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Original Article

suPAR as a marker of diabetic nephropathy in patients with type 2 diabetes

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Abstract: Objective: The aim of this study was to investigate the association of serum levels of soluble urokinase plasminogen activator receptor (suPAR) with early stages of diabetic nephropathy in patients with type 2 diabetes. Methods: A total of 106 patients with type 2 diabetes and 13 healthy controls were recruited, from January 2016 to December 2017. Serum suPAR, estimated glomerular filtration rates (eGFR), and urine albumin-creatinine ratios (UACR) were determined in all participants. Other clinical risk factors for diabetic nephropathy were collected. Results: Serum levels of suPAR in patients with type 2 diabetes were significantly higher than in healthy controls (620.2±456.4 pg/mL vs. 390.3±60.4 pg/mL, $P < 0.05$). UACR and eGFR were significantly increased from the lowest to highest quartile of suPAR (p for trend < 0.05). After adjusting for other clinical risk factors via multivariate regression analysis, serum levels of suPAR proved to be an independent contributor to eGFR ($\beta = -3.614$, $t = -4.281$, $P < 0.001$). Conclusion: Serum levels of suPAR were independently associated with eGFR, suggesting the role of a potential biomarker predicting early stages of diabetic nephropathy.

Keywords: Soluble urokinase plasminogen activator receptor (suPAR), type 2 diabetic nephropathy, biomarker

Introduction

Chronic kidney disease (CKD) is an important public health problem, with prevalence estimated at 8%-16% worldwide [1]. Diabetes is the leading cause of CKD in the USA, Japan, and many other developed countries. It is also rapidly becoming the leading cause in developing countries, including China, as a result of worldwide global increase in type 2 diabetes [2, 3]. Diabetic nephropathy occurs in up to 40% of people with type 1 or type 2 diabetes [4]. People with diabetic nephropathy are at significant risk of progression to end-stage renal disease (ESRD). There is also a concomitant increase in cardiovascular morbidity and mortality [4]. Hence, it is important to identify patients at risk for diabetic nephropathy and those at high risk for progression to ESRD.

Unfortunately, there is paucity of sensitive and specific biomarkers for indication of the early development of diabetic nephropathy, as well as progression to ESRD. Albuminuria has long been used to monitor onset and progression of diabetic nephropathy [4]. Microalbuminuria has been historically considered a strong predictor of progression to proteinuria. However, pathological abnormalities have been reported to occur before the onset of microalbuminuria [5]. Therefore, a new biomarker is needed to indicate the early development and progression of diabetic nephropathy.

Soluble urokinase plasminogen activator receptor (suPAR) is the circulating form of a glycosyl-phosphatidylinositol-anchored three-domain membrane protein. It is expressed on a variety of cells, including immunologically active cells, endothelial cells, and podocytes [6-8]. Both the circulating and membrane-bound forms are directly involved in the regulation of cell adhesion and migration through binding of integrins [6]. The circulating form is produced by cleavage of membrane-bound urokinase-type plasminogen activator and is readily detected in plasma and serum [9, 10]. Elevated suPAR levels have been associated with poor outcomes

in many patient populations, such as different types of cancers, inflammation and infection diseases, and diabetes [11-14]. In addition, suPAR has been implicated in the pathogenesis of kidney disease, specifically focal segmental glomerulosclerosis (FSGS), through interference with podocyte migration and apoptosis [7, 15]. Moreover, serum suPAR levels were elevated in patients with diabetic kidney disease (DKD) [16]. Therefore, suPAR appears to be an emerging biomarker of DKD. This present study hypothesized that serum suPAR might increase earlier in the progression of DKD, before microalbuminuria becomes evident. If this is the case, then serum DKD might be a novel biomarker for early identification of DKD. This study aimed to explore the association of serum suPAR with diabetic nephropathy, stratified according to levels of albuminuria and kidney function.

Materials and methods

Study population

This cross-sectional observational study enrolled consecutive patients with type 2 diabetes mellitus (T2DM). They visited the Department of Endocrinology and Metabolism of The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China, between January 2016 and December 2017.

Diagnostic criteria of diabetes mellitus (DM)

Levels of glycosylated hemoglobin ≥ 6.5%, fasting blood glucose ≥ 7 mmol/l, or oral glucose tolerance test 2-h blood glucose ≥ 11.1 mmol/l were the diagnostic criteria of DM.

Diagnostic criteria of diabetic kidney disease (DKD) [2]

Criteria include CKD patients with macroalbuminuria and CKD patients with microalbuminuria in the presence of diabetic retinopathy or in type 1 diabetes of at least 10 year's duration. Other causes of CKD should be considered in the presence of any of the following circumstances: Absence of diabetic retinopathy; Low or rapidly decreasing GFR; Rapidly increasing proteinuria or nephrotic syndrome; Refractory hypertension; Presence of active urinary sediment; Signs or symptoms of other systemic disease or > 30% reduction in GFR within 2-3 months after initiation of an ACE inhibitor or ARB.

Staging of diabetic nephropathy with reference to Mogensen criterion [17]

Stage 1 is characterized by early hyperfunction and hypertrophy. These changes are found at diagnosis, before insulin treatment. Increased urinary albumin excretion, aggravated during physical exercise, is also a characteristic finding. Changes are at least partly reversible by insulin treatment.

Stage 2 develops silently over many years and is characterized by morphologic lesions, without signs of clinical disease. However, kidney function tests and morphometry on biopsy specimens reveal changes. Function is characterized by increased GFR.

Stage 3, incipient diabetic nephropathy, is the forerunner of overt diabetic nephropathy. Its main manifestation is abnormally elevated urinary albumin excretion, as measured by radioimmunoassay. A level higher than the values found in normal subjects, but lower than in clinical disease, is the main characteristic of this stage, which appears to be between 15 and 300 micrograms/min in the baseline situation. GFR is still supra-normal. Antihypertensive treatment in this phase is under investigation, using the physical exercise test.

Stage 4 is overt diabetic nephropathy, the classic entity characterized by persistent proteinuria (greater than 0.5 g/24 h). When associated high blood pressure is left untreated, renal function (GFR) declines, with mean fall rates around 1 mL/min/mo. Long-term antihypertensive treatment reduces the fall rate by about 60%, postponing uremia considerably.

Stage 5 is end-stage renal failure with uremia due to diabetic nephropathy. As many as 25% of the population presently entering end-stage renal failure programs in the United States are diabetic. Diabetic nephropathy and diabetic vasculopathy constitute a major medical problem in society today.

All patients with T2DM were assessed by the Modification of Diet in Renal Disease (MDRD) equation [18]. Patients with T2DM were excluded when renal diseases attributable to other causes were suspected. Therefore, exclusion

criteria included the presence of hematuria, renal insufficiency of unexplained origin, urinary tract infections and history of rapidly progressive renal failure, glomerulonephritis, and polycystic kidney disease. According to the urine albumin-creatinine ratio (UACR) [19] and eGFR, investigating the role of serum suPAR in the different stages of DKD, patients with T2DM were stratified into a normal albuminuria group (UACR < 30 mg/g), microalbuminuria group (30 mg/g ≤ UACR < 300 mg/g), macroalbuminuria group (UACR ≥ 300 mg/g, eGFR > 15 mL/min per 1.73 m^2), and ESRD group (eGFR < 15 mL/min per 1.73 m^2). The normal albuminuria group was further divided into a normal eGFR group (eGFR < 120 mL/min per 1.73 m^2) and a higher eGFR group (eGFR ≥ 120 mL/min per 1.73 m^2).

The current study also recruited healthy volunteers visiting the clinic for routine examinations. All study participants provided written informed consent before recruitment into the study.

eGFR calculation

eGFR was estimated using the Schwartz formula for patients < 18 years of age [20] and the CKD-Epidemiology Collaboration (CKD-EPI) formula for those ≥ 18 years of age [21].

Anthropometric and biochemical measurements

Standard anthropometric (height, weight, body mass index (BMI)), clinical (systolic and diastolic blood pressures), and laboratory biochemical analyses were performed. All participants were required to fast for 12 hours overnight before blood and urine samples were taken. Blood (5 mL) was drawn under aseptic conditions from the cubital vein in the morning after the overnight fast. Serum (2-3 mL) was separated in a -4°C centrifuge at 3,000 g for 20 minutes (GDXL-16D; Kaihang Instrument Company, Changzhou, China). Urine samples (10 mL) were collected in the morning after the overnight fast. Serum and urine samples were stored at -80°C until processing. Serum samples were analyzed for total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), glucose, creatinine (Cr), and blood urea nitrogen (BUN), using an automated biochemical analyzer (ADVIA 1800 Clinical Chemistry System; Erlangen, Germany). Urine samples were analyzed for albuminuria and urine creatinine using an automated biochemical analyzer (ADVIA 1800 Clinical Chemistry System). UACR was calculated by dividing the concentration of urine albumin by the concentration of urine creatinine. eGFR was calculated using the formula of CKD-EPI. A commercially available ELISA kit was used for the serum suPAR assay, following manufacturer protocol. All samples were measured in duplicate (Quantikine Human uPAR Immunoassay; R&D, Minneapolis, MN).

Statistical analyses

All statistical analyses were performed using SPSS statistical package, version 22.0 (SPSS, Inc., Chicago, IL, USA) for Windows. A descriptive statistical analysis was performed for all studied variables. Normally distributed data were analyzed using the Kolmogorov-Smirnov test. Data are expressed as the mean ± SD, median and interquartile range (IQR) (25th and 75th percentiles), or the number (percentage) of patients for categorical variables. Differences in means with normal distribution were compared using a *t*-test. Paired variables were compared using the paired samples *t*-test or Wilcoxon matched-paired sign-rank test.

Logistic regression analyses were used to identify steroid response and clinical measures in patients with DKD. suPAR levels were log-transformed with regression analyses.

The two groups were compared with independent sample t-test and approximate t-test if the variance was uneven. Single factor analysis of variance (LSD) was used for multigroup comparisons. If the variance was uneven, Kruskal-Wallis rank sum test was employed.

Receiver-operating characteristic (ROC) curve analysis and area under the curve (AUC) statistics provided a composite score for prediction of an event. The ROC curve was generated by plotting sensitivity against 1-specificity. Two-sided *P* values < 0.05 indicate statistically significant differences.

Results

Clinical characteristics of participants

Baseline characteristics of the 119 participants, according to the suPAR quartiles, are shown in **Table 1.**

Table 1. Clinical characteristics according to suPAR quartiles (*p* values for continuous variables and categorical variables were determined by ANOVA and the Chi-squaredd test, respectively)

Variables	Total	Q1	Q2	Q3	Q4	*p* value for the trend
suPAR, pg/ml (range)	130.17~3496.83	130.17~351.83	351.83~450.17	450.17~711.50	711.50~3496.83	-
n	119	30	30	30	29	-
Age (year)	55.37±15.29	54.77±15.85	46.00±15.74	58.43±13.59	62.52±10.96	< 0.001
Female, n (%)	45 (37.8)	12 (40.0)	10 (33.3)	14 (46.7)	9 (31.0)	0.597
BMI (kg/m^2)	24.20±3.21	24.83±3.22	24.26±2.84	24.17±3.38	23.51±3.41	0.479
SBP (mmHg)	139.77±19.26	135.70±23.33	132.20±13.17	137.00±14.58	154.69±16.76	< 0.001
DBP (mmHg)	80.27±12.11	75.47±13.43	81.53±12.36	79.13±9.67	85.10±11.17	0.017
Diabetic duration (years)	114.48±85.54	78.33±69.10	108.67±110.19	117.60±64.98	154.66±76.33	0.006
Antidiabetic treatment						
Hypoglycemic drug-naive, n (%)	28 (23.5)	12 (40.0)	7 (23.3)	6 (20.0)	3 (10.3)	0.057
Insulin treatments, n (%)	54 (45.4)	11 (36.7)	11 (36.7)	12 (40.0)	20 (69.0)	0.034
Insulin-secretagogues, n (%)	31 (26.1)	15 (50.0)	5 (16.7)	7 (23.3)	4 (13.8)	0.005
Insulin-sensitizers, n (%)	4 (3.4)	3 (10.0)	0 (0.0)	1 (3.3)	0 (0.0)	0.106
Hypertension, n (%)	56 (47.1)	10 (33.3)	10 (33.3)	16 (53.3)	20 (69.0)	0.014
Hypertensive duration (years)	4.26±6.09	2.80±5.54	2.81±5.56	5.15±6.11	6.34±6.64	0.056
Hypertensive treatment						
ACEI/ARB, n (%)	26 (21.8)	5 (16.7)	3 (10.0)	8 (26.7)	10 (34.5)	0.109
CCB, n (%)	22 (18.5)	4 (13.3)	2 (6.7)	7 (23.3)	9 (31.0)	0.078
Beta receptor blocker, n (%)	14 (11.8)	2 (6.7)	2 (6.7)	3 (10.0)	7 (24.1)	0.118
Statins medication, n (%)	6 (5.0)	3 (10.0)	1 (3.3)	1 (3.3)	1 (3.4)	0.56
Smoking, n (%)	20 (16.8)	8 (26.7)	5 (16.7)	4 (13.3)	3 (10.3)	0.36
Drinking, n (%)	13 (10.9)	6 (20.0)	4 (13.3)	1 (3.3)	2 (10.9)	0.173
CHD, n (%)	11 (9.2)	1 (3.3)	3 (10.0)	4 (13.3)	3 (10.3)	0.591
Cerebral Infarction, n (%)	41 (34.5)	7 (23.3)	3 (10.0)	7 (23.3)	24 (82.8)	< 0.001
TG (mmol/L)	1.69±1.22	1.53±0.94	0.97±0.52	2.02±1.34	2.07±1.47	0.004
TC (mmol/L)	4.70±1.39	4.39±1.20	4.70±1.13	5.05±1.41	4.66±1.69	0.386
HDLC (mmol/L)	1.15±0.27	1.05±0.22	1.17±0.21	1.18±0.32	1.20±0.30	0.16
LDLC (mmol/L)	2.93±0.95	2.84±0.96	3.05±0.83	3.34±0.69	2.54±1.10	0.016
Scr (μmol/L)	145.11±204.78	61.72±18.81	59.40±16.21	82.15±42.67	344.23±308.02	< 0.001
UA (μmol/L)	327.33±118.14	309.73±95.86	272.76±51.15	313.66±132.97	397.36±131.02	0.001
eGFR (ml/min/1.73 m^2)	85.03±37.23	101.58±19.28	110.65±19.91	85.84±28.32	30.73±26.87	< 0.001
HbA1c (%)	8.32±2.15	8.68±2.42	8.83±2.53	8.34±1.83	7.42±1.38	0.052
UACR	56.98±89.70	17.15±38.01	42.87±98.88	80.29±104.89	105.35±79.64	0.008

Table 2. Changes of serum suPAR concentrations in the stage of diabetic nephropathy in manifest type 2 diabetes

Group	N	suPAR (pg/mL)
Blank control	14	390.04±604.10
Stage 5 DKD with dialysis	12	1519.06±697.02[a]
Stage 5 DKD without dialysis	6	1052.33±181.72[a]
Stage 4 DKD	16	672.47±207.56[a,b,c]
Stage 3 DKD	16	609.35±223.21[a,b,c]
Stage 2 DKD	9	394.37±73.16[b,c,d]
Stage 1 DKD	27	362.45±109.21[b,c,d,e]
DM	20	367.95±110.84[b,c,d,e]
Welch F	-	19.707
P	-	< 0.001

a, P < 0.05 vs. blank control; b, P < 0.05 vs. stage 5 DKD with dialysis; c, P < 0.05 vs. stage 5 DKD without dialysis; d, P < 0.05 vs. stage 4 DKD; e, P < 0.05 vs. stage 3 DKD.

Relationship between suPAR and other clinical variables

In unadjusted analyses including all participants, suPAR was correlated with red blood cells (r = -0.611), hemoglobin (r = -0.588), lymphocytes (r = -0.381), neutrophil/lymphocyte ratio (r = 0.527), urinary microalbumin (r = 0.480), urine protein (r = 0.532), hematuria (r = 0.215), creatinine (r = 0.663), urea (r = 0.309), uric acid (r = 0.243), albumin (r =0.176), total bilirubin (r = -0.390), direct bilirubin (r = -0.407), indirect bilirubin (r = -0.374), LDL (r = -0.232), RBP (r = 0.570), 24-hour urinary protein quantification (r = 0.394), eGRF (r = -0.646), and systolic blood pressure (r = 0.20) (P < 0.001 for all). It was weakly associated with total cholesterol (r = 0.09, P = 0.021).

Table 3. Results of multivariate linear stepwise regression model affecting suPAR levels

Variable quantity	β	Standard error	Standardized regression coefficient	t	P	Confidence interval of β	
						Lower limit	Upper limit
Constant term	644.696	146.47		4.401	< 0.001	351.987	937.406
UACR	0.874	0.263	0.338	3.325	0.001	0.352	1.397
eGFR	-3.614	0.844	-0.495	-4.281	< 0.001	-5.300	-1.928
Urinary microalbumin	0.868	0.217	0.409	3.993	< 0.001	0.434	1.303
Serum creatinine	1.147	0.203	0.589	5.656	< 0.001	0.742	1.552
Hemoglobin	-2.840	0.912	-0.222	-3.114	0.003	-4.663	-1.018

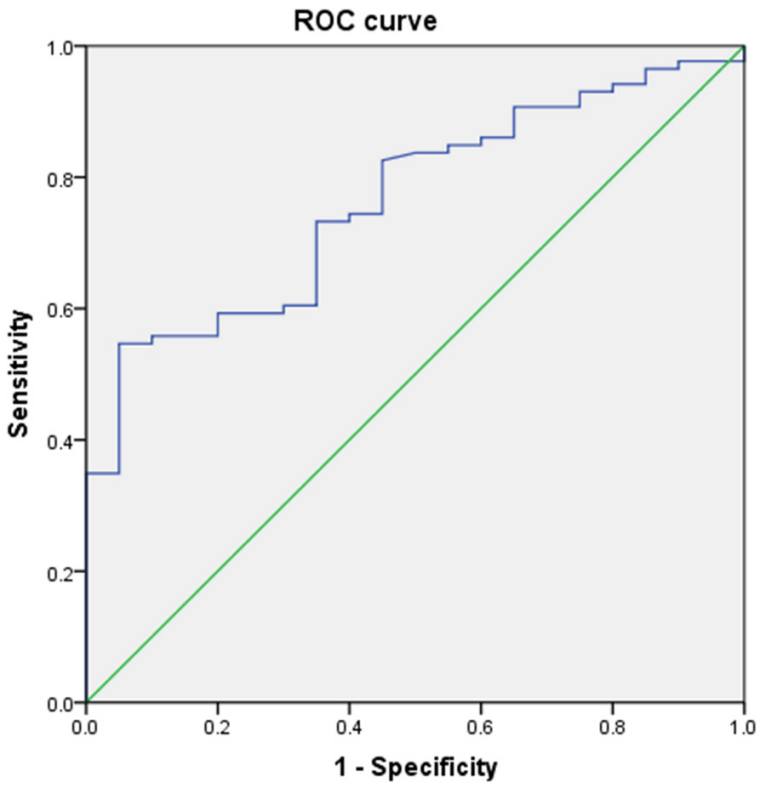

Figure 1. ROC analysis used to explore the cutoff suPAR value to predict early stages of diabetic nephropathy.

suPAR was not correlated with leukocytes (r = -0.162), neutrophils (r = 0.081), monocytes (r = -0.17), eosinophils (r = -0.077), basophils (r = -0.175), platelets (r = -0.07), urine PH (r = 0.08), urine specific gravity (r = -0.026), and urinary tube type (r = -0.018).

*Correlation of serum suPAR concentrations and stage of diabetic nephropathy in manifest type 2 diabetes (**Table 2**)*

In an independent cross-section of patients with manifest type 2 diabetes, there were significantly differences of suPAR concentrations in the stages of diabetic nephropathy (P < 0.001). Higher suPAR levels appeared in stage 3 DKD, compared with healthy control subjects (P < 0.05). Next, the effects of the stage of diabetic nephropathy on serum suPAR concentrations were tested using linear regression analysis. Results showed that serum suPAR concentrations increased with an increase in the stage of diabetic nephropathy (P < 0.05).

*Multiple linear stepwise regression analysis with suPAR as the dependent variable (**Table 3**)*

In view of the results of the single factor mentioned above, classification of variables with statistical significance was incorporated into the multivariate linear regression equation.

ROC analysis to explore the cutoff suPAR value to predict early stages of diabetic nephropathy

Serum suPAR is a strong independent contributor to DPN. Therefore, ROC analysis was further applied to explore the cutoff suPAR value to indicate early stages of diabetic nephropathy. The optimal cutoff value of suPAR to predict early diabetic nephropathy was 499.33. The corresponding AUC was 0.763 (95% CI 0.663-0.863). Its Youden index was 0.497, sensitivity was 0.547, and specificity was 0.950 (**Figure 1**).

Discussion

Current opinion considers the risk of developing diabetic nephropathy a continuum, starting at urinary albumin excretion still within the nor-

mal range [22, 23]. Detection of incipient diabetic nephropathy at earlier time points is essential. Early identification of patients at risk of developing diabetic nephropathy is essential. The current study explored the possibility that suPAR is a potential novel risk marker for early stages of diabetic nephropathy, in patients with type 2 diabetes mellitus (T2DM). Present results showed that suPAR was associated with stage of diabetic nephropathy, with significant differences appearing in stage 3 DKD in the subjects. ROC analysis further demonstrated that suPAR was a sensitive indicator for the stage of DKD, suggesting that serum suPAR might be a potentially useful biomarker for early diagnosis of diabetic nephropathy in patients with T2DM.

In accord with present results, a recent study [24] reported that serum suPAR associates with new-onset microalbuminuria in subjects at increased risk for type 2 diabetes independently. Furthermore, they demonstrated that suPAR may allow for earlier risk stratification than microalbuminuria. The current study further classified subjects according to the stage of diabetic nephropathy with type 2 diabetes mellitus (T2DM), finding the role of suPAR in the specific stage of diabetes mellitus (T2DM). However, the current study compared the absolute value of suPAR in these two different samples, finding that the absolute values of suPAR were lower than theirs as a whole. Two possible reasons should be considered. ELISA reagent kits used were different from their study and present subjects were different from theirs.

SuPAR is a potential novel risk marker for management of diabetes. It has been demonstrated that suPAR levels are higher in patients with type 1 diabetes and are associated with diabetes duration and complications independent of other risk factors [25]. Recent studies have described suPAR as a circulating factor implicated in FSGS [26]. Hayek showed that elevated levels of suPAR independently associated with incident chronic kidney disease and an accelerated decline in eGRF in the groups studied [27].

suPAR has been demonstrated to bind to and activate β3-integrin, resulting in podocyte effacement and alteration of glomerular permselectivity [15]. Moreover, blocking ligand occupancy of the αVβ3 integrin could inhibit the pathophysiologic changes that occur in the early stages of diabetic nephropathy [28]. Hence, this study suggests suPAR as a candidate biomarker for early stages of diabetic nephropathy in patients with type 2 diabetes mellitus (T2DM). Except for β3-integrin activation signaling, the effects of suPAR on podocyte function may be modulated by other pathways in a disease-specific manner. Expression of sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) is high in diabetic nephropathy, shifting suPAR mediated podocyte injury from a migratory (FSGS) to an apoptotic phenotype (diabetic nephropathy) [29].

In conclusion, suPAR appears to be a promising biomarker, indicating early diagnosis of diabetic nephropathy in patients with T2DM. The current study demonstrates an association of suPAR with early diagnosis of diabetic nephropathy in patients with T2DM.

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Disclosure of conflict of interest

None.

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