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Tissue expression of tubular injury markers is associated with renal function decline in diabetic nephropathy

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ABSTRACT

Aims: The pathogenesis of diabetic kidney disease (DKD) is complex and multifactorial; increasing evidence suggests that tubular injury and inflammatory process are involved in disease progression. We investigated the potential association of renal expression of tubular injury markers, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and inflammatory markers, tumor necrosis factor receptor (TNFR) 1 and 2 with renal progression in pathologically proven diabetic nephropathy (DN).
Methods: We identified 122 patients with confirmed DN. After excluding patients with other coexisting renal disease or estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2, 35 patients were included. Annual decline of (GFR decline slope) was calculated using linear regression analysis. Tissue tubular and glomerular expressions of NGAL, KIM-1, TNFR1, and TNFR2 were assessed using immunohistochemistry.
Results: Median baseline urinary protein to creatinine ratio (uPCR) was 6.76 (2.18–7.61) mg/mg Cr, median baseline eGFR was 50 (43–66) mL/min per 1.73 m^2, and median GFR decline slope was 15.6 (4.4–35.1) mL/min per 1.73 m^2 per year. Positive correlations were observed between tubular expressions of NGAL and KIM-1, and GFR decline slopes ($r = 0.601$, $p < 0.001$; $r = 0.516$, $p = 0.001$, respectively), and between tubular expressions of KIM-1 and uPCR ($r = 0.596$, $p < 0.001$), and between NGAL and interstitial fibrosis and tubular atrophy (IFTA) score ($r = 0.391$, $p = 0.024$). No correlations were found between glomerular or tubular expressions of TNFRs, and clinical parameters including GFR decline slopes. On multivariate analysis, the association between tubular expressions of KIM-1 and GFR decline slopes was dependent on uPCR. Tubular expressions of NGAL were independently associated with GFR decline slopes, with an adjusted coefficient factor of 0.290 (95% confidence interval, 0.009–0.202, $p = 0.038$).
Conclusions: These findings suggest that tubular injury plays a key role in the pathogenesis of DKD in high-risk patients. Further studies are warranted to determine whether tubular injury could be a therapeutic target in DKD.

1. Introduction

Despite major implementation of renoprotective treatment in recent decades, diabetic kidney disease (DKD) continues to rank as the leading cause of end-stage renal disease (ESRD). Paradoxically, improvements in cardiovascular outcomes in patients with diabetes have allowed ample time for the development of kidney dysfunction and ESRD.[1] Therefore, prevention and delay of DKD progression is becoming increasingly important to reduce public health burdens. Susceptibilities to DKD and outcomes are highly variable. The amount of proteinuria, elevated blood pressure (BP), decreased kidney function, hyperglycemia, and episodes of acute kidney injury (AKI) have been identified as risk factors for the progression of DKD.[2–10]

Classically, glomeruli have been considered the primary injury site for diabetic nephropathy (DN), and albuminuria, which reflects glomerular damage, has been a key prognostic marker. Recently, several biomarkers related to inflammation or tubular injury have been identified as potent predictors of renal outcome. Circulating tumor necrosis factor receptor (TNFR) 1 and 2 have been reported to be strongly associated with subsequent progression to ESRD in patients with diabetes.[11,12] Furthermore, urinary and plasma levels of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) may function as early markers of DN.[13–16]

Conflict of Interest Statement: All authors declare that they have no conflict of interest.

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Accumulating evidence indicates that the inflammatory process and tubular damage are early events in the course of DN and are not merely secondary to glomerular damage.[17–19]

In the present study, we investigated whether renal expression of NGAL, KIM-1, TNFR1, and TNFR2 predict subsequent decline of kidney function in subjects with pathologically proven DN.

2. Materials and methods

2.1. Population

We identified 122 patients with diabetes mellitus (DM) who underwent renal biopsy and were confirmed to have DN between January 2000 and December 2014 at a 2000-bed tertiary referral center in Seoul, Korea. We excluded subjects with other coexisting renal disease based on pathologic findings (other types of glomerulonephritis, acute tubular necrosis, or acute interstitial nephritis) (n = 24), those with estimated GFR (eGFR) <30 mL/min/1.73 m^2 at the time of biopsy (n = 54), those who received treatment with immunosuppressants (n = 2), and those who underwent follow-up for <-6 months or <3 visits after renal biopsy (n = 7). Finally, 35 subjects were included in the analyses. All included subjects had provided written informed consent at the time of biopsy for the collection and use of their remnant biopsy specimens, and serum and urinary samples for research regarding biomarkers. This study was approved by the institutional review board of Samsung Medical Center. As this study was retrospective and the subjects were de-identified, the institutional review board waived the need for additional written consent from the subjects.

2.2. Clinical and laboratory data

Data regarding age, sex, duration and type of diabetes, presence of diabetic retinopathy, anti-hypertensive treatment including angiotensin-converting enzyme inhibitor (ACE-I) or angiotensin receptor blocker (ARB), body mass index (BMI), smoking status, and systolic and diastolic (BP) at the time of renal biopsy were obtained from electronic medical records of the patients. Hemoglobin A1c (HbA1c) levels at the time of renal biopsy were also obtained. The average of the urinary protein to creatinine ratio (uPCR) in spot urine samples obtained during the 6 months after renal biopsy was used as the baseline time-averaged uPCR. The primary outcome was the GFR decline slope (in mL/min/1.73 m^2 per year). To estimate the GFR decline slopes for each patient, we collected all serum creatinine measurements from 3 months before renal biopsy to the day of the last follow-up. We then calculated eGFR using the Chronic Kidney Disease Epidemiology Collaboration formula.[20] Next, a model for time versus eGFR was created using a linear regression analysis, and the absolute values of the slope of the regression line were regarded as GFR decline slopes over time, with a positive value representing a decreasing trajectory of GFR.

2.3. Renal biopsy and pathologic classification

Kidney histology was retrospectively reviewed. The tissues had been obtained by needle biopsy, and the specimens had been processed for light microscopy and immunofluorescence, as well as for electron microscopy. Classification of DN and histological scoring were performed according to the criteria of Mooyaar et al.[21] Renal pathological findings were also included as categorical covariates: glomerular class (I, IIa, IIb, III, IV); interstitial fibrosis and tubular atrophy (IFTA) score (0, 1, 2, 3); interstitial inflammation score (0, 1, 2); arteriolar hyalinosis score (0, 1, 2); and arteriosclerosis score (0, 1, 2).

2.4. Immunohistochemical (IHC) study

Serial sections of formalin-fixed paraffin-embedded kidney tissues were prepared and placed on a microscope slide. After deparaffinization and rehydration, tissue sections were washed, followed by blocking of endogenous peroxidase through incubation with Dako REAL Peroxidase Blocking solution (DAKO, Seoul, South Korea) for 30 min at room temperature. In addition, tissues prepared for NGAL were incubated with avidin and biotin for 15 min. Then, all tissues were treated with Dako Protein Block Serum-Free agent (DAKO) overnight at 4 °C to prevent background staining. Subsequently, tissue sections were incubated in primary antibodies KIM-1 (Human TIM-1/KIM-1/HAVCR Ab; R&D Systems, Boston, MA, USA; 1:300), NGAL (Human Lipocalin-2/NGAL Antibody; R&D Systems; 1:100), and TNFR1 and TNFR2 (Anti-TNF Receptor I Ab and II Ab; Abcam, Cambridge, UK; 1:500 and 1:50, respectively) for 1 h at room temperature. Thereafter, the tissue sections were incubated with a secondary antibody (EnVision for KIM-1, TNFR1, and TNFR2; rabbit polyclonal antibody to rat I immunoglobulin G-biotin for NGAL) for 30 min. Then, streptavidin horseradish peroxidase (HRP) for tissues that reacted with NGAL antibody was added, along with 3,3′ diaminobenzidine (DAB) chromogenic for visualization in a color reaction, followed by hematoxylin dye. Brown staining showed positive results.

Staining levels were scored as follows. The IHC staining was evaluated by the percentage and intensity of positive tubular or glomerular cells. The percentage of positive cells was recorded as 0 (0–10%), 1 (11–25%), 2 (26–50%), 3 (51%–75%), or 4 (>75%). The staining intensity of positive cells was recorded as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). The total score (0 − 12) was calculated by multiplying the two parameters. Positive immunoreactivity for NGAL and KIM-1 was predominantly detected in renal tubules, while immunoreactivity for TNFR1 and TNFR2 was detected in both tubules and glomeruli. Therefore, the glomerular and tubular expressions of TNFRs were separately assessed.

2.5. Serum and urine NGAL and KIM-1 measurements

Serum NGAL (sNGAL), serum KIM-1 (sKIM-1), urinary creatinine, urinary NGAL (uNGAL), and urinary KIM-1 (uKIM-1) concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (NGAL: Lipocalin-2/NGAL, R&D Systems; KIM-1: TIM-1/KIM-1/HAVCR, R&D Systems), according to the manufacturer's instructions. Measurements were performed in duplicate and triplicate for serum and urine, respectively.

2.6. Statistical analyses

Data were expressed as percentages for categorical variables and as medians (interquartile ranges, IQRs) for continuous variables. As some variables (GFR decline slope, HbA1c, and uPCR) showed skewed distributions, log transformation was carried out before performing correlation and regression analysis. The Mann–Whitney rank sum test was used for comparisons of continuous variables, and Fisher's exact test was used for categorical variables. Correlations among continuous variables were assessed using the Spearman rank correlation coefficient. To investigate the variables that could predict rapid progression of DN, multivariate linear regression analysis was conducted to explore the association of GFR decline slopes with baseline clinical and histopathological variables, and tubular expression of NGAL and KIM-1 following univariate linear regression with $p < 0.2$, and the significant explanatory parameters were chosen in an enter stepwise manner. Results were expressed as the regression coefficient (β).

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