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ORIGINAL ARTICLE

Urinary tubular biomarkers in short-term type 2 diabetes mellitus patients: a cross-sectional study

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Abstract The purpose of this study was to investigate the prevalence of tubular damage in short-term (less than five years) type 2 diabetes mellitus (T2DM) patients and to explore the correlation between tubular markers and their relationship with renal indices at different stages of diabetic nephropathy. A group of 101 short-term T2DM patients and 28 control subjects were recruited. Tubular markers, such as neutrophil gelatinase-associated lipocalin (NGAL), *N*-acetyl-β-D-glucosaminidase (NAG), and kidney injury molecule 1 (KIM-1), as well as urinary albumin excretion were measured in voided urine. Glomerular filtration rate (GFR) was estimated via Macisaac's formula. The patients were further categorized into three groups, namely, the normoalbuminuria, microalbuminuria, and macroalbuminuria groups, according to their urine albumin/creatinine ratio (UACR). Urinary tubular markers were compared and their correlations with renal indices [UACR and estimated GFR (eGFR)] were analyzed among the different diabetic groups. Compared with the control group, Urinary NGAL [median (IQR)][83.6(41.4−138.7) μg/gcr vs. 32.9(26.1−64.5) μg/gcr], NAG [13.5(8.7−17.9) U/gcr vs. 7.6(6.5−13.0) U/gcr] and KIM-1 [120.0(98.4−139.9) ng/gcr vs. 103.1(86.8−106.2) ng/gcr] in the T2DM were all markedly increased. For all patients, urinary NGAL had stronger positive correlations with UACR than NAG ($R = 0.556$ vs. 0.305, both $P < 0.05$). In addition, only urinary NGAL showed a negative correlation with eGFR ($R = -0.215$, $P < 0.05$). Urinary KIM-1, however, showed no significant difference among the three T2DM groups and did not correlate with either UACR or eGFR. As UACR increased from the normoalbuminuria to the last macroalbuminuria group, all of the markers increased. However, only the concentrations of NGAL were statistically different among the three diabetic groups. The correlation between the tubular markers and their relationships with the renal indices differed markedly among the three T2DM groups. In conclusion, these results suggest that tubular damage is common in short-term T2DM patients. Urinary NGAL may be a promising early marker for monitoring renal impairment in short-term T2DM patients.

Keywords Tubular markers · Type 2 diabetes mellitus · Diabetic nephropathy · Microalbuminuria

Shi-long Xiong and Wen-jin Fu contributed equally to this study.

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Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus (DM), and a main cause of mortality and morbidity in DM patients [1, 2]. As the global prevalence of type 2 DM

(T2DM) is increasing markedly, the incidence of DN is expected to continue to rise. Therefore, predicting and preventing the development of DN are of great clinical significance.

Early diagnosis and clinical interventions are critical for the prevention of DN. Currently, the earliest and the most commonly used clinical index of DN is elevated urinary albumin excretion (AER), referred to as microalbuminuria [3, 4]. As an indicator of renal damage, however, AER has some limitations. First, some patients do not progress to macroalbuminuria but remain at microalbuminuria or even regress to normoalbuminuria [5]. Second, some patients follow a non-albuminuric pathway to renal impairment, wherein the increases in AER and decreases in glomerular filtration rate (GFR) are not closely related [6]. Thus, more sensitive and specific renal biomarkers than AER will be valuable in predicting early kidney injury and the progression or regression of renal damage in T2DM patients.

Many studies have shown that tubular damage occurs early in the course of DN and is not merely secondary to glomerular damage as previously thought [7, 8]. In addition, evidence supporting the prognostic and diagnostic value of tubular damage markers in various renal disorders is increasing [9–11]. Tubular markers such as neutrophil gelatinase-associated lipocalin (NGAL), *N*-acetyl-β-D-glucosaminidase (NAG), and kidney injury molecule-1 (KIM-1) have recently gained much attention because they are considered as sensitive and specific biomarkers in detecting kidney damage.

NGAL is a 25 kDa protein belonging to the lipocalin family and is secreted in small amounts in the lung, kidney, trachea, stomach, and colon tissue [12]. NGAL is hyper produced in the kidney tubules within a few hours after deleterious stimuli such as ischemia–reperfusion. Therefore, NGAL is regarded as an excellent early predictor of acute renal damage [13]. NAG is a 140 kDa lysosomal brush border enzyme found in the proximal tubular cells [14, 15], which is released into the urine after renal proximal tubule injury. As a clinical tubular damage marker, urinary NAG is elevated in acute and chronic kidney disease and is of diagnostic value for the early detection of acute kidney injury [15, 16]. Finally, KIM-1 is a transmembrane protein, which seems to play a role in the pathogenesis of tubular cell damage and repair in human kidneys [17]. KIM-1 can be upregulated in proximal tubular cells during various states, including ischemia, toxic renal injury, and polycystic kidney [18–21], and is associated with the extent of tubulointerstitial damage and fibrosis.

Although DM duration is considered a risk factor for DN, some studies have indicated that renal injury occurs even in patients with impaired glucose tolerance [22]. Thus, renal injury may occur in short-term T2DM patients, and understanding the renal damage in these patients will benefit the prevention of DN. Furthermore, little is known of the relationship of tubular markers with renal indices during the different stages of DN. However, elucidating these parameters is important to determine their diagnostic values in DN. Therefore, this cross-sectional study was conducted to explore the correlation between these markers and the degree of kidney impairment in short-term T2DM.

Materials and methods

Subjects

101 Chinese T2DM patients (according to the 1999 WHO criteria) were recruited from the diabetes clinic at affiliated Houjie Hospital of Guangdong medical university, China, between September 2009 and January 2010. The duration of T2DM was less than 5 years in all patients. Those with fever, infection, surgery, trauma or suffering from systemic, cardiac, or hepatic, other renal diseases were excluded. In addition, 28 healthy volunteers served as controls. They included 13 males and 15 females with mean age of 48.3 $\pm$ 12.3 years. All the Participants didn't take any antihypertensive drugs or had 1 week withdrawal of antihypertensive drugs before the study. The study protocol was approved by the Ethics Research Committee at Houjie Hospital (Dongguan, China). Written informed consent was obtained from all participants. The clinical features of the study subjects were summarized in Table 1.

Based on UACR, diabetic patients were divided into three groups: 61 with normoalbuminuria (UACR $<$ 30 mg/g), 24 with microalbuminuria (UACR between 30 and 300 mg/g) and 16 with macroalbuminuria (UACR $\geq$ 300 mg/g).

Measurements

Fasting blood sample was tested for creatinine, glucose, hemoglobin A1C (HbA1c), Cystatin C (CysC), and other chemistry laboratory parameters. CysC was measured on BECKMAN COULTER DXC800 automatic analyzer (BECKMAN, USA) by latex particle-enhanced turbidimetric immunoassays (Leadman, china). Serum and urine creatinine levels were measured by automatic picric colorimetry (BECKMAN, USA). For all subjects, estimated GFR (eGFR) was calculated by Macisaac's formulae: eGFR = 86.7/CycC-4.2, where eGFR was expressed as ml/min 1.73 m^2 and serum CycC as mg/l [23].

First-voided morning urine samples were collected and centrifuged at 2500 rpm for 10 min, then Urine aliquots were stored at -70°C until analysis. Urinary albumin was measured by immune turbidimetry (BECKMAN

Table 1 Clinic characteristics of the subjects

Variable	T2DM group ($n = 101$)	Controls ($n = 28$)	P-value
Age (years)	53.9 ± 13.8	49.9 ± 15.9	0.230
Gender (male/female)	58/45	13/15	0.352
Diabetes duration (years)	2.7 ± 1.5	NA	NA
Systolic BP (mmHG)	134 ± 13	125 ± 6.5	0.062
Diastolic BP (mmHG)	80.1 ± 7.9	79.4 ± 3.4	0.771
FBG (mmol/l)	9.5 ± 4.3	5.4 ± 0.6	<0.001
HbA1c (%) (mmol/mol)	7.4 ± 2.0 (57 ± 22)	4.1 ± 0.7 (21 ± 7)	<0.001
Renal indices			
Serum creatinine (μmol/l)	78.5 ± 27.5	61.3 ± 14.1	0.017
Serum cysc (mg/l)	1.001 ± 0.283	0.714 ± 0.153	<0.001
eGFR (ml/min/1.73 m^2)	85.9 ± 22.6	109.6 ± 14.2	<0.001
UACR	17.9 (7.1−55)	5.4 (3.5−7.8)	<0.001
Urinary tubular markers			
NGAL (μg/g cr)	83.6 (41.4−138.7)	32.9 (26.1−64.5)	0.003
NAG (U/g cr)	13.5 (8.7−17.9)	7.6 (6.5−13.0)	0.033
KIM-1 (ng/g cr)	120.0 (98.4−139.9)	103.1 (86.8−106.2)	0.006

BP blood pressure, *eGFR* estimated glomerular filtration rate, *NA* Not applicable, *FBG*, fasting blood glucose, *KIM-1* kidney injury molecule 1, *NAG N*-acetyl-b-D-glucosaminidase, *HbA1*$_C$ hemoglobin $A1_C$, *NGAL* neutrophil gelatinase associated lipocalin, *UACR* urine albumin/creatinine ratio. Clinical characteristics are presented as mean ± standard deviation except for UACR: median (25–75th percentile). Urinary markers with creatinine adjustment presented as median (IQR)

COULTER, USA). Both KIM-1 and NGAL were measured by commercial sandwich ELISA kits (Quantikine R&D Systems Inc. Abingdon, UK). These assays were done according to the manufacturers' instructions. Assays for both NGAL and KIM-1 demonstrated near linearity with the squared correlation coefficient $R^2 = 0.995$, 0.990, respectively. The intra-assay coefficients of variation were 3.6% (range: 3.1–4.4%) for NGAL and 3.1% (range: 1.2–4.0%) for KIM-1. NAG was detected by colorimetric assay as suggested by the manufacturer (O&D BIOTECH, china). TO account for the influence of urinary dilution on the biomarker concentration, all of the tubular markers were normalized to the urinary creatinine concentration.

Statistical analysis

Data were given as mean ± SD when normally distributed, as median and IQR when distribution was skewed, as frequencies and percentages for categorical variables. Differences between patients and controls were tested using Mann–Whitney U or Student testing. Two-sided analysis of variance or Kruskale-Wallis was used for multiple comparisons. Spearman's correlation coefficients were calculated between KIM-1, NAG, NGAL, and other variables. All Statistical analyses were performed using the SPSS® statistical package, version 13.0 (SPSS Inc. Chicago, IL, USA) for Windows®. Statistical significance was defined as $P < 0.05$.

Results

Overall characteristics of the subjects

Table 1 details the general characteristics and clinical parameters of the study groups. The T2DM and control groups were well matched according to age, blood pressure, and gender. Compared with the control group, the T2DM patients had higher urinary NGAL, NAG, KIM-1, UACR, serum cystatin C, serum creatinine, fasting blood glucose (FBG), HbA1c, but lower eGFR. Even after adjusting the difference in eGFR, the levels of the first three values were still significantly higher in the T2DM patients than the control.

Correlations of tubular markers in the T2DM patients

The relationships between tubular markers were analyzed in all T2DM patients, which were summarized in Table 2. Urinary NAG was directly correlated with urinary NGAL ($R = 0.358$, $P < 0.05$) and KIM-1 ($R = 0.281$, $P < 0.05$). However, urinary NGAL showed a weak positive but non-significant association with urinary KIM-1.

Table 2 also shows that both urinary NGAL ($R = 0.556$, $P < 0.005$) and urinary NAG ($R = 0.305$, $P < 0.005$) were positively associated with UACR. However, only urinary NGAL showed a negative correlation with the residual

Table 2 Correlation with tubular markers in T2DM ($N = 101$)

Variable	Univariate correlation coefficient		
	NGAL	KIM-1	NAG
Renal function			
eGFR	−0.215*	−0.103	−0.114
UACR	0.556*	0.163	0.305*
Tubular markers			
NGAL	NA	0.038	0.358*
KIM-1	0.038	NA	0.281*
NAG	0.358*	0.281*	NA

* $P < 0.05$. *KIM-1* kidney injury molecule 1, *NAG N*-acetyl-b-D-glucosaminidase, *NGAL* neutrophil gelatinase associated lipocalin, *UACR* urine albumin/creatinine ratio, *NA* Not applicable

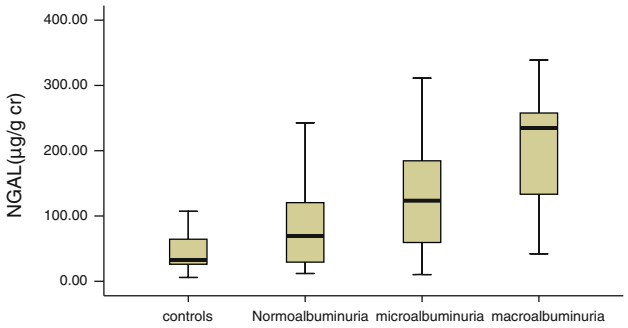

Fig. 1 Urinary NGAL in different groups. *NGAL*, neutrophil gelatinase associated lipocalin. The central line represents the median, the boxes span from the 25th to 75th percentiles, and the *error bars* extend from the 10th to 90th percentiles

eGFR. No significant correlations between KIM-1 and UACR, eGFR were found.

Urinary tubular markers in the different study groups

Urinary NGAL in the normoalbuminuria group was higher than that in the control. In the microalbuminuric group, urinary NGAL also increased compared with the control and the normoalbuminuria group [median (IQR) 123.6(59.4–184.6) vs. 32.9(26.1–64.5) vs. 69.2(29.3–120.4) μg/gCr]. Finally, patients with macroalbuminuria [235(133.3–257.7) μg/gCr] showed higher NGAL levels than all the other groups, as shown in Table 3 and Fig. 1.

The urinary NAG level was significantly elevated in all three diabetic groups compared with the control. The microalbuminuric and macroalbuminuric patients had higher urinary NAG than the normoalbuminuric patients [15.5(12.6–20.5) and 17.8(14.6–31.8) vs. 10.5(8.2–16.2) U/g Cr], but there was no significant difference between the micro- and the macroalbuminuria groups.

Although the urinary KIM-1 levels in three T2DM groups were elevated compared with the control, no significant differences were found among the three diabetic groups.

Correlation with the tubular markers in the different diabetic groups

In the normoalbuminuria group, both urinary NGAL and NAG were positively correlated with UACR, but the former has stronger correlation than the latter ($R = 0.603$ vs. 0.298), as shown in Table 4. The association between KIM-1 and UACR, however, was not significant. Moreover, none of the tubular markers correlated with eGFR. Except for KIM-1 and NAG that were directly correlated, no other statistical correlations was found among the tubular markers in this group.

In the microalbuminuria group, only NGAL was correlated with NAG among the markers. No other statistical correlation was found between the markers and the kidney indices, except for the positive association between urinary NGAL and UACR.

However, in the macroalbuminuria group, a significant inverse correlation between NGAL and eGFR ($R = -0.81$, $P < 0.05$) and a weak but significantly negative correlation between NAG and eGFR ($R = -0.227$, $P < 0.05$) were found. None of the markers correlated statistically with UACR and with each other.

Table 3 Tubular markers and renal function in different groups

	Control ($n = 28$)	Normoalbuminuria ($n = 61$)	Microalbuminuria ($n = 24$)	Macroalbuminuria ($n = 16$)
Renal function				
eGFR (ml/min/1.73 m^2)	109.6 ± 14.2	98.4 ± 20.6	88.6 ± 19.7*	73.9 ± 24.4*#
UACR	5.4 (3.5−7.8)	10.1 (6.1−18.6)*	76.6 (49.3−148.0)*	500.0 (429.1−1469.7)*
Tubular markers				
NGAL (μg/g cr)	32.9 (26.1−64.5)	69.2* (29.3−120.4)	123.6*# (59.4−184.6)	235.0*#& (133.3−257.7)
NAG (U/g cr)	7.6 (6.5−13.0)	10.5* (8.2−16.2)	15.5*# (12.6−20.5)	17.8*# (14.6−31.8)
KIM-1 (ng/g cr)	103.1 (86.8−106.2)	119.7 (97.7−139.3)	127.8* (115.9−150.9)	117.7* (98.4−127.7)

KIM-1 kidney injury molecule 1, *NAG N*-acetyl-b-D-glucosaminidase, *NGAL* neutrophil gelatinase associated lipocalin, *UACR* urine albumin/creatinine ratio. For difference between the individual four groups: * $P < 0.05$ vs. controls, # $P < 0.05$ vs. Normoalbuminuria, & $P < 0.05$ vs. microalbuminuria

Table 4 Association with tubular markers in the different diabetic groups

Variable	Normoalbuminuria ($N = 61$)			Microalbuminuria ($N = 24$)			Macroalbuminuria ($N = 16$)		
	NGAL	KIM-1	NAG	NGAL	KIM-1	NAG	NGAL	KIM-1	NAG
Renal function									
eGFR	−0.158	0.156	−0.122	0.294	−0.123	0.122	−0.81*	0.168	−0.227*
UACR	0.603*	0.191	0.298*	0.204*	−0.253	0.077	−0.120	−0.024	0.150
Tubular markers									
NGAL	NA	0.013	0.180	NA	0.093	0.626*	NA	−0.082	0.050
KIM-1	0.013	NA	0.374*	0.093	NA	−0.175	−0.082	NA	0.033
NAG	0.180	0.374*	NA	0.626*	−0.175	NA	0.050	0.033	NA

* $P < 0.05$. *KIM-1* kidney injury molecule 1, *NAG N*-acetyl-b-D-glucosaminidase, *NGAL* neutrophil gelatinase associated lipocalin, *UACR* urine albumin/creatinine ratio, *NA* Not applicable

Discussion

In this cross-sectional study, the three urinary markers of tubular damage were considerably elevated in the short-term T2DM patients compared with the healthy control subjects. They were already elevated even in the normoalbuminuric T2DM patients before the increase in UACR. In addition, these markers were elevated from normoalbuminuric to microalbuminuric, and further to macroalbuminuric patients, demonstrating increased tubular damage with increasing levels of urinary albumin. Furthermore, the three markers exhibited different correlations with the renal indices in the three T2DM groups.

Generally, albuminuria is assumed to be caused by glomerular injury. However, less than one-third of diabetic patients with microalbuminuria have the typical glomerulopathy [24, 25]. Moreover, animal studies revealed that albuminuria is a sensitive marker for early tubular toxicity [26, 27].In the latest study, the lower urinary tubular markers at baseline were strongly associated with the regression of microalbuminuria during 2-year follow-up in type 1 DM [5]. Therefore, although the early albuminuria in DM patients may be partially attributed to tubular damage, the tubular markers might have the potential to reflect early changes in urinary albumin in T2DM patients.

In this study, renal damage was evaluated in terms of UACR and eGFR. The eGFR was calculated via Macisaac's formulae based on the serum cystatin C. That is because as a marker of renal function in DM patients, cystatin C is superior to serum creatinine, and again, the commonly used modification of diet in renal disease formula is not suitable for the Chinese population [28].

In this study, all three tubular markers were elevated in the normoalbuminuric T2DM patients. The urine concentrations of NGAL, NAG, and KIM-1 were approximately 2, 1.5, and 1.2 times of those in the controls, respectively. Different mechanisms, including reduced clearance, increased production by injured tubular cells, or both, could account for the elevation of urinary NGAL. However, only increased tubular production could account for the elevation of urinary NAG and KIM-1 because their relatively large molecular weights precluded glomerular filtration [18, 29]. The injured tubules likely resulted in both reduced reabsorption and increased production of NGAL, which may explain the higher increase in urinary NGAL than in NAG and KIM-1 in the normoalbuminuria group. At the same time, UACR had a modest correlation with urinary NGAL ($R = 0.556$) and a mild but statistical significant correlation with NAG ($R = 0.358$). These findings further support an early "tubular phase" in DN, i.e., impairment of tubular reabsorption resulting in the early UACR elevation. Thus, tubular injury, rather than glomerular injury, might be the earliest kidney lesion and the primary cause of albuminuria in T2DM. Moreover, this finding also indicates that urinary NGAL and NAG might have potential for predicting microalbuminuria and DN in the early stages of DM and for evaluating renal prognosis and efficacy of intervention. This hypothesis needs longitudinal studies in T2DM. However, none of the markers showed any statistical association with eGFR in the normoalbuminuria group. Considering that these markers were mainly a result of tubular injury, this phenomenon could be explained by the fact that the patients have only had DM for a short period and their eGFR have not declined markedly (Table 3).

In the microalbuminuria group, however, only urinary NGAL displayed a weak but statistically significant correlation with UACR. The glomerular injury in this group is likely a more important factor for increased UACR than tubular damage, even though tubule damage indicates further deterioration, as implied by the greater increase in NGAL and NAG than in the normoalbuminuria group. In this group, urinary NGAL showed a stronger association with NAG than that in the normoalbuminuria group ($R = 0.626$ vs. 0.180), this might indicate that urinary NGAL is principally produced as NAG by the injured tubule cells in this group, and both are mainly induced by

the same factors. None of the markers correlated with eGFR as in the normoalbuminuria group.

In the macroalbuminuria group, both urinary NGAL and NAG were inversely related with eGFR, however, the former was stronger than the latter one ($R = -0.81$ vs. -0.227), which was similar to the findings of a Type 1 DM study [30]. None of the markers were correlated with UACR. Thus, in this group, the increase in UACR might be mainly mediated by glomerular injury rather than increased tubular damage. At the same time, increased tubular damage was accompanied by the loss of renal function. Furthermore, urinary NGAL might be a sensitive and early marker for the loss of renal function in DN as in acute renal failure, even though eGFR is only mildly reduced in this group (the lowest eGFR was 50 ml/min/1.73 m^2). This viewpoint, however, disagrees with a recent research, wherein the author demonstrated that although high levels of urinary NGAL and KIM-1 are associated with a faster decline in GFR, they did not add information about the progression or treatment effect beyond known progression promoters in type 1 DM patients with overt nephropathy [31].

Different correlations were found among the tubular markers in the three diabetic groups. The discrepancies imply that these biomarkers reflect different pathophysiological mechanisms in tubulointerstitial damage at different stages of DN as that found in the different etiologies of acute kidney injury [29]. Similar to the findings in type 1 DM patients [30], KIM-1 is not correlated with either UACR or eGFR in all three groups. This is in agreement with the idea that KIM-1 only reflects tubular damage in the more advanced stages of renal disease [9].

To our knowledge, this is the first study on the tubular marker in short-term T2DM. In accordance with the other studies on DM with longer durations [27, 30], tubular damage occurred in the normoalbuminuric short-term T2DM patients. Thus, the evaluation of renal impairment and the corresponding measures for preventing DN should be implemented even in newly diagnosed DM patients.

There are several limitations to the current study. First, the number of patients with macroalbuminuria and microalbuminuria was small, which might underestimate the association of the tubular markers and the renal indices. Second, only one urine sample was made available for the tubular marker and UACR measurement. Third, this was only one cross-sectional study; hence, the value of these markers should be confirmed with a longitudinal study.

In conclusion, we have demonstrated that tubular damage occurs in the short-term T2DM patients, and that urinary NGAL may be a more promising early marker than UACR for detecting and predicting renal injury in T2DM patients.

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Conflict of interest Nothing to declare.

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