serum amyloid A-1	up	0.7	8	0.02
Q9BRK3	MXRA8	Matrix-remodeling-associated protein 8	down	0.7	5	0.005

*. Results were filtered for 1% false discovery rate (FDR).

**. AUC: area under curve.

#. Fold changes are based on peak intensities from mass spectrometry.

†. Significance level was set as <0.5.

Logistic regression was performed to evaluate the combination of proteins and identify biomarker panels. Panels with AUC values greater than 0.9 were considered reliable panels for discrimination of AR patients from stable patients. ROC analysis of protein combination results demonstrated that six protein panels have acceptable potential to differentiate patients from stable recipients (Table 3).

Table 3 Panel of combined protein with AUCs* more than 0.9 resulted from ROC** analysis of logistic regression.

Panel	Proteins	AUC	Sensitivity	Specificity
1	MFAP5 + EGF	100	100	100
2	MFAP5 + EGF + FABP4	100	100	100
3	EGF + C1QB	93	100	90
4	MFAP5 + C1QB	92	100	85
5	MFAP5 + CDH2	96	100	88
6	MFAP5 + CUB	100	100	100

*. AUC: area under curve.

**. ROC: receiver operating characteristic curve.

3.5 EGF Quantification in Urine Samples

Biomarker panels were comprised of a combination of candidate biomarkers. MFAP5[1] and EGF from Panel 1 were candidates to be validated. Although these two biomarkers had the same AUCs, EGF was validated in an independent cohort because of a bigger fold change and lower P-value than MFAP5. Forty-six allograft recipients (63% male) aged between 14 to 67 years old (average: 39 years) were included in an independent cohort. EGF concentration was measured in urine samples of 28 biopsy-proven (AMR: 19, TCMR: 9) and 18 stable patients using an ELISA kit and normalized with

[1] . Microfibrillar-associated protein 5

urine creatinine (uEGF/uCr). Statistical analysis showed that urinary EGF (uEGF) was significantly lower in the AR group compared to the control group (P-value = 0.001). There were no differences in urinary creatinine levels between groups (P-value = 0.473). Normalized values of uEGF (uEGF/uCr) were statistically lower (P-value = 0.001) in AR in comparison to the control group (Table 4). Although ROC analysis showed similar specificity for both uEGF and uEGF/uCr (71%), higher sensitivity was observed for uEGF/uCr (Figure 4). The P-value was less than 0.05 with a confidence interval of 95% for both ROC analyses.

Table 4 Urinary EGF, creatinine concentrations and uEGF/creatinine ratio in patients with AR and stable graft function in validation cohort.

Groups	**uEGF (pg/ml)** median ± IR	**Creatinine (mg/ml)** mean ± SD	**uEGF/Creatinine (pg/mg)** median ± IR
AR (n = 28)	1080.2 ± 1709.7*	1.7 ± 1.01	754.8 ± 708.6*
Stable graft (n = 18)	2747.1 ± 2022.5	1.9 ± 0.97	1361.3 ± 1172.9

*. Represents significant difference with stable group (P-value < 0.05).

IR: interquartile range.

SD: standard deviation.

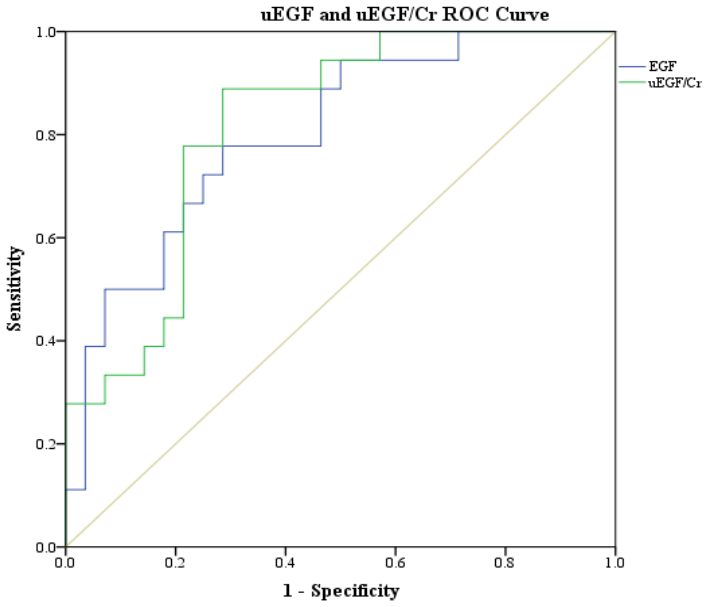

Figure 4 ROC curve for uEGF and uEGF/Cr in AR patients. Calculated AUCs were 0.8 and 0.81 for for uEGF (P-value: 0.001, 95% CI: 67-93%) and uEGF/Cr respectively (P-value: 0.000, 95% CI: 69-93%) Sensitivity was 77% for uEGF and 88% for uEGF/Cr. Bothe parameters had similar specificity of 71%.

3.6 Gene Ontology and Pathway Analysis

Differential proteins were searched in the DAVID database and clusters with Benjamini index lower than 0.05 were considered significant. Gene ontology analysis showed that proteolysis was

the major biological process of the 83 differentially expressed proteins. Sixty-three proteins showed endopeptidase activity. Proteins were mainly distributed in cellular membranes and cell junctions (Figure 5).

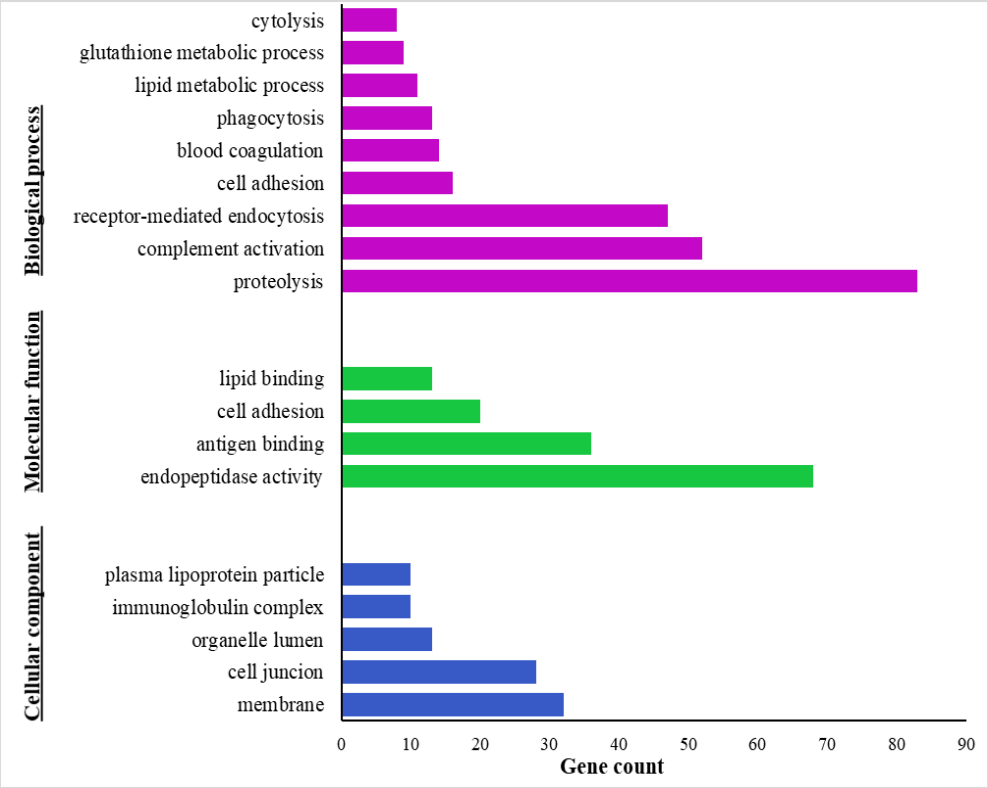

Figure 5 Gene ontology analysis chart for differentially proteins between studied groups. Differential proteins were mainly participated in biological process of proteolysis and complement activation with 83 and 52 proteins respectively. Endopeptidase activity and antigen binding were the most molecular function of differential proteins. The majority of proteins were distributed in cellular membrane and cell junctions. Benjamini index less than 0.05 was considered statistically significant.

Pathway analysis in the KEGG database showed that the "complement and coagulation pathway" is the major pathway in differential proteins (Figure 6).

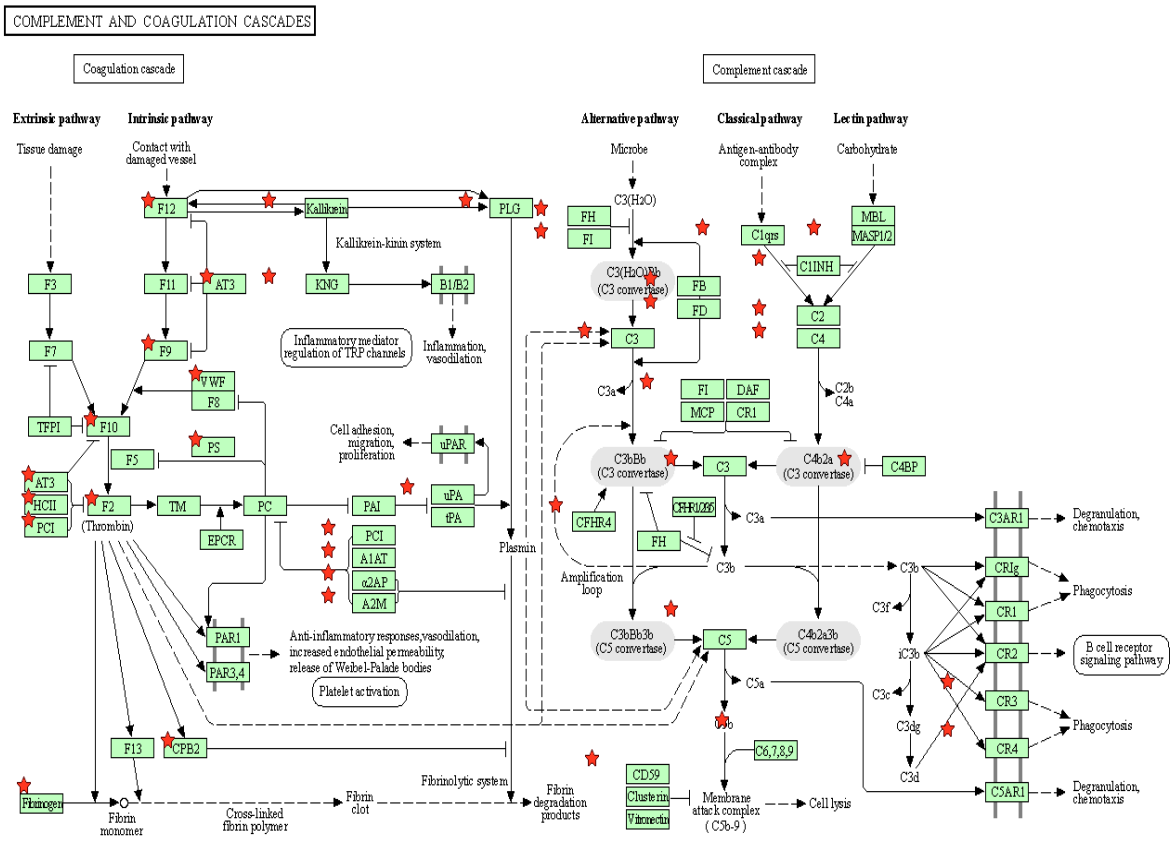

Figure 6 Pathway analysis of differentially expressed proteins using KEGG online database. Complement activation and coagulation pathways were the main pathway that differential proteins participated in with 42 proteins. Red Stars are proteins differentially expressed in this pathway. Complement activation and coagulation pathways are connected with plasmin and thrombin, which affect the complement factors and activate complement cascade to membrane attack complex formation. P-value was less than 0.000001.

Protein network analysis in the STRING database showed the association of five proteins between candidate proteins: Fibrinogen beta chain, epidermal growth factor, Cadherin-2, Matrix remodeling-associated protein 8 and Complement C1q subcomponent subunit B. There were no interactions between other proteins (Figure 7).

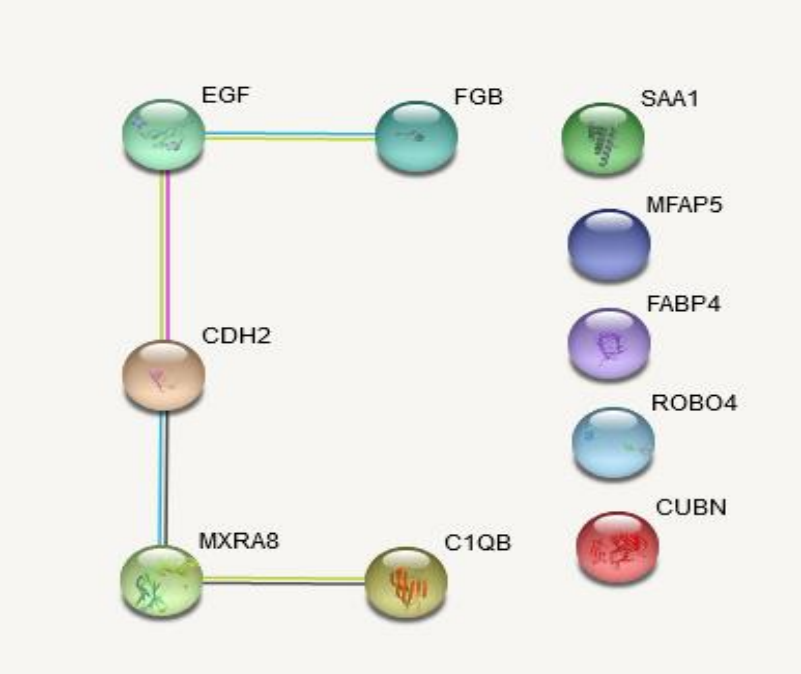

Figure 7 Protein network analysis of candidate biomarkers in protein-protein Interaction Networks Functional Enrichment Analysis (STRING). Protein interactions were detected in five proteins including Fibrinogen beta chain, epidermal growth factor, Cadherin-2, Matrix remodeling-associated protein 8 and Complement C1q subcomponent subunit B. Proteins are expressed as nodes. Connection lines indicators are as follow: blue line: database evidence, black line: co-expressed genes, green line: neighborhood genes, purple: experimental evidence.

4. Discussion

Acute rejections are considered the main obstacle to successful transplantation. Patients who experience acute rejection are at higher risk of chronic allograft nephropathies and graft loss. As surrogate markers of rejection, serum creatinine and proteinuria, are neither accurate nor specific, and continuous monitoring of graft by protocol biopsies is not applicable. A sensitive, accurate, and easily obtainable diagnostic method is needed to improve graft survival and outcomes [22]. There has been growing interest in developing noninvasive diagnostic biomarkers in recent years. In this study, the urine profile of AR patients was investigated with LFQ to find diagnostic biomarkers of AR.

Proteomics analysis of the patient's urines revealed significant changes in 453 proteins, mostly downregulated in the AR group. Among differential proteins, 10 proteins with high sensitivity and specificity were identified as possible candidate biomarkers for the prediction and/or diagnosis of AR patients.

In this study, FABP4 was upregulated in the urine proteome of AR patients. FABP4 is expressed not only on adipocytes and macrophages but also on endothelial cells of capillaries, including glomerular endothelial cells and peritubular capillaries [23]. Its urinary excretion has been suggested as a biomarker of glomerular injury [24]. The major sites of injury in acute antibody-mediated rejections (ABMR) are capillaries. According to Banff 2107 classification of allograft rejection, microvascular inflammation (glomerulitis and/or peritubular capillaritis) and tubulitis are

the main histologic evidence of ABMR and T-cell mediated rejection, respectively [21]. FABP4 has been demonstrated to be expressed following endothelial injury and during endothelial regeneration in proliferative endothelial lesions [24]. It has previously been observed that urinary FABP4 is upregulated in proximal tubular epithelial cell injury. The increased urinary excretion of FABP4 during rejection, both cellular and antibody-mediated rejection, indicates its role in injury and repair. However it might result in neointima formation, endothelial dysfunction and inflammation in vascular smooth muscles and lead to chronic vascular lesions [23]. Thus, FABP4 inhibitors might play a role in preventing chronic vascular lesions.

Elevated plasma serum amyloid A (SAA) level has long been known as a biomarker of allograft rejection. Previous proteomic studies also showed the changes in SAA in AR patients [14, 25, 26]. SAA is an apolipoprotein mainly produced by macrophages in the liver and binds to high-density lipoprotein (HDL) in circulation [27]. SAA was considered a sensitive though not specific marker of acute rejection [28, 29]. It can be used as an indirect evidence of macrophage activation. The severity of acute rejection both cellular and antibody-mediated rejection and graft outcome has been proposed to correlate with the number of infiltrating macrophages [30]. Therefore, elevated urinary excretion of SAA in our patients is an indicator of the severity of inflammation in the AR group.

In addition to its role in inflammation, SAA induces enzymes that degrade extracellular matrix (ECM) such as matrix metalloproteinase and collagenase from fibroblasts, leading to ECM remodeling [27]. Therefore, upregulation of SAA in AR patients might result in ECM alterations and the intensification of inflammatory responses. ECM accumulation and fibrosis are the major adaptive responses to acute inflammatory injuries such as AR. This response becomes maladaptive in the presence of chronic or repetitive injuries [31]. The maladaptive response may lead to chronic antibody-mediated rejection, which pathologically manifests as transplant glomerulopathy, peritubular capillary basement membrane multilayering and arterial intimal fibrosis [21]. A recent work by Clotet-Freixas demonstrated a significant decrease in ECM proteins in glomeruli and tubulointerstitium [13]. Accordingly, our findings showed the elevation of MFAP5 and the reduction of MXRA8 and Cadherin-2 in the urine profile of AR patients. MFAP5 is the main glycoprotein of ECM in arteries' walls and mediates angiogenesis and ECM remodeling [32]. A positive correlation between MFAP5 and epithelial-mesenchymal transition (EMT) has been reported [33]. Therefore, it seems that the elevation of MFAP5 in our patients is a sign of initiation of EMT, although there was no sign of chronicity in histologic reports.

MXRA8 is a transmembrane protein that modulates signaling pathways through binding to integrin in ECM and mediates cell-cell interaction [34]. MXRA8 function is not precisely understood in the kidney. However, a study on a family member of this protein showed a negative association between MXRA5 and kidney function [35]. Considering the co-expression of MXRA8 and C1QB in protein network analysis, it seems that MXRA8 alteration is partly related to the immune response in AR patients.

Cadherins are transmembrane glycoproteins and are essential for the maintenance of epithelial polarity and cell-cell adhesion. Cadherin-2 expression is restricted to proximal tubules [36] and plays an important role in epithelial cell adhesion. In line with our results, several studies noted the downregulation of cadherins and other adhesion molecules in acute kidney injuries and acute rejections [37, 38].

Considering the role of MFAP5, MXRA8 and Cadherin-2 in cell connections and remodeling of

ECM, changes in the expression of these urine proteomic proteins in acute rejection is possibly associated with the remodeling of ECM, which together with impaired cell adhesion could influence the cell signaling in graft tissue.

We identified biomarker panels by a combination of candidate biomarkers using logistic regression. Among the six obtained panels, panels 1 (MFAP5 + EGF) and 2 (MFAP5 + EGF + FABP4) were both the best diagnostic panels with the highest AUC values of 100%. However, due to affordability, we chose EGF for validation between the two panels because this protein had more fold changes than MFAP5 and FABP4 with less P-value in the urinary profile of the studied groups in the proteomics step.

The kidney mainly produces EGF and facilitates the restoration of epithelial cells in the glomerular basement membrane in acute inflammation, [39] however persistent signaling of EGF in chronic injuries can cause activation of fibroblast cells, EMT, ECM deposition and finally renal fibrosis [40]. It is possible to hypothesize that ECM remodeling is initiated in the early stages of AR, resulting in chronic changes and graft loss if left untreated. In this study, proteomics analysis showed the EGF reduction in AR patients. In order to confirm this reduction, EGF levels were measured in urine samples of AR in an independent set of patients. Validation results showed that uEGF and uEGF/Cr were significantly lower in AR patients. However, urine creatinine could not differentiate patients from the stable group. These results confirm that uEGF and uEGF/Cr are more reliable biomarkers for AR diagnosis than urine creatinine. Several lines of evidence suggested that downregulated urinary EGF is an indicator of tubulointerstitial injuries and a higher level of EGF is positively associated with better renal function [41-43]. In our previous study, we showed that uEGF was reduced in patients with antibody-mediated rejection [12]. In animal models, EGF gene expression is reduced in acute and chronic renal rejection [43]. Reduction of EGF in urine profile has been reported in AR patients [44]. Similarly, in line with previous reports, EGF validation in this study demonstrated that EGF reduction might be an appropriate indicator of AR progression, the improper function of graft tissue and failure in the recovery process and initiation of ECM remodeling. Considering the role of EGF in kidney injuries, it seems that EGF reduction in kidney transplant patients could be of the pathology of acute rejections and EGF increase could indicate success in recovery. It should be noted that validation of all biomarkers in panel 1 or 2 would probably provide more acceptable results in the diagnosis of AR patients.

In our previous investigation on biomarker discovery in AMR patients, we observed that ECM proteins including collagens and nidogen-1 downregulated in urine profile [12]. Proteomics study on glomeruli and tubulointerstitial revealed downregulation of collagens, nidogen and laminin subunits and elevation of ECM proteolytic enzymes in AMR patients [13]. In this study, we found that ECM degradation progression and EMT signaling factors (MFAP5, MXRA8, Cadherin-2, SAA and EGF) changed in acute rejection. Protein network analysis of candidate biomarkers also showed interactions between some proteins. Therefore, these alterations in the urine protein profile of AR patients strengthen this hypothesis that ECM remodeling and initiation of EMT occurs in early acute rejection in response to immunologic processes. These proteins could be reliable biomarkers in precise detection of AR. However, caution must be applied with a small sample size, as the findings might need further validation in larger population.

5. Conclusion

Proteomic analysis of urine samples is a useful method to discover important AR process changes and identify non-invasive diagnostic biomarkers in AR. We identified a panel of 10 candidate biomarkers with high sensitivity and specificity in the detection of AR. According to the validation results of EGF, we hypothesize that ECM remodeling and EMT, apart from immune response, are major processes in AR and could be used in the timely and precise detection of AR and to identify novel therapeutic targets.

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Author Contributions

Mohsen Nafar participated in performance of the research, research design and writing of the paper. Shiva Samavat participated in sample preparation and writing of the paper. Shiva Kalantari participated in sample preparation, data analysis and writing of the paper. Leonard Foster participated in performance of laboratory analysis and writing of the paper. Kyung-Mee Moon (Jenny) participated in performance laboratory analysis. Somaye-Sadat Heidari participated in sample preparation, data analysis and writing of the paper.

Competing Interests

Authors declare no conflict of interest.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Supplementary 1.
2. Supplementary 2.

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