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ORIGINAL ARTICLE

Urinary chemokine C-X-C motif ligand 16 and endostatin as predictors of tubulointerstitial fibrosis in patients with advanced diabetic kidney disease

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ABSTRACT

Background. Interstitial fibrosis and tubular atrophy (IFTA) is a well-recognized risk factor for poor renal outcome in patients with diabetic kidney disease (DKD). However, a noninvasive biomarker for IFTA is currently lacking. The purpose of this study was to identify urinary markers of IFTA and to determine their clinical relevance as predictors of renal prognosis.

Methods. Seventy patients with biopsy-proven isolated DKD were enrolled in this study. We measured multiple urinary inflammatory cytokines and chemokines by multiplex enzyme-linked immunosorbent assay in these patients and evaluated their association with various pathologic features and renal outcomes.

Results. Patients enrolled in this study exhibited advanced DKD at the time of renal biopsy, characterized by moderate to severe renal dysfunction [mean estimated glomerular filtration rate (eGFR) 36.1 mL/min/1.73 m^2] and heavy proteinuria (mean urinary protein:creatinine ratio 7.8 g/g creatinine). Clinicopathologic analysis revealed that higher IFTA scores were associated with worse baseline eGFR ($P < 0.001$) and poor renal outcome ($P = 0.002$), whereas glomerular injury scores were not. Among measured urinary inflammatory markers, C-X-C motif ligand 16 (CXCL16) and endostatin showed strong correlations with IFTA scores ($P = 0.001$ and $P < 0.001$, respectively), and patients with higher levels of urinary CXCL16 and/or endostatin experienced significantly rapid renal progression compared with other patients ($P < 0.001$). Finally, increased urinary CXCL16 and endostatin were independent risk factors for poor renal outcome after multivariate adjustments (95% confidence interval 1.070–3.455, $P = 0.029$).

Conclusions. Urinary CXCL16 and endostatin could reflect the degree of IFTA and serve as biomarkers of renal outcome in patients with advanced DKD.

Keywords: CXCL16, diabetic kidney disease, endostatin, interstitial fibrosis and tubular atrophy, pathologic classification

INTRODUCTION

Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease (ESRD) in South Korea [1]. In the past, the natural course of DKD was described as predictable and the level of proteinuria was considered one of the most important prognostic indicators for the decline in renal function, especially in patients with early stages of diabetes mellitus (DM) [2–4]. Recently, however, a large epidemiological study demonstrated that among diabetic patients, the prevalence of albuminuria has been decreasing while the prevalence of estimated glomerular filtration rate (eGFR) has been increasing steadily over the last three decades [5]. Moreover, studies suggest that proteinuria alone could not accurately predict the clinical course and renal prognosis of diabetic patients [6]. Changing paradigms of DKD

necessitate redefining the natural history of DKD as well as discovering novel biomarkers for the accurate prediction of renal progression.

The Renal Pathology Society proposed new pathologic classifications of DKD in 2010, consisting of glomerular, tubulointerstitial and vascular components [7]. Although several studies have validated the clinical use of this system for predicting renal outcomes [8–11], a major limitation is that diabetic patients exhibiting no clinical evidence of nondiabetic renal disease still do not undergo routine renal biopsy in most hospitals. Therefore biomarkers that can accurately reflect the pathologic status of the kidney would have the potential to substitute for invasive renal biopsy.

To date, there has been considerable research on developing biomarkers that help predict the renal prognosis of diabetic patients [12]. Proinflammatory cytokines and chemokines are of particular interest among them since sustained low-grade inflammation is a major contributing factor to the development and progression of DKD [13]. Indeed, evidence has demonstrated that several urinary inflammatory markers are associated with a rapid decline in renal function [14–18]. However, the diagnosis of DKD was made based on clinical evidence in these studies. Therefore the presence of unexpected nondiabetic renal diseases could not be excluded and, more importantly, the relationship between various urinary markers and the pathologic features of DKD has not been provided. The aim of this study was to identify potential urinary inflammatory markers that can reflect intrarenal pathologic status in patients with biopsy-proven DKD. We also evaluated the association among various urinary biomarkers, the rate of decline in renal function and the progression to ESRD to determine the clinical relevance of these inflammatory markers as predictors of renal prognosis.

MATERIALS AND METHODS

Patient selection and study design

An overview of the study design and patient recruitment strategy is provided in Figure 1. First, we screened 220 type 2 diabetic patients who underwent renal biopsy at six different hospitals from January 2012 to December 2016 to determine eligibility for this study. Upon pathologic review, 114 patients were diagnosed with isolated DKD with no evidence of other nondiabetic renal disease. Of these, 70 patients were ultimately included in this study after excluding 44 patients (see Figure 1 for detailed reasons). Indications for renal biopsy were described previously [19]. We also recruited three independent control groups for the comparison of urinary inflammatory markers; 6 age- and sex-matched individuals at high risk of DM, defined as having impaired fasting glucose and/or impaired glucose tolerance, 10 type 2 diabetic patients maintaining normal renal function and normoalbuminuria and 15 age-, sex- and eGFR-matched nondiabetic patients with biopsy-proven hypertensive nephrosclerosis.

Clinical and laboratory data were collected at the time of renal biopsy. Renal function was assessed by eGFR, calculated by the Chronic Kidney Disease Epidemiology Collaboration formula [20]. The level of proteinuria was measured by 24-h urine collection, if possible, or calculated as spot urinary protein:creatinine ratio [PCR, g/g creatinine (Cr)]. Renal outcomes were defined as progression to ESRD requiring either dialysis or kidney transplantation. The institutional review board from each hospital approved this study (IRB no. KHNMC 2008-030) and informed consent was obtained from all patients.

Pathologic diagnoses and classifications of patients

All biopsy specimens were processed by standard methods and examined by pathologists at each hospital. Pathologic diagnosis of DKD was made and categorized by pathologic classification of the Renal Pathology Society (Supplementary data, Table S1) [7]. In brief, five different pathologic parameters were used: glomerular classification, interstitial fibrosis and tubular atrophy (IFTA), interstitial inflammation, arteriolar hyalinosis and arteriosclerosis. Hypertensive nephrosclerosis was diagnosed based on their typical pathologic findings [21] and the exclusion of other primary glomerular or tubulointerstitial nephropathy. All pathologic data were reviewed and confirmed by another pathologist at Kyung Hee University (LSJ) for the reliability and reproducibility of the diagnoses and classification.

Urine sample collection and measurements of urinary inflammatory markers by multiplex enzyme-linked immunosorbent assay (ELISA)

Urine samples were collected and processed as previously described [22]. Multiple urinary inflammatory markers were simultaneously measured by multiplex ELISA using a customized Magnetic Luminex Screening Assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). We reviewed the previous literature and selected 10 candidate urinary inflammatory markers: monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), interferon γ-induced protein-10 (IP-10), C-X-C motif ligand 16 (CXCL16), endostatin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), CXCL9 and growth/differentiation factor-15 (GDF-15) [18, 23–27]. Each target cytokine or chemokine was detected by Luminex 200 (Luminex, Austin, TX, USA), and its concentration was calculated based on the standard curves generated from the reference sample. The levels of these markers were expressed relative to the urine creatinine concentration (ng/g Cr).

Immunohistochemical staining of CD68, CXCL16 and endostatin

Detailed immunohistochemistry procedures have been provided previously [28]. The following primary antibodies were used for immunohistochemistry experiments: CD68 (catalog no. MS-397; Thermo Fisher Scientific, Waltham, MA, USA), CXCL16 (catalog no. ab101404; Abcam, Cambridge, MA, USA) and endostatin (catalog no. ab3453; Abcam). The number of macrophages infiltrating the tubulointerstitium was counted in whole biopsy tissue and expressed as the number of CD68-positive cells per square millimeter. To quantify CXCL16 and endostatin expression, the average percentage of positively

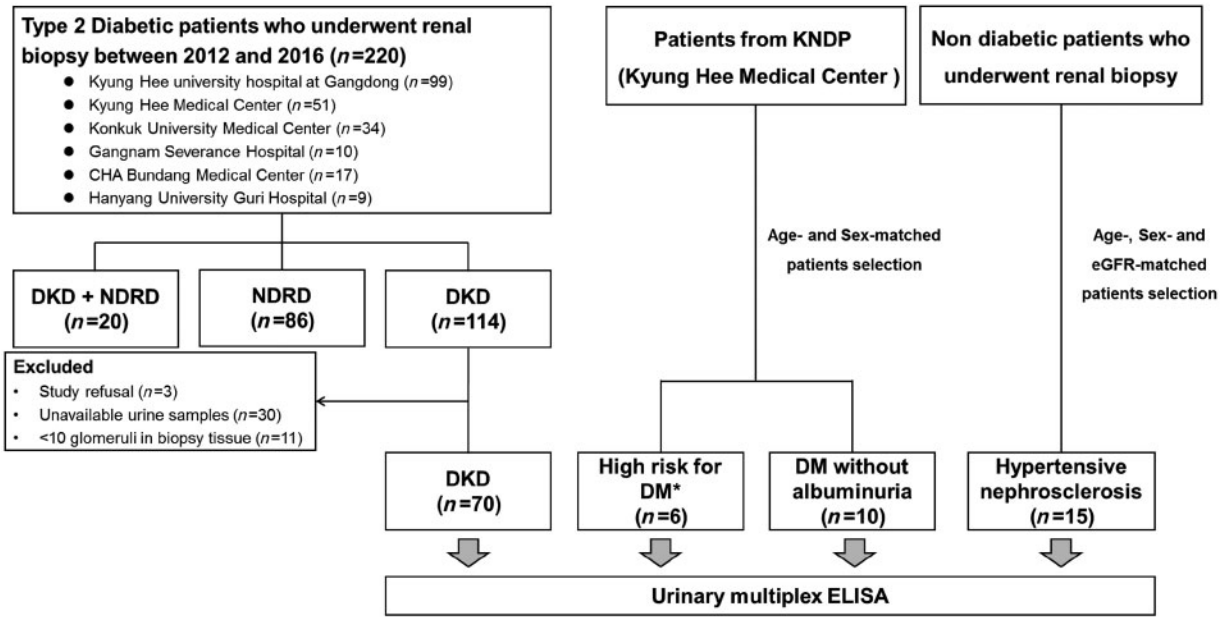

FIGURE 1: An overview of the study design and patient selection. NDRD, nondiabetic renal disease; KNDP, Korea National Diabetes Program. *Defined as having impaired fasting glucose and/or impaired glucose tolerance.

Table 1. Clinical characteristics and laboratory parameters of 70 patients with biopsy-proven DKD

Characteristics	Values
Age (years)	57.3 ± 11.4
Sex (male), *n* (%)	45 (64.3)
BMI (kg/m^2)	25.2 ± 3.4
Duration of diabetes (years)	11.4 ± 8.3
Hypertension, *n* (%)	62 (88.6)
Hemoglobin A1c (%)	7.8 ± 2.0
Hemoglobin (g/dL)	10.4 ± 2.0
eGFR (mL/min/1.73 m^2)	36.1 ± 23.7
Albumin (g/dL)	3.3 ± 0.7
Total cholesterol (mg/dL)	195.8 ± 64.7
Urine PCR (g/g Cr)	7.77 ± 6.80
Duration of follow-up (months)	20.2 ± 14.7
Progression to ESRD, *n* (%)	36 (51.4)

Values are presented as mean ± SD unless stated otherwise.
BMI, body mass index.

stained renal tubular cells in five randomly selected corticomedullary areas from each section at 400× magnification was calculated in each sample.

Statistical analyses

All statistical analyses were performed with SPSS for Windows, version 20.0 (IBM, Armonk, NY, USA). Baseline characteristics and clinical parameters were expressed as the mean ± SD or as the number of patients and percentage. Analysis of variance and chi-square tests were used to compare these variables. Urinary inflammatory markers were described as medians [first and third interquartile ranges (IQRs)] and compared among the subgroups by the Kruskal–Wallis test for overall comparisons and the Mann–Whitney test for comparisons of each group since these data were nonnormally distributed. We used Pearson's correlation analyses to compare urinary inflammatory markers, urinary PCR and the rate of decline in eGFR. Kaplan–Meier curves were generated to assess the probabilities of the renal outcomes according to the various indicators and the Cox proportional hazards model was used for further multivariate analysis. The combined scores of CXCL16 and endostatin were determined by calculating the predicted probabilities of ESRD progression for each patient using logistic regression analysis. Patients were then divided into tertiles according to their values of calculated probability. Finally, the percentage of positively stained cells was expressed as the mean ± standard error and compared by the Kruskal–Wallis and the Mann–Whitney tests. P-values <0.05 were considered statistically significant.

RESULTS

Baseline clinical characteristics of patients with biopsy-proven DKD

The baseline demographics of the enrolled patients are shown in Table 1. The mean age was 57.3 years, 64.3% (45/70) of the patients were male and the mean duration of diabetes was 11.4 years. Most patients showed moderate to severe renal dysfunction, with a mean eGFR of 36.1 mL/min/1.73 m^2 and a mean urinary PCR of 7.77 g/g Cr. The mean duration of follow-up was 20.2 months and 36 patients (51.4%) progressed to ESRD during the follow-up period.

Clinical characteristics of patients with DKD according to pathologic classification

Next we categorized patients according to the pathologic classification of DKD. The results of the comparisons of the clinical demographics across groups are shown in Table 2. We found that the degree of glomerular injury was not associated with eGFR (38.5 ± 21.7 versus 39.0 ± 24.6 versus

Table 2. Clinical characteristics and laboratory parameters of patients according to pathologic classification of diabetic kidney disease

Characteristics	Glomerular lesions				IFTA				Interstitial inflammation			
	Class II ($n=14$)	Class III ($n=32$)	Class IV ($n=24$)	P-value	1 ($n=40$)	2 ($n=20$)	3 ($n=10$)	P-value	0 ($n=12$)	1 ($n=43$)	2 ($n=15$)	P-value
Age (years)	63.9 ± 11.1	54.3 ± 11.6	57.5 ± 9.9	0.030	58.5 ± 10.2	56.1 ± 12.3	55.0 ± 14.6	0.591	57.2 ± 12.1	57.5 ± 10.7	56.8 ± 13.4	0.997
Sex (male), *n* (%)	7 (50.0)	23 (71.9)	15 (62.5)	0.353	25 (62.5)	12 (60.0)	8 (80.0)	0.524	5 (41.7)	31 (72.1)	9 (60.0)	0.140
BMI (kg/m^2)	25.3 ± 3.2	24.5 ± 3.1	26.0 ± 3.9	0.262	25.5 ± 3.5	24.5 ± 3.6	25.4 ± 2.7	0.575	25.7 ± 3.3	25.1 ± 3.7	25.1 ± 2.7	0.845
Duration of diabetes (years)	15.3 ± 10.5	11.0 ± 8.1	9.6 ± 6.6	0.116	11.7 ± 8.5	9.7 ± 6.8	13.5 ± 10.3	0.459	10.7 ± 8.1	11.1 ± 8.0	12.6 ± 9.4	0.803
Hypertension, *n* (%)	13 (92.9)	28 (87.5)	21 (87.5)	0.853	33 (82.5)	19 (95.0)	10 (100)	0.067	10 (83.3)	39 (90.7)	13 (86.7)	0.836
HbA1c (%)	7.7 ± 1.9	7.8 ± 2.0	7.7 ± 2.1	0.941	7.7 ± 1.7	7.8 ± 2.3	7.9 ± 2.6	0.943	8.2 ± 2.3	7.7 ± 1.9	7.4 ± 1.9	0.667
Hemoglobin (g/dL)	10.7 ± 2.3	10.3 ± 1.6	10.4 ± 2.3	0.758	11.0 ± 2.1	9.7 ± 1.4	9.4 ± 2.2	0.012	10.6 ± 2.1	10.5 ± 2.0	9.9 ± 2.1	0.548
eGFR (mL/min/1.73 m^2)	38.5 ± 21.7	39.0 ± 24.6	30.8 ± 23.5	0.408	44.5 ± 22.6	30.12 ± 22.8	14.3 ± 8.1	<0.001	42.2 ± 29.4	38.8 ± 23.4	23.4 ± 14.7	0.057
Albumin (g/dL)	3.7 ± 0.5	3.1 ± 0.6	3.2 ± 0.8	0.032	3.4 ± 0.7	3.0 ± 0.5	3.0 ± 0.6	0.036	3.3 ± 0.8	3.3 ± 0.7	3.1 ± 0.4	0.664
Total cholesterol (mg/dL)	189 ± 27	213 ± 76	182 ± 61	0.145	200 ± 64	180 ± 62	209 ± 75	0.412	213 ± 79	180 ± 55	227 ± 69	0.030
Urinary PCR (g/g Cr)	5.46 ± 5.12	9.55 ± 7.98	6.77 ± 5.42	0.114	5.70 ± 3.97	11.37 ± 9.97	8.92 ± 5.19	0.007	6.15 ± 5.34	7.83 ± 7.52	8.93 ± 5.65	0.576

Characteristics	Arteriolar hyalinosis				Arteriosclerosis			
	0 ($n=7$)	1 ($n=32$)	2 ($n=31$)	P-value	0 ($n=8$)	1 ($n=28$)	2 ($n=34$)	P-value
Age (years)	65.9 ± 8.6	56.0 ± 11.1	56.8 ± 11.7	0.106	58.1 ± 9.9	56.4 ± 8.1	57.9 ± 14.0	0.870
Sex (male), *n* (%)	3 (42.9)	19 (59.4)	23 (74.2)	0.216	6 (75.0)	16 (57.1)	23 (67.6)	0.552
BMI (kg/m^2)	27.2 ± 4.1	24.5 ± 3.0	25.5 ± 3.5	0.140	25.5 ± 2.7	25.9 ± 3.6	24.6 ± 3.3	0.348
Duration of diabetes (years)	10.1 ± 9.9	11.6 ± 8.1	11.3 ± 8.3	0.902	8.9 ± 7.6	11.1 ± 6.7	12.2 ± 9.6	0.582
Hypertension, *n* (%)	7 (100)	27 (84.4)	28 (90.3)	0.883	6 (75.0)	26 (92.9)	30 (88.2)	0.594
Hemoglobin A1c (%)	7.8 ± 2.2	7.6 ± 1.8	7.9 ± 2.1	0.823	7.4 ± 1.7	8.1 ± 1.9	7.6 ± 2.1	0.528
Hemoglobin (g/dL)	10.9 ± 2.2	10.3 ± 1.9	10.4 ± 2.2	0.829	10.9 ± 2.3	10.6 ± 2.1	10.1 ± 1.9	0.475
eGFR (mL/min/1.73 m^2)	38.6 ± 30.3	36.4 ± 22.8	35.2 ± 23.8	0.943	40.7 ± 27.6	33.5 ± 18.7	37.2 ± 26.6	0.704
Albumin (g/dL)	3.4 ± 0.6	3.3 ± 0.7	3.2 ± 0.6	0.511	3.3 ± 0.7	3.3 ± 0.7	3.2 ± 0.6	0.679
Total cholesterol (mg/dL)	184 ± 47	204 ± 74	189 ± 58	0.584	224 ± 62	186 ± 55	198 ± 72	0.365
Urine PCR (g/g Cr)	3.95 ± 1.37	8.28 ± 5.00	10.18 ± 8.27	0.020	5.60 ± 3.21	6.91 ± 5.33	9.00 ± 8.24	0.311

Values are presented as mean ± SD unless stated otherwise.
BMI, body mass index.

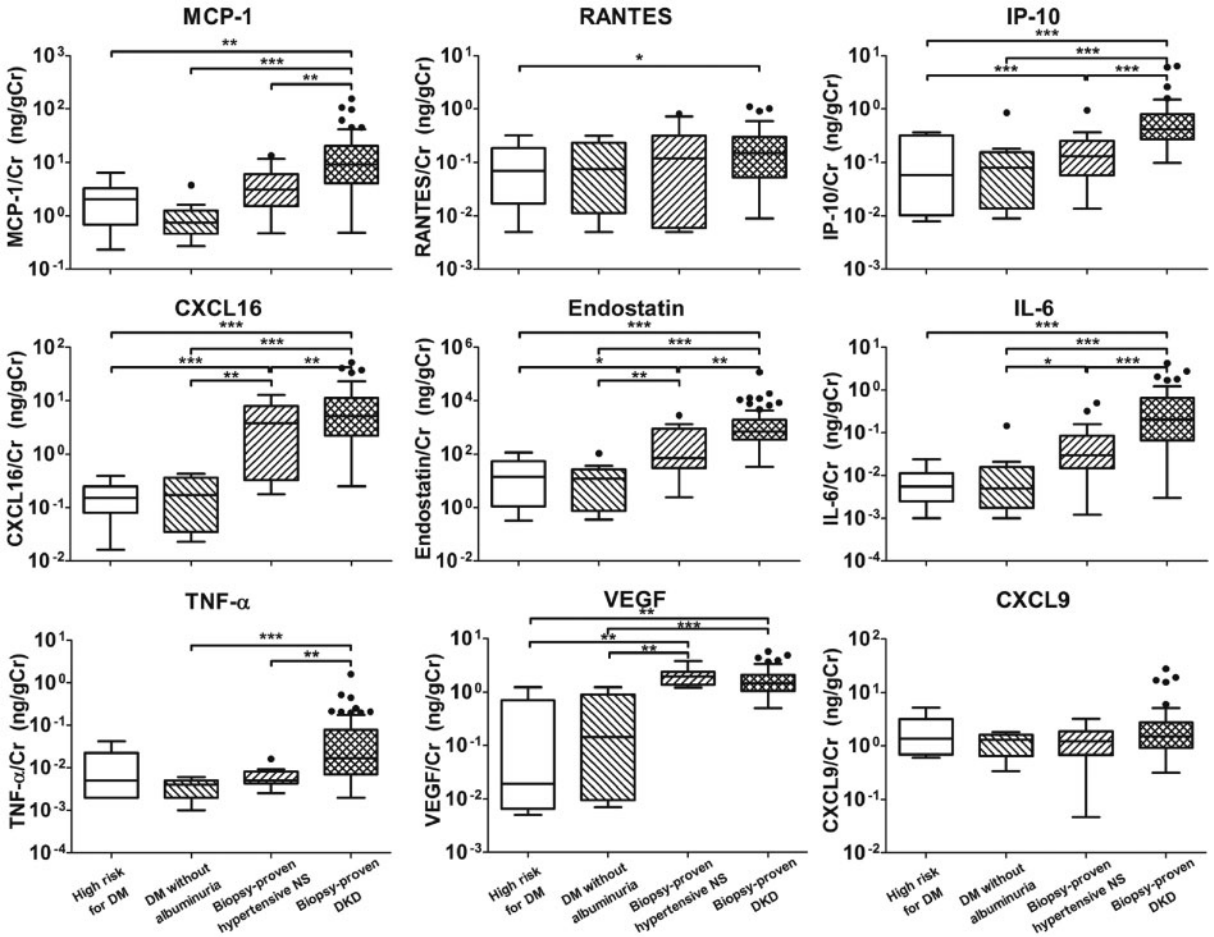

FIGURE 2: The levels of urinary inflammatory markers in individuals at high risk for diabetes, diabetic patients without albuminuria, those with biopsy-proven hypertensive nephrosclerosis and those with biopsy-proven DKD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as box-and-whisker plots and the ends of the whiskers represent the 1.5 IQRs of the value. Dots indicate outliers. NS, nephrosclerosis.

30.8 ± 23.5 mL/min/1.73 m^2 for Class II versus Class III versus Class IV, respectively; $P = 0.408$). In contrast, patients who exhibited severe IFTA showed significantly lower levels of eGFR (44.5 ± 22.6 versus 30.1 ± 22.8 versus 14.3 ± 8.1 mL/min/1.73 m^2 for an IFTA score of 1 versus 2 versus 3, respectively; $P < 0.001$). Those with higher interstitial inflammation scores showed a tendency to have lower eGFR, although the difference did not reach statistical significance (42.2 ± 29.4 versus 38.8 ± 23.4 versus 23.4 ± 14.7 mL/min/1.73 m^2 for an interstitial inflammation score of 0 versus 1 versus 2, respectively; $P = 0.057$). Neither score was correlated with baseline eGFR in our study.

Intriguingly, urinary PCR was not associated with the degree of glomerular injury (5.46 ± 5.12 versus 9.55 ± 7.98 versus 6.77 ± 5.42 for Class II versus Class III versus Class IV, respectively; $P = 0.114$), interstitial inflammation or arteriosclerosis scores. Patients with a higher degree of IFTA (scores of 2 and 3) showed more proteinuria than those with a lower grade of IFTA (scores of 1), but patients with IFTA scores of 3 showed lower levels of proteinuria than those with IFTA scores of 2. Finally, the level of proteinuria was higher in patients with advanced arteriolar hyalinosis (3.95 ± 1.37 versus 8.28 ± 5.00 versus 10.18 ± 8.27 for an arteriolar hyalinosis score of 0 versus 1 versus 2, respectively; $P = 0.020$).

The levels of urinary inflammatory markers in patients with DKD according to pathologic classification

The levels of urinary inflammatory markers were compared across patients with DKD and three independent control groups (Figure 2). The baseline characteristics of the control groups are provided in Supplementary data, Table S2. We observed that the levels of eight urinary inflammatory markers, except CXCL9 and GDF-15, were significantly higher in patients with DKD than in the individuals at high risk for DM and diabetic patients without albuminuria. Moreover, these patients also exhibited elevated levels of most urinary inflammatory markers, as compared with nondiabetic patients with biopsy-proven hypertensive nephrosclerosis, even though the renal function was similar between the two groups (eGFR 36.1 ± 23.7 versus 34.3 ± 29.6 mL/min/1.73 m^2 for DKD versus hypertensive nephrosclerosis; $P = 0.801$ by independent t-test). These results indicate that intrarenal inflammation is a predominant feature of DKD and induced by the combined effects of the diabetic milieu and decreased renal function. GDF-15 was excluded from the analysis since its levels were outside the measurable range in >50% of samples (data not shown).

Subsequently we examined the association between the pathologic classification of DKD and urinary inflammatory

markers (Table 3). Glomerular classification, arteriolar hyalinosis or arteriosclerosis scores were not associated with the level of any urinary inflammatory marker. In contrast, three urinary inflammatory markers—MCP-1, CXCL16 and endostatin—showed significant correlation with the severity of IFTA ($P = 0.003$, $P = 0.001$ and $P < 0.001$, respectively). Urinary endostatin was also elevated in patients exhibiting severe interstitial inflammation ($P = 0.034$).

Collectively these data suggest that the degree of IFTA showed a strong correlation with residual renal function in patients with biopsy-proven advanced DKD, while the glomerular injury scores were not. In addition, the levels of urinary MCP-1, CXCL16 and endostatin may reflect the severity of IFTA better than that of interstitial inflammation.

Association between urinary inflammatory markers and the annual decline in renal function

Next we investigated the association between urinary inflammatory markers and the annual decline in renal function, calculated as the percentage decrease in eGFR from baseline per year. Correlation analyses revealed that five urinary inflammatory markers, including MCP-1, CXCL16, endostatin, IL-6 and CXCL9, showed a negative correlation with the rate of decline in renal function (Table 4). Among these, urinary endostatin and CXCL16 were the two most strongly correlated inflammatory markers ($R = -0.602$, $P < 0.001$ and $R = -0.520$, $P < 0.001$, respectively). Urinary PCR was associated with a decline in eGFR, but the correlation coefficient was lower than those of the abovementioned urinary markers ($R = -0.315$ and $P = 0.045$).

Renal survival in patients with advanced DKD according to the pathologic classification and the levels of urinary inflammatory markers

Figure 3 shows unadjusted Kaplan–Meier survival curves of patients according to the pathologic classification and the levels of urinary inflammatory markers. Renal survival was significantly worse in patients with severe IFTA ($P = 0.002$; Figure 3B) and/or interstitial inflammation ($P = 0.008$; Figure 3C), presumably as a result of poor residual renal function. Notably, neither pathologic feature was associated with renal outcome after adjusting for multivariate variables, including eGFR (Table 5). Other pathologic parameters, including glomerular lesions, arteriolar hyalinosis and arteriosclerosis, were not related to renal survival (Figure 3A, 3D and 3E). We also found that urinary PCR was not associated with renal outcome ($P = 0.515$; Figure 3F). Patients with higher levels of urinary MCP-1 had marginally worse renal outcomes ($P = 0.064$; Figure 3G). Importantly, higher levels of urinary CXCL16, urinary endostatin and the combination of urinary CXCL16 and endostatin were all associated with a higher rate of reaching ESRD ($P < 0.001$ for all comparisons; Figure 3H–J). Furthermore, Cox regression analysis revealed that the combination of urinary CXCL16 and endostatin was an independent risk factor for ESRD after multivariate adjustment, including baseline renal function [hazard ratio 1.923 (95% confidence interval 1.070–3.455), $P = 0.029$; Table 5].

Taken together, these results show that the levels of urinary CXCL16 and endostatin could serve as a predictor of rapid progression to ESRD in patients with advanced DKD due to their relevant correlations with the severity of IFTA and the rate of decline in renal function. On the other hand, glomerular injury scores and urinary PCR, a traditional indicator of rapid renal progression, were not associated with the rate of progression to ESRD in those patients.

Association between macrophage infiltration, interstitial inflammation and renal survival

Previous studies documented the relationship between tubulointerstitial macrophage infiltration and worse renal outcomes [29–32]. Therefore we additionally performed immunohistochemical staining of CD68, a marker for macrophages, and the representative findings are shown in Supplementary data, Figure S1. In contrast to the normal tissues of patients with renal cell carcinoma, where macrophages were rarely found (Supplementary data, Figure S1A), tissues obtained from those with DKD revealed prominent macrophage infiltration in the tubulointerstitium (Supplementary data, Figure S1B and S1C). Renal outcomes were inversely correlated with the degree of macrophage infiltration, although the statistical significance was not found after multivariate adjustments (Table 5).

Immunohistochemical staining of CXCL16 and endostatin in renal biopsy tissue

Finally, we performed immunohistochemical staining to evaluate the expression of CXCL16 and endostatin in renal biopsy tissue. CXCL16 was rarely detected in normal tissues of patients obtained from renal cell carcinoma (Figure 4A). In contrast, we observed CXCL16 expression exclusively in nonatrophic proximal tubular cells in tissues from patients with DKD (Figure 4B). Endostatin was normally detected in peritubular capillaries but rarely in renal tubular cells (Figure 4C). The immunoreactivity of endostatin was significantly increased in tissues from patients with DKD; most atrophic and some nonatrophic renal tubular cells were positive for endostatin staining (Figure 4D). Furthermore, the percentage of renal tubular cells staining for CXCL16 and endostatin was correlated with the degree of the severity of IFTA ($P = 0.01$ and $P = 0.003$, respectively; Figure 4E and F).

DISCUSSION

In this study we extensively analyzed clinicopathologic data and various urinary inflammatory markers to discover novel biomarkers that could predict IFTA in patients with advanced DKD. By recruiting patients whose diagnosis of DKD was confirmed by biopsy, we could eliminate the possibility of unrevealed nondiabetic renal disease, which is frequently difficult to exclude based on clinical information [19, 33], and examine the relationship between various markers and pathological features. Furthermore, patients enrolled in this study had a unique feature, in that most showed moderate to severe renal dysfunction (mean eGFR of 36.1 mL/min/1.73 m^2; Table 1). A considerable concern is that most predictors of renal progression in DKD were assessed in patients with early stages of DKD, raising a

Table 3. Levels of urinary inflammatory markers according to pathologic classification of DKD

Urinary levels	Glomerular lesions				IFTA				Interstitial inflammation			
	Class II ($n=14$)	Class III ($n=32$)	Class IV ($n=24$)	P-value	1 ($n=40$)	2 ($n=20$)	3 ($n=10$)	P-value	0 ($n=12$)	1 ($n=43$)	2 ($n=15$)	P-value
MCP-1 (ng/g Cr)	6.4 (2.9, 15.2)	12.7 (5.7, 25.0)	8.9 (4.0, 20.9)	0.180	6.5 (2.9, 14.3)	12.8 (7.5, 26.7)	20.7 (9.0, 35.5)	0.003	10.0 (4.1, 19.6)	8.2 (3.7, 17.7)	17.4 (7.3, 28.5)	0.149
RANTES (ng/g Cr)	0.1 (0, 0.3)	0.2 (0.1, 0.3)	0.1 (0, 0.3)	0.310	0.2 (0.1, 0.3)	0.1 (0, 0.3)	0.2 (0.1, 0.3)	0.681	0.1 (0.1, 0.3)	0.1 (0, 0.3)	0.2 (0.1, 0.3)	0.884
IP-10 (ng/g Cr)	0.4 (0.2, 1.1)	0.6 (0.3, 0.9)	0.3 (0.2, 0.5)	0.106	0.4 (0.2, 0.8)	0.5 (0.3, 0.8)	0.4 (0.4, 0.7)	0.852	0.5 (0.2, 0.9)	0.4 (0.3, 0.7)	0.5 (0.3, 1.1)	0.556
CXCL16 (ng/g Cr)	3.6 (1.6, 9.3)	4.6 (2.5, 8.6)	7.7 (3.8, 16.6)	0.280	3.8 (1.7, 6.5)	8.1 (3.6, 17.4)	16.5 (6.1, 23.0)	0.001	6.0 (1.0, 8.4)	4.6 (2.1, 10.1)	7.6 (3.4, 17.3)	0.248
Endostatin (ng/g Cr)	447.1 (114.9, 1172.0)	694.5 (356.8, 1957.2)	1119.1 (364.1, 3093.7)	0.452	443.7 (171.5, 986.3)	1679.9 (474.8, 4180.5)	3967.9 (1403.2, 7827.5)	<0.001	640.9 (90.2, 1196.4)	515.6 (359.9, 1586.5)	1699.0 (515.6, 8320.6)	0.034
IL-6 (ng/g Cr)	0.08 (0.03, 0.24)	0.28 (0.13, 0.83)	0.25 (0.05, 0.49)	0.154	0.13 (0.04, 0.47)	0.34 (0.13, 0.65)	0.51 (0.13, 0.87)	0.090	0.24 (0.05, 0.49)	0.18 (0.06, 0.61)	0.43 (0.13, 0.77)	0.415
TNF-α (ng/g Cr)	0.01 (0, 0.04)	0.03 (0.01, 0.08)	0.02 (0.01, 0.09)	0.100	0.02 (0.01, 0.07)	0.02 (0.01, 0.07)	0.16 (0.01, 0.32)	0.179	0.01 (0.01, 0.08)	0.02 (0.01, 0.07)	0.01 (0.01, 0.17)	0.556
VEGF (ng/g Cr)	1.2 (1.0, 1.8)	1.7 (1.2, 2.3)	1.4 (1.0, 1.8)	0.075	1.4 (1.0, 2.0)	1.5 (1.2, 2.8)	1.5 (1.0, 3.8)	0.403	1.4 (0.9, 2.1)	1.5 (1.1, 2.1)	1.4 (0.8, 3.0)	0.793
CXCL9 (ng/g Cr)	1.1 (0.8, 2.3)	1.8 (1.1, 3.2)	1.6 (0.9, 2.3)	0.202	1.3 (0.8, 2.1)	2.9 (1.0, 3.5)	2.1 (1.2, 2.8)	0.296	1.4 (0.6, 3.3)	1.4 (0.9, 2.8)	2.1 (1.3, 2.7)	0.353

Urinary levels	Arteriolar hyalinosis				Arteriosclerosis			
	0 ($n=7$)	1 ($n=32$)	2 ($n=31$)	P-value	0 ($n=8$)	1 ($n=28$)	2 ($n=34$)	P-value
MCP-1 (ng/g Cr)	3.9 (2.6, 19.7)	8.2 (3.5, 16.6)	13.4 (7.0, 32.3)	0.026	6.2 (2.6, 28.4)	9.9 (3.8, 16.7)	8.5 (5.5, 21.3)	0.609
RANTES (ng/g Cr)	0.1 (0, 0.2)	0.2 (0, 0.3)	0.2 (0.1, 0.5)	0.316	0.2 (0, 0.5)	0.1 (0, 0.3)	0.2 (0.1, 0.3)	0.484
IP-10 (ng/g Cr)	0.4 (0.1, 0.9)	0.3 (0.2, 0.8)	0.5 (0.3, 0.7)	0.507	0.3 (0.2, 1.1)	0.4 (0.3, 0.6)	0.4 (0.3, 0.8)	0.942
CXCL16 (ng/g Cr)	3.0 (0.8, 5.4)	5.7 (2.3, 9.9)	5.7 (2.5, 17.0)	0.292	3.6 (0.9, 5.3)	6.9 (1.8, 10.2)	5.1 (2.5, 16.9)	0.328
Endostatin (ng/g Cr)	438.3 (264.7, 549.7)	632.9 (329.9, 1643.2)	1100.1 (366.4, 3648.0)	0.292	419.1 (152.3, 1039.9)	1071.9 (381.9, 1911.0)	610.1 (358.3, 2458.6)	0.487
IL-6 (ng/g Cr)	0.04 (0.02, 0.36)	0.14 (0.06, 0.51)	0.40 (0.13, 0.85)	0.031	0.10 (0.04, 0.85)	0.22 (0.06, 0.51)	0.21 (0.10, 0.79)	0.817
TNF-α (ng/g Cr)	0.01 (0.01, 0.05)	0.02 (0.01, 0.07)	0.02 (0.01, 0.16)	0.199	0.01 (0.01, 0.13)	0.03 (0.01, 0.09)	0.02 (0.01, 0.07)	0.867
VEGF (ng/g Cr)	1.0 (0.8, 1.5)	1.5 (1.2, 2.0)	1.6 (1.1, 2.4)	0.338	1.4 (0.8, 2.1)	1.4 (1.1, 1.8)	1.5 (1.1, 2.4)	0.481
CXCL9 (ng/g Cr)	2.1 (1.1, 2.7)	1.5 (0.9, 2.1)	1.4 (1.0, 3.2)	0.433	1.6 (0.9, 2.4)	1.3 (0.7, 2.5)	1.8 (1.0, 3.0)	0.139

All urinary markers are expressed as the median (25th, 75th percentiles).

question of whether these markers could still be useful among those with advanced DKD [12]. By focusing on patients with only moderate to severe renal dysfunction, we could minimize unwanted selection bias resulting from variable baseline renal function.

Our data show that neither the pathologic severity of glomerular injury nor the level of proteinuria was correlated with residual kidney function and renal outcomes, which was an unexpected finding. Indeed, studies that assessed the clinical relevance of the new pathologic classification of DKD also revealed conflicting results [8–11, 34–36]. Notably, in a large retrospective study, patients exhibiting severe diabetic glomerulopathy were found to have significantly worse renal outcomes [8]. However, the severity of glomerular injury was no longer related to poor renal outcomes when patients exhibiting glomerular classification Class I and IIa were excluded from the outcome analyses [8]. This result is consistent with our findings in that most patients enrolled in our study also showed glomerular classification Class IIb or higher [68/70 (97.1%)]. In contrast to glomerular classification, a relevant correlation between IFTA scores and residual renal function was found in this study. Moreover, renal outcome was worse in patients with higher IFTA scores. These findings are in line with previous studies that showed severe tubulointerstitial fibrosis in patients with DKD was associated with both low eGFR and a rapid decline in renal function [8, 11, 35, 37]. Given these results, we concluded that tubulointerstitial fibrosis, but not glomerular injury, was the major determinant of renal outcomes in patients with advanced DKD.

Further urinary multiplex ELISA demonstrated that the levels of urinary MCP-1, CXCL16 and endostatin were correlated with IFTA scores but not with other pathologic variables, including glomerular and vascular injury scores, suggesting the potential as surrogate markers of chronic renal tubulointerstitial fibrosis. Moreover, these urinary inflammatory markers were also significantly associated with the rate of annual decline in eGFR, and the combination of urinary CXCL16 and endostatin remained a predictor of worse renal outcome after multivariate adjustments, suggesting the independent roles of these molecules in renal progression. Confirming whether renal tubular expression of CXCL16 and endostatin demonstrated by immunohistochemistry implies tubular secretion into urine or reabsorption from urine was difficult in this study. Based on previous experimental studies [38–40], we speculate that urinary CXCL16 and endostatin might be secreted from renal tubular cells and actively participate in tubulointerstitial injuries.

A number of studies have documented the role of CXCL16 and endostatin in kidney diseases. CXCL16 is a chemokine that interacts with CXCR6 in immune cells to promote chemotaxis or cell adhesion [41]. In the kidney, CXCL16 is known to be secreted from renal tubular cells [39, 42], and studies suggest that renal CXCL16 plays an important role in the development of renal tubulointerstitial inflammation and fibrosis [39, 43, 44]. Elewa *et al.* [27] also reported that plasma CXCL16 levels were higher in patients with advanced stages of DKD and/or overt albuminuria, suggesting a possible link between the progression of DKD and CXCL16.

Endostatin is a C-terminal proteolytic fragment of collagen XVIII that functions as a specific endogenous angiogenesis

Table 4. Correlation between urinary inflammatory markers and the annual decline in renal function

Urinary levels	Decline in eGFR (% per year)	
	R	P-value
MCP-1 (log_{10}, ng/g Cr)	−0.496	0.001
RANTES (log_{10}, ng/g Cr)	−0.255	0.108
IP-10 (log_{10}, ng/g Cr)	−0.207	0.194
CXCL16 (log_{10}, ng/g Cr)	−0.520	<0.001
Endostatin (log_{10}, ng/g Cr)	−0.602	<0.001
IL-6 (log_{10}, ng/g Cr)	−0.476	0.002
TNF-α (log_{10}, ng/g Cr)	−0.281	0.075
VEGF (log_{10}, ng/g Cr)	−0.299	0.057
CXCL9 (log_{10}, ng/g Cr)	−0.351	0.024
Urinary PCR (log_{10}, ng/g Cr)	−0.315	0.045

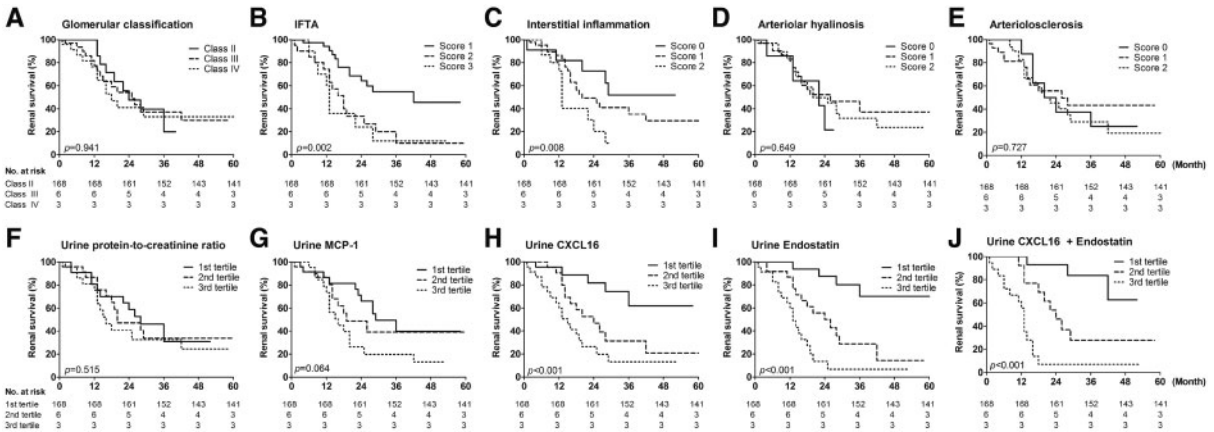

FIGURE 3: Renal survival of patients with biopsy-proven DKD based on different pathologic classifications (**A–E**) and urinary inflammatory markers (**F–J**). Renal survival according to the scores of (A) glomerular lesions, (B) IFTA, (C) interstitial inflammation, (D) arteriolar hyalinosis, (E) arteriosclerosis, (F) urine PCR, (G) MCP-1, (H) CXCL16, (I) endostatin and (J) the combination of CXLC16 and endostatin.

Table 5. Multivariate Cox regression analysis for renal outcome

Categories	Univariate		Multivariate[a]	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Glomerular classification	1.135 (0.736–1.752)	0.566		
IFTA	1.734 (1.151–2.612)	0.008	1.403 (0.902–2.182)	0.133
Interstitial inflammation	2.052 (1.168–3.605)	0.012	1.457 (0.824–2.576)	0.195
Arteriolar hyalinosis	1.076 (0.511–2.267)	0.847		
Arteriosclerosis	1.282 (0.805–2.044)	0.296		
Macrophage infiltration[b]	2.430 (1.115–5.295)	0.025	2.503 (0.809–7.750)	0.111
Urinary PCR[c]	1.059 (0.700–1.602)	0.786		
MCP-1[c]	1.462 (0.990–1.599)	0.056		
CXCL16[c]	2.123 (1.369–3.291)	0.001	1.010 (0.542–1.885)	0.974
Endostatin[c]	2.581 (1.633–4.079)	<0.001	1.377 (0.657–2.886)	0.396
CXCL16 + endostatin[c]	3.058 (1.907–4.903)	<0.001	1.923 (1.070–3.455)	0.029

[a]Adjusted by age, sex, eGFR, the amount of proteinuria and the presence of hypertension.
[b]HR for each increase in tertile of the density of tubulointerstitial macrophage infiltration.
[c]HR for each increase in tertile.
HR, hazard ratio; CI, confidence interval.

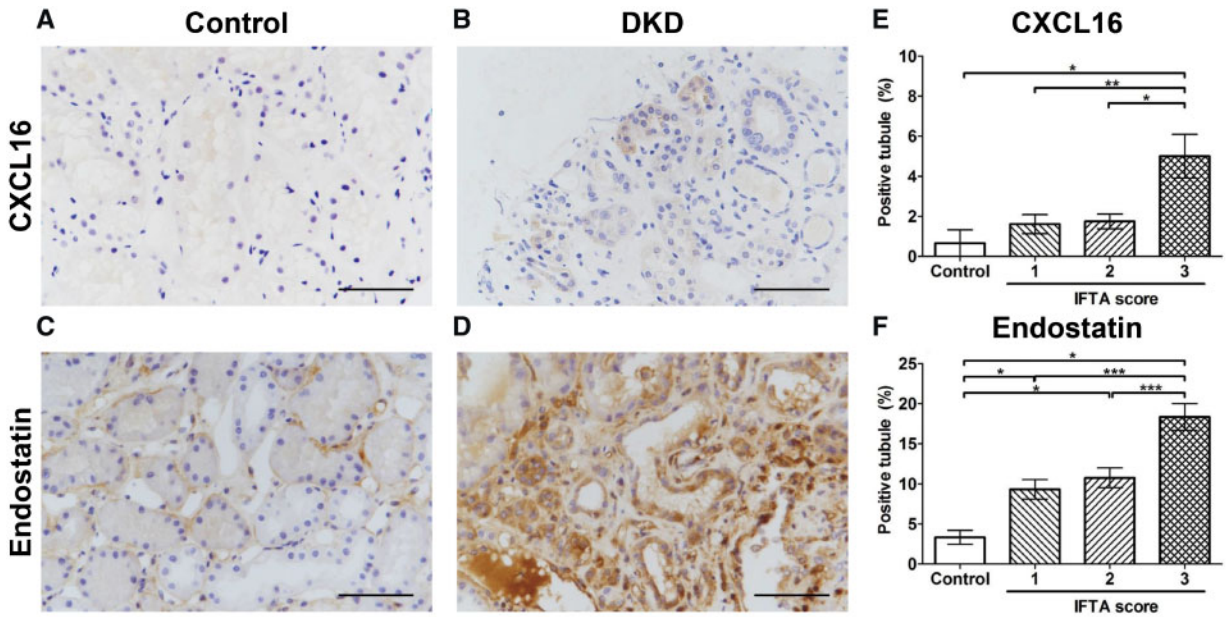

FIGURE 4: Representative immunohistochemical staining of CXCL16 and endostatin. Normal tissues obtained from patients with clear cell renal cell carcinoma were selected as controls. (**A** and **B**) CXCL16 expression in controls and DKD. (**C** and **D**) Endostatin expression in controls and DKD. (**E** and **F**) The percentage of CXCL16 and endostatin-positive renal tubular cells in controls and patients with different IFTA scores. *P<0.05, **P<0.01, ***P<0.001. Scale bars: (A–D) 50 μm, (E and F) data are expressed mean ± standard error.

inhibitor, as its name implies [45]. Renal tubular cells are known to secrete endostatin in response to both proinflammatory and profibrotic stimuli [38, 46] and the overexpression of endostatin induces tubulointerstitial fibrosis [46]. Several clinical studies have also shown that the levels of circulating endostatin were higher [47, 48] and were associated with rapid renal progression [26, 49] in various CKD populations. Interestingly, a very recent study revealed that higher levels of plasma endostatin were strong predictors of nonrecovery after acute kidney injury, showing its negative impact on the regenerative capacity of the kidney [50].

The limitations of this study should be mentioned. The number of enrolled patients was relatively small, and most patients enrolled in our study had advanced kidney disease. Therefore our results could not be generalized to entire DKD populations. Whether urinary CXCL16 and endostatin can be useful markers in early diabetic patients should be elucidated in further studies. Additionally, we could not obtain data regarding changes in the levels of urinary inflammatory markers over the clinical course of the patients.

In conclusion, our study demonstrated that urinary CXCL16 and endostatin could reflect the degree of IFTA and serve as biomarkers of renal outcome in patients with advanced DKD. We expect that further prospective trials will confirm whether these urinary markers could guide renal prognosis in clinical practice.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

Y.H.L. drafted the article and performed the analysis and interpretation of the data. Y.G.K., J.Y.M., S.W.J., J.S.K., K.H.J., S.Y.L., D.H.Y., J.T.W., S.Y.L., S.C., H.Y.C., H.C.P., Y.I.J., J.H.P. and S.W.H. were responsible for analysis and interpretation of the work. K.P.K., S.H.P., D.J.K. and S.J.L. performed the pathologic data analysis and interpretation. S.H.L. was responsible for the study concept and design and analysis and interpretation of the data.

CONFLICT OF INTEREST STATEMENT

None declared.

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Targeted broad-based genetic testing by next-generation sequencing informs diagnosis and facilitates management in patients with kidney diseases

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ABSTRACT

Background. The clinical diagnosis of genetic renal diseases may be limited by the overlapping spectrum of manifestations between diseases or by the advancement of disease where clues to the original process are absent. The objective of this study was to determine whether genetic testing informs diagnosis and facilitates management of kidney disease patients.

Methods. We developed a comprehensive genetic testing panel (KidneySeq) to evaluate patients with various phenotypes including cystic diseases, congenital anomalies of the kidney and urinary tract (CAKUT), tubulointerstitial diseases, transport disorders and glomerular diseases. We evaluated this panel in 127 consecutive patients ranging in age from newborns to 81 years who had samples sent in for genetic testing.

Results. The performance of the sequencing pipeline for single-nucleotide variants was validated using CEPH (Centre de'Etude du Polymorphism) controls and for indels using Genome-in-a-Bottle. To test the reliability of the copy number variant (CNV) analysis, positive samples were re-sequenced and analyzed. For patient samples, a multidisciplinary review board interpreted genetic results in the context of clinical data. A genetic diagnosis was made in 54 (43%) patients and ranged from 54% for CAKUT, 53% for ciliopathies/tubulointerstitial diseases, 45% for transport disorders to 33% for glomerulopathies. Pathogenic and likely pathogenic variants included 46% missense, 11% nonsense, 6% splice site variants, 23% insertion–deletions and 14% CNVs. In 13 cases, the genetic result changed the clinical diagnosis.

Conclusion. Broad genetic testing should be considered in the evaluation of renal patients as it complements other tests and provides insight into the underlying disease and its management.