ORIGINAL RESEARCH

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Evaluation of the Urinary Kidney Injury Molecule-1 Levels in Patients With Diabetic Nephropathy

Abstract

Purpose: Kidney Injury Molecule-1 is a protein that increases in urine following tubular damage. Kidney Injury Molecule-1 levels were correlated with the level of chronic kidney disease secondary to diabetic nephropathy in patients with type 2 Diabetes Mellitus.

Methods: Clinical and laboratory findings of 142 patients with diabetic nephropathy and 34 control subjects were analysed. Creatinine and HbA1c levels in blood samples and albumin, creatinine and Kidney Injury Molecule-1 levels in urine samples were assessed.

Results: Urinary Kidney Injury Molecule-1 levels were significantly increased both in subgroups of diabetic nephropathy (normo-/micro-/macro-albuminuria) and in chronic kidney disease (stage 2-4) compared with controls. Urinary Kidney Injury Molecule-1 levels in stage 2 chronic kidney disease patients were significantly higher than those of the patients with stage 3-4 chronic kidney disease. Urinary Kidney Injury Molecule-1 levels, along with urinary albumin excretion and the duration of diabetes, were found to be independent risk factors associated with low glomerular filtration rates.

Conclusion: Urinary Kidney Injury Molecule-1 levels seems to predict renal injury secondary to diabetic nephropathy in early period independent of albuminuria, because urinary Kidney Injury Molecule-1 was elevated despite normal urinary albumin excretion in the normoalbuminuric subgroup. Urinary Kidney Injury Molecule-1 levels, which are elevated in primarily in stage 2, shows a gradual decrease in patients with chronic kidney disease stages 3 and 4; thus, urinary Kidney Injury Molecule-1 levels may be useful in tracking the progression of kidney disease.

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Diabetic nephropathy (DN) is the most common and increasing cause of chronic kidney disease (CKD) [1]. Early diagnosis of DN in diabetic patients is critically important to avoid end stage renal disease (ESRD). Both American Diabetes Association (ADA) and National Kidney Foundation (NKF) recommend assessment of urinary albumin excretion (UAE) and glomerular filtration rate (GFR) in diagnosis and follow up of CKD in diabetic subjects [2,3]; however, evaluation of GFR and UAE are not ideal for determining renal damage in diabetics. Serum creatinine (sCr) levels are affected by a wide range of factors, including age, gender, race and muscle mass [4]. Furthermore, approximately 20% of patients with DN remain normoalbuminuric, despite a reduction in GFR [5]. Finally, UAE, which is used for early diagnosis of DN, primarily reflects glomerular damage; however, the level of tubulointerstitial damage is known to be an even more accurate marker of long term prognosis and disease progression in all types of CKD [6]. New biomarkers are needed to identify early renal tubulointerstitial damage in diabetic patients.

Kidney Injury Molecule-1 (KIM-1) is a transmembrane protein isolated from rat and human renal tubular cells by Ichimura *et al.* [7]. KIM-1 is found at low levels in normal kidneys; however, its expression increases after toxic, ischemic and/or proteinuric kidney damage [8-10]. Tissue KIM-1 expression and urinary KIM-1 levels have been reported to be increased in renal damage models of experimental animal studies and in several human studies. The results of these studies are promising, especially for early detection of tubulointerstitial damage [11-13]. In contrast, there are conflicting reports on the relationship between urinary KIM-1 concentrations and GFR [14-15]. There are no data correlating levels of urinary KIM-1 and extent of proteinuria in type 2 diabetes mellitus (DM).

Materials and Methods

Study population

Type 2 diabetic patients with DN who were admitted to the Nephrology Department of our institution during the period August 2013 to November 2013 were evaluated. DN was defined as low GFR or increased albumin excretion in urine or determination of proliferative changes in fundus, in patients with worse glycemic and hypertensive control. Creatinine clearance (CrCl) was used as a measurement of GFR. To estimate the CrCl, 24 hours urine samples were collected and CrCl was calculated using a standard formula [16]: $(CrCl = (U \cdot V) / (P \cdot t))$; U=urine creatinine, P= plasma creatinine, V= volume of urine during 24 hours period, t= duration

of which urine was collected. NKF/DOQI guidelines were used to define CKD stages [2]: the patients with GFR of 60-90 ml/min were defined as stage 2, 30-60 ml / min were defined as stage 3 and 15-30 ml/min were defined as stage 4 CKD [2]. In the classification of DN, patients with albumin excretion below 30 mg/day were defined as normoalbuminuric, 30-300 mg/day were defined as microalbuminuric and more than 300 mg/day were defined as macroalbuminuric [17]. Patients with stage 2-4 CKD were enrolled in the study. The control group consisted of 34 diabetic patients with no evidence of DN (no micro-or macroalbuminuria, decline in GFR or proliferative changes in the fundus). Exclusion criteria were as follows: age (less than 18 or over 70 years), type 1 DM, active infection, heart failure, endocrinopathies other than type 2 DM, CKD from other causes than type 2 DM, GFR lower than 15 and higher than 90, 24 hours urine sample collected incorrectly and/or recent urinary tract infections. Patients with a history of use of nephrotoxic agents and non-steroidal anti-inflammatory drug use in last 30 days, urinary stone disease, urinary tract surgery or malignant disease were also excluded. Patients with acute renal damage (including acute exacerbation of CKD) and dehydration were also excluded from the study cohort. To avoid the effects of contrast nephropathy, patients who had received contrast-enhanced imaging studies within the last two weeks were excluded from the study. Of the initial 216 DN patients evaluated, 53 were excluded from the study based on these exclusion criteria and a further 21 patients did not give informed consent; thus, 142 patients with DN were enrolled in the study. The study was approved by local ethics committee of Abant İzzet Baysal University.

Blood and urine samples and measurements

Blood samples obtained in the morning after an overnight fasting into dry tubes. Urine specimens (24 hour samples) were obtained using disposable urine cups that did not contain any preservatives. Both blood and urine samples were centrifuged at 1000 g for 10 minutes. HbA1c, serum creatinine, urinary albumin and urinary creatinine levels were measured by auto-analyzers according to the manufacturer's instructions on the same day the samples were obtained (Architect c 8000, Abbott Laboratories, Chicago, USA). Urinary supernatants were stored in -70 °C until analysis.

ELISA was used in assessment of urinary KIM-1 levels according to the manufacturer's instructions (Aviscera Bioscience, Santa Clara, USA). Detection range of the kit used was 31-2000 pg/ml. Precision of intra and inter assay were coefficient of variation (%): 4-8%, 6-8%, respectively. Minimum detectable dose of KIM-1 was 3 pg/mL. Urinary KIM-1 analyses

of the samples were performed simultaneously. Urinary KIM-1 (uKIM-1) was calculated from 24 hours KIM-1 concentration and urine volume. uKIM-1 (ng/day) levels were used for statistical analyses.

Statistical analyses

For statistical analysis, SPSS software version 15.0 (SPSS; Chicago, USA) was used. Continuous variables were presented as mean $\pm$ standard deviation while categorical variables were presented as percentage. The normal distribution of all variables was tested by the Shapiro-Wilk test. One-way ANOVA test was applied to compare continuous variables, and the difference between subgroups was assessed with the post hoc Tukey's test. χ^2 test was used to compare for categorical data. Nonparametric data were compared using the Kruskal-Wallis test. A multiple linear regression model was used to identify independent predictors of decreased GFR (<90 mL/min). A value of $p < 0.05$ was considered statistically significant.

Results

Clinical and laboratory parameters according to DN stages

Table 1 shows clinical and laboratory measurements of 34 controls and 142 patients grouped according to diabetic nephropathy level. There were no statistically significant differences among the four groups in terms of age and gender (ANOVA $p > 0.05$, $\chi^2 p > 0.05$; respectively). Duration of DM was significantly different between DN groups (Kruskal-Wallis test, $p = 0.003$). Mean HbA1c and systolic-diastolic blood pressure levels were significantly higher in patients with DN compared with control subjects; however, there were no statistically significant differences within the subgroups of DN (all *post hoc* $p > 0.05$). There were significant differences among groups in terms of sCr, GFR, UAE and uKIM-1 levels. Further comparisons between groups were performed with *post hoc* and Mann Whitney U tests and are presented in Table 1.

Clinical and laboratory parameters according to CKD stages

Table 2 shows clinical and laboratory measurements of 34 controls and 70 patients grouped according to CKD stage. There were no statistically significant differences among the groups in terms of age and gender (ANOVA $p > 0.05$, $\chi^2 p > 0.05$; respectively). Duration of DM was significantly different between the CKD groups (Kruskal-Wallis test, $p = 0.006$). HbA1c and systolic-diastolic blood pressure levels were significantly higher in CKD patients compared with the control subjects (*post hoc* $p = 0.019$, $p = 0.030$, $p = 0.047$; respectively); however, there were

no statistically significant differences within the CKD groups (all *post hoc* $p > 0.05$). There were significant differences among the groups in terms of sCr, GFR, UAE and uKIM-1 levels. Advanced *post hoc* analyses for uKIM-1 are presented in Table 2.

The results of multivariate (backward) analysis are listed in Table 3 and indicate risk factors for decreased GFR (<90 mL/min). Duration of diabetes mellitus, UAE, and uKIM-1 were found to be independent positive predictors of GFR decline in the multivariate analysis. The other parameters (age, gender, systolic BP, diastolic BP and HbA1C) in multivariate analysis were not found to be significantly associated with GFR.

Assessment of clinical parameters associated with uKIM-1

Results of the bivariate correlation analyses of uKIM1 levels and clinical parameters of the patients are presented in Table 4. There were positive correlations among uKIM-1 levels and duration of diabetes, HbA1c levels, systolic and diastolic blood pressures and UAE.

Discussion

The results obtained from this study may be summarized as follows: (1) uKIM-1 levels in patients in all three DN stages and in patients with stage 2-4 CKD is increased compared with control subjects; (2) the highest uKIM-1 levels were seen in patients with stage 2 (Cr Cl 60-89 mL/min) CKD; (3) uKIM-1 in macroalbuminuric patients was significantly increased compared with other DN groups; and, (4) uKIM-1 levels, UAE and the duration of diabetes, were all found to be independent risk factors associated with low GFR.

Reports in the literature suggest that not only glomerular but also tubular damage may play important roles in the pathophysiology and the course of DN [6]. Even more than glomerular damage, tubulointerstitial damage reflects the progression and long term prognosis of renal damage [18]. UAE, which is used for early diagnosis of DN, indicates glomerular damage but does not provide information about the extent of tubular injury [19]. Nielsen *et al.* found that urinary KIM-1 levels increased in normo-, micro- and macro-albuminuric type 1 diabetic patients compared with non-diabetic control subjects; however, the authors did not see significant differences among diabetic subgroups. It was suggested that elevated urinary KIM-1, which can be detected before albuminuria, is a marker of the tubular injury that develops before glomerular damage [20]. In the present study, uKIM-1 levels were found to increase with an increase in albuminuria. The significant differences in uKIM-1 levels in the various DN subgroups appear to indicate albuminuria is not associated only with glom-

TABLE 1. Clinical and laboratory measurements according to the stages of diabetic nephropathy

Parametre	Control group (n=34)	Group 1 Normoalbuminuria (n=39)	Group 2 Microalbuminuria (n=42)	Group 3 Macroalbuminuria (n=61)	p
Age (year)	59 ± 8	62 ± 7	61 ± 9	66 ± 10	>0.05 *
Gender (female/male)	18/16	21/18	19/23	32/29	>0.05 †
DM duration (years)	4.9 ± 1.2	7.6 ± 2.9	8.9 ± 4.1	13.7 ± 5.8	0.003 ‡
Systolic BP (mmHg)	118 ± 12.8	133.8 ± 15.3	139.2 ± 17.1	146.1 ± 18.0	0.026 *
Diastolic BP (mmHg)	75.2 ± 11.8	79.4 ± 13.1	86.7 ± 12.8	95.9 ± 14.2	0.039 *
HbA1C (%)	5.2 ± 0.6	7.9 ± 1.7	7.7 ± 1.6	8.6 ± 1.9	0.020 *
sCr (mg/dL)	0.73 ± 0.28	1.01 ± 0.32	1.28 ± 0.37	2.93 ± 0.76	0.009 *
CrCl (mL/min)	108 ± 14	89 ± 11	81 ± 17	52 ± 21	0.007 ‡
UAE (mg/day)	11 ± 8	16 ± 9	108 ± 67	1786 ± 483	0.011 ‡
24 Hours Urine Volume (mL)	1428 ± 548	1305 ± 456	1210 ± 574	1171 ± 603	>0.05‡
uKIM-1 (ng/day)	1124 ± 168	1847 ± 486§	2325 ± 645¶	2889 ± 776	0.006 *

* One way ANOVA (for parametric data)

† Chi-square test test (for categorical variables)

‡ Kruskal Wallis test (for nonparametric data).

§ uKIM-1 difference between group 1 *versus* controls, *post-hoc* $P=0.025$ (Tukey's test).

¶ uKIM-1 difference between group 2 *versus* controls, *post-hoc* $P=0.019$; group 2 *versus* group 1, *post-hoc* $P=0.039$ (Tukey's test).

|| uKIM-1 difference between group 3 *versus* controls, *post-hoc* $P=0.007$; group 3 *versus* group 1, *post-hoc* $P=0.022$; group 3 *versus* group 2, *post-hoc* $P=0.044$; group 2 *versus* group 1, $P=0.041$; group 1 *versus* controls, $P=0.031$ (Tukey's test).

DM duration difference between group 3 *versus* controls, $P=0.002$; group 3 *versus* group 1, $P=0.021$; group 3 *versus* group 2, $P=0.038$; group 2 *versus* group 1, $P=0.094$; group 1 *versus* controls, $P=0.026$ (Mann-Whitney U test).

Systolic BP difference between group 3 *versus* controls, $P=0.017$; group 3 *versus* group 1, *post-hoc* $P=0.056$; group 3 *versus* group 2, *post-hoc* $P=0.085$; group 2 *versus* group 1, *post-hoc* $P=0.225$; group 1 *versus* controls, $P=0.033$ (Tukey's test).

Diastolic BP difference between group 3 *versus* controls, $P=0.029$; group 3 *versus* group 1, *post-hoc* $P=0.068$; group 3 *versus* group 2, *post-hoc* $P=0.114$; group 2 *versus* group 1, *post-hoc* $P=0.189$; group 1 *versus* controls, $P=0.047$ (Tukey's test).

sCr difference between group 3 *versus* controls, $P=0.007$; group 3 *versus* group 1, *post-hoc* $P=0.016$; group 3 *versus* group 2, *post-hoc* $P=0.026$; group 2 *versus* group 1, *post-hoc* $P=0.036$; group 1 *versus* controls, $P=0.041$ (Tukey's test).

DM: Diabetes mellitus, BP: Blood pressure, sCr: Serum Creatinine, CrCl: Creatinine clearance, UAE: Urinary albumin excretion, uKIM-1: Urinary KIM-1

erular damage but also with tubular damage. In addition, elevated uKIM-1 levels, even in the normoalbuminuric phase, suggest that the process of diabetic nephropathy is an early diagnostic finding.

Many complex processes have been outlined in patients with diabetes that is caused by impaired insulin resistance [21]. Carlsson *et al.* found an independent and inverse relationship between uKIM-1 and insulin sensitivity [22]. In light of this, a

change in the concentration of KIM-1 may be detectable in patients with type 2 diabetes even before the development of DN; therefore, in our study our control group was a group of diabetic patients who did not yet have evidence of DN (micro- or macroalbuminuria, decline in GFR or proliferative changes in the fundus). Thus, we prevented our results from the effects of insulin resistance and by a different mechanism may occur on KIM-1 before the development of DN. To eliminate the

TABLE 2. Clinical and laboratory measurements according to the CKD stages

Parametre	Control group (n=34)	Stage 2 CKD (n=28)	Stage 3 CKD (n=23)	Stage 4 CKD (n=19)	P
Age (years)	58 ± 8	61 ± 11	64 ± 9	68 ± 12	>0.05 *
Gender (female/male)	18/16	12/16	13/10	8/11	>0.05 †
DM duration (years)	4.9 ± 1.2	10.2 ± 4.9	12.3 ± 5.1	15.8 ± 6.7	0.003 ‡
Systolic BP (mmHg)	118 ± 12.8	138.4 ± 10.2	146.2 ± 15.4	152.3 ± 12.8	0.026 *
Diastolic BP (mmHg)	75.2 ± 11.8	91.3 ± 9.6	95.0 ± 14.2	102.3 ± 13.1	0.035 *
HbA1C (%)	6.1 ± 0.6	8.4 ± 0.8	8.1 ± 1.1	9.1 ± 2.3	0.042 *
sCr (mg/dL)	0.71 ± 0.28	1.53 ± 0.37	2.34 ± 0.52	3.21 ± 0.76	0.011 *
CrCl (mL/min)	108 ± 14	72 ± 10	47 ± 12	23 ± 5	0.002 ‡
UAE (mg/day)	11 ± 8	576 ± 217	1610 ± 880	2588 ± 1021	0.019 ‡
24 Hours Urine Volume (mL)	1428 ± 548	1286 ± 462	1190 ± 538	1154 ± 589	>0.05‡
uKIM-1 (ng/day)	1124 ± 168	2962 ± 724§	2168 ± 642¶	1765 ± 483	0.009 *

* One way ANOVA (for parametric data)

† Chi-square test test (for categorical variables)

‡ Kruskal Wallis test (for nonparametric data)

§ uKIM-1 difference between CKD (stage 2) *versus* controls, *post-hoc* $P=0.002$ (Tukey's test).¶ uKIM-1 difference between CKD (stage 3) *versus* controls, *post-hoc* $P=0.012$; CKD (stage 3) *versus* CKD (stage 2), *post-hoc* $P=0.025$ (Tukey's test).|| uKIM-1 difference between CKD (stage 4) *versus* controls, *post-hoc* $P=0.031$; CKD (stage 4) *versus* CKD (stage 2), *post-hoc* $P=0.021$; CKD (stage 4) *versus* CKD (stage 3), *post-hoc* $P=0.043$ (Tukey's test).

DM: Diabetes mellitus, BP: Blood pressure, sCr: Serum Creatinine, CrCl: Creatinine clearance, UAE: Urinary albumin excretion, uKIM-1: Urinary KIM-1.

possible effects of insulin resistance on KIM-1 levels in our study group, we compared our results with KIM-1 levels of a control group that included diabetic patients with without DN.

How does impairment in renal function affect KIM-1 levels? A rat study suggested that chronic KIM-1 expression stimulated hypoxia by tubulointerstitial inflammation and loss of capillaries and, at the same time, KIM-1 was induced by hypoxia and inflammation [23]. This vicious circle resulted in tubulointerstitial fibrosis. Clinical studies showed that KIM-1 was very sensitive to proximal tubular damage and was associated with the degree of fibrosis [24]. Timmeran *et al.* reported that tissue expression of KIM-1 increased and tissue KIM-1 levels were associated with urinary KIM-1 levels in renal biopsy specimens of the patients with renal damage from various causes [11]. They also showed that expression of KIM-1 was located especially in tissue with active tubular damage but not in atrophic or fibrotic regions. In the present study, uKIM-1

levels, which are elevated primarily in stage 2 but also in other stages of CKD compared with healthy controls, show a gradual decrease in patients with CKD in stages 3 and 4. We believe that the factors underlying these findings is the advancement of tubular atrophy and fibrosis development and the consequent reduction in the expression of KIM-1 that is seen throughout the progression of the CKD stages.

Another important study in this field was performed by Conway *et al.* [25] who observed the prognostic value of KIM-1 in predicting renal damage in 978 type 2 diabetic patients. They found that urinary KIM-1 levels were correlated with UAE and, throughout 4 years of follow up, they showed that elevated levels of urinary KIM-1 were associated with a rapid decline in renal function. The authors concluded that urinary KIM-1 did not provide additional prognostic value (other than UAE) in determining the prognosis of type 2 diabetic patients. Although the results of this study were not consistent with our results, these differences can be explained by differences in ex-

TABLE 3. Backward linear regression analyses of factors related with decreased GFR (< 90 mL/min).

	Beta	t	p
Duration of DM	0.189	1.911	0.044
UAE (mg/day)	0.241	2.405	0.029
uKIM-1 (ng/day)	0.525	5.918	0.014

DM: Diabetes mellitus, UAE: Urinary albumin excretion, uKIM-1: Urinary KIM-1

TABLE 4. Bivariate correlation analysis between uKIM-1 levels and clinical parameters.

Parametre	r	p
Age (years)	0.122	0.091 *
Gender (female/male)	0.085	0.112 †
DM duration (years)	0.402	0.019 †
Systolic BP (mmHg)	0.339	0.022 *
Diastolic BP (mmHg)	0.192	0.041 *
HbA1C (%)	0.255	0.031 *
sCr (mg/dL)	0.102	0.143 *
CrCl (mL/min)	0.130	0.122 †
UAE (mg/day)	0.161	0.039 †

* Pearson correlation test

† Spearman's correlation test

DM: Diabetes mellitus, BP: Blood pressure, sCr: Serum creatinine, CrCl: Creatinine clearance, UAE: Urinary albumin excretion, uKIM-1: Urinary KIM-1.

perimental design. First, Conway grouped CKD patients according to GFR: GFR >60 mL/min, GFR 60-30 mL/min and GFR <30 mL/min. Second, although the total study population of Conway's study was larger than our study population, macroalbuminuric patients, whose KIM-1 levels tend to be higher, were much more common in our study (61 vs. 15). Third, GFR calculations were performed differently in the two studies: the CKD-EPI formula was used in the GFR calculation in Conway's study. And finally, urinary KIM-1 levels were assessed in spot urine samples in Conway *et al.*'s study. Many of the analytes used in laboratory assessments have diurnal variation; however, there are no data in the literature that suggest that KIM-1 has such a variation. Thus it is not known yet whether uKIM-1 levels in spot urine sample reflect the total excretion of uKIM-1 in 24 hours. The differences listed above

may be responsible of different results between Conway *et al.*'s and our study.

Similar to what was reported in the Conway's study, we found a correlation between uKIM-1 and HbA1c%; however, we showed further correlations among uKIM-1 and systolic and diastolic blood pressures. The underlying reason for this difference could be that the proportion of subjects with macroalbuminuria and poor glycemic control, and, thus, patients with poor blood pressure control were much more common in our study than in the study by Conway (4-fold more common). This population reflects the patients with more advanced renal injury and denser tubulointerstitial damage. These findings strengthen our hypothesis that expression of uKIM-1 declines with the advancement of renal failure.

Our study reflects the results of a single center cross sectional study in a limited number of CKD patients with DN. We have not confirmed our speculations about renal tubular damage with renal biopsy; however, clinical guidelines limit renal biopsy in conditions with an overt cause of proteinuria, such as DN. Markers of diabetic renal damage used in clinical practice such as GFR and UAE were compared with uKIM-1 levels, suggesting that uKIM-1 could be useful in patient management.

In addition to diagnosing early renal damage caused by DN, uKIM-1 levels may also prove useful in monitoring the progression of kidney disease in patients with type 2 DM. Prospective, multicenter studies, which would include tissue evaluation, are needed.

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