

Levels and Clinical Significances of Glypican-5 in Urine of Type 2 Diabetic Nephropathy Cases

Ruizhao Li,* Li Zhang,* Shu Zhang, Huan Yang, Bingfeng Yao, Wei Dong, Bin Zhang, Yuanhan Chen, Shuangxin Liu, Xingchen Zhao, Qianmei Zhang, Wei Shi, Xinling Liang

Division of Nephrology,
Guangdong Provincial
People's Hospital, Guangdong
Academy of Medical Sciences,
Guangzhou, China

*Ruizhao Li and Li Zhang have
contributed equally to this
study.

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Introduction. Increased glypican-5 expression in podocytes induces podocyte injury. Glypican-5 is shed into the urine, but its value in predicting progression of diabetic nephropathy (DN) has not been investigated.

Methods. Glypican-5 was determined in spot urine from 20 type 2 diabetes mellitus (T2DM) patients, and 37 type 2 DN patients, and 20 healthy controls by enzyme-linked immunosorbent assay. The association of urinary glypican-5 with markers of renal function was evaluated.

Results. Urinary glypican-5 was significantly higher in DN patients than in both DM patients and controls. Glypican-5 level was not associated with baseline 24-hour urine protein/albumin excretion, serum creatinine, estimated glomerular filtration rate (eGFR), systolic blood pressure (SBP), or hemoglobin (Hb) A1c values in the DN patients. After 52 weeks follow-up, urinary glypican-5 level was associated with significant increases in urine protein and albumin excretion and a significant decline in eGFR in the DN patients. The decline in eGFR was independent of changes in urine protein and albumin excretion, SBP, or HbA1c. The results indicate that urinary glypican-5 was not only a biomarker but also contributed to the pathogenesis of DN in these patients.

Conclusion. Urinary glypican-5 was specifically elevated in type 2 diabetes patients with DN and it was associated with disease progression. Urinary glypican-5 may serve as a useful non-invasive marker for the progression of DN.

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INTRODUCTION

The prevalence of diabetic nephropathy (DN) has increased dramatically around the world and has become a leading cause of end-stage renal disease (ESRD).¹ It is characterized clinically by progressively increasing albuminuria and decreasing glomerular filtration rate. Presently, effective treatment strategies to slow or even halt the progression of CKD are still limited.^{2,3} As a result, much effort has been devoted to understanding the mechanisms that may be involved in the

pathogenesis of renal injury as well as biomarkers in DN. Albuminuria in diabetic patients is commonly used as a non-invasive biomarker predicting the progression to ESRD,⁴ but not specific for DN and is highly variable within an individual.⁵ In addition, not all type 2 diabetic patients with albuminuria will develop progressive renal dysfunction.^{6,7} More sensitive and specific urinary biomarkers predicting the progression to ESRD might help treat type 2 diabetic patients with DN to prevent the progression to ESRD. Podocytes are terminally differentiated

cells reside on the outer surface of the glomerular basement membrane (GBM) and play a key role in maintaining the structure and function of the glomerular filtration barrier. There is evidence that glomerular podocytes are involved in the pathogenesis of DN,^{8,9} including apoptosis, and the loss of podocytes in T2DM patients.¹⁰ Podocytes are located on the urinary lumen side of the GBM, making urine a logical place to look for markers of podocyte injury and for the quantitation of podocyte damage in urinary sediment.¹¹⁻¹³ However, urinary assay of podocyte damage is difficult and the results may not be associated with the progression of renal injury.^{14,15} The use of glypican-5, a podocyte cell surface heparan sulfate proteoglycan, may overcome some of the shortcomings of previous urinary markers of DN progression.¹⁶ Glypican-5 is a co-receptor or modulator of the activity of heparin-dependent growth and adhesion factors.¹⁷ It is expressed predominantly in podocytes, is released depending on intraglomerular conditions. It enhances basic fibroblast growth factor (FGF) 2 signaling, influences albumin permeability by its effects on podocyte differentiation and function, and increases the susceptibility of the diabetic kidney to nephrotic damage.¹⁸ Urinary glypican-5 is presumed to originate in the podocytes because podocyte-specific GPC5 knockdown mice had the minimally detectable urine Gpc5 protein concentration compared with wild-type mice.¹⁶ This study evaluated urinary glypican-5 as a biomarker of renal injury progression. Glypican-5 expression was assayed in urine obtained from T2DM patients and healthy controls, and the association of urinary glypican-5 expression and renal function, including urinary protein/albumin excretion and eGFR, was evaluated.

MATERIALS AND METHODS

Study Participants

Fifty-seven T2DM patients diagnosed by 1999 World Health Organization study group criteria and with normoalbuminuria (urinary albumin excretion < 30 mg/24h) or macro albuminuria (urinary albumin excretion < 300 mg/24 h), and 20 healthy volunteers were enrolled at Guangdong general hospital between October 2008 and June 2011. Eligible patients were 18-70 years of age with serum creatinine \leq 265.2 μ mol/L and not treated with angiotensin converting enzyme inhibitors

(ACEIs) or angiotensin receptor blockers (ARBs) within the previous 4 weeks. Patients with type 1 diabetes, bilateral renal-artery stenosis, coronary heart disease, cardiomyopathy, serious arrhythmia, cerebrovascular disease, urinary tract infection, or acute or severe chronic liver disease were excluded. The investigators interviewed patients and volunteers individually, and all participants gave written informed consent.

The 37 type 2 diabetes patients with macro albuminuria began treatment with valsartan 160 mg/d on enrolment. Patients with serum potassium > 5.5 mmol/L, a > 30% increase in serum creatinine, or intolerable adverse effects related to valsartan therapy after 4 weeks were to be excluded. No patient had intolerable adverse effects, and all complied with the treatment and were followed-up every 12 weeks for 52 weeks. During follow-up, patients ate a protein-restricted diet limited to 0.8 g/kg/d and a total energy intake 30 kcal/kg/d. A mild dietary sodium restriction of \leq 90 mmol/d was advised. Blood glucose was controlled by sulfonylureas (glibenclamide and tolbutamide), α -glucosidase, pioglitazone, and insulin. Blood pressure was controlled by α -adrenergic and β -adrenergic antagonists, diuretics, and calcium antagonists if necessary, and without ACEIs or ARBs. The blood pressure and laboratory data of patients at the end of the follow-up period or through their last follow-up visit were recorded and included in the analysis.

Patient Monitoring and Sample Collection

Physical examinations and laboratory evaluations were performed. Urine samples were centrifuged at 3,000 g for 10 min at room temperature to remove cell debris. Venous blood samples were collected after a 12-h fast and the serum was separated by centrifugation at 4°C at 4000 g for 10 min. Both urine and blood samples were stored at -80 °C until assayed. Blood pressure (BP), serum creatinine (Scr), serum glycated hemoglobin (HbA1c), 24-hour urine albumin, 24-hour urine protein, and estimated glomerular filtration rate (eGFR) calculated by the modification of diet in renal disease (MDRD) equation²³ were performed at baseline and at each study and follow-up visit.

Urinary Glypican-5 Concentration

Glypican-5 concentration was determined

using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Groundwork Biotechnology Diagnostics, Campanile Drive San Diego, CA, USA) following the manufacturer's instructions. In brief, the assay was a three-step procedure conducted in 96-well polystyrene microplates precoated with mouse monoclonal antibody against glypican-5. Urine samples and horseradish peroxidase-conjugated polyclonal antibodies against glypican-5 were added to each well, mixed, and incubated for 1 hour at 37 °C. After incubation and washing, substrate solution was added to each well and incubated for 15 minutes at room temperature in the dark. Stop solution was added to each well and the absorbance was read at 450 nm using an ELISA plate reader. The glypican-5 concentration of each sample was calculated using Curve Expert 1.3. Duplicate measurements were obtained for all samples. The lower limit of detection was 0.1 ng/mL, and the glypican-5 level was expressed as a ratio relative to the creatinine concentration (ng/g creatinine).

Statistical Analysis

Continuous variables were expressed as means \pm standard deviation, and were compared by the t-test or analysis of variance (ANOVA), or Mann–Whitney rank sum test as appropriate. Categorical variables were reported as numbers and percentages, and were compared using the chi-squared test. ANOVA was used to compare baseline parameters. Student's t-test was used to compare baseline and follow-up values. Spearman rank order correlation analysis was used to determine the significance of the relationships of experimental variables. All statistical tests were two-sided, with $P < .05$ considered significant. The

statistical analysis was performed using SPSS for Windows version 17 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Participant Characteristics

The characteristics of the T2DM patients and healthy controls are shown in Table 1. Diabetes patients with microalbuminuria were enrolled in the DN group and those with normoalbuminuria were enrolled in the DM group. The HbA1c in the DM ($8.23 \pm 1.63\%$) and DN ($8.11 \pm 2.14\%$) group were not significantly different ($P > .05$), but both were significantly higher than in the healthy controls ($5.53 \pm 0.24\%$), both $P < .001$. Scr ($132.01 \pm 49.86 \mu\text{mol/L}$ vs. $71.73 \pm 16.59 \mu\text{mol/L}$; and $132.01 \pm 49.86 \mu\text{mol/L}$ vs. $66.27 \pm 14.16 \mu\text{mol/L}$) was significantly higher, and eGFR ($55.52 \pm 27.94 \text{ mL/min } 1.73\text{m}^2$ vs. $91.31 \pm 21.41 \text{ mL/min } 1.73\text{m}^2$; and $55.52 \pm 27.94 \text{ mL/min } 1.73\text{m}^2$ vs. $94.09 \pm 14.13 \text{ mL/min } 1.73\text{m}^2$), were significantly lower in the DN group than in DM group and healthy controls, respectively (all $P < .001$). DN group had significantly higher 24-hour urine protein excretion (2688.00 mg (1330.50 mg , 4119.00 mg) vs. 54.25 mg (36.98 mg , 78.15 mg), $P < .001$) and albumin excretion (1818.00 mg (1102.00 mg , 3411.50 mg) vs. 8.45 mg (4.20 mg , 16.13 mg), $P < .001$) relative to DM group. The age ($P > .05$) and sex ratios ($P > .05$) of the three groups were not significantly different.

Urinary glypican-5 was higher in DN than in DM patients and healthy controls. The glypican-5 concentration in spot urine was determined not only by the amount released from podocytes but also by the urine flow rate. Therefore, urinary glypican-5 was normalized by the creatinine concentration to correct for variations in the flow rate, as is done with other urinary biomarkers. Glypican-5 levels, reported as the ratio of urinary glypican-5

Table 1. Baseline Clinical Characteristics.

Indices	Healthy Control Group	DM Group	DN Group
N	20	20	37
Sex (male/female)	10/10	10/10	21/16
Age, y	56.00 ± 13.30	56.65 ± 13.69	55.27 ± 9.34
Body Weight (kg)	64.89 ± 8.62	67.51 ± 9.00	63.30 ± 10.78
Body-mass Index (kg/m ²)	22.42 ± 1.99	$24.68 \pm 2.20^*$	$24.06 \pm 2.92^*$
Systolic Blood Pressure (mmHg)	125.95 ± 11.61	$128.50 \pm 11.76^*$	$137.41 \pm 20.57^*$
Diastolic Blood Pressure (mmHg)	68.05 ± 6.68	70.70 ± 10.64	$78.51 \pm 10.07^{*\#}$

Data presented as mean \pm standard deviation of the means. Categorical variables were described as numbers.

A value of $P < .05$ was considered significant.

*indicate significant difference relative to healthy controls group.

#indicate significant difference relative to DM group.

Table 2. Baseline Laboratory Profiles

Indices	Healthy Group (n = 20)	DM Group (n = 20)	DN Group (n = 37)
FBG (mmol/L)	5.17 ± 0.58	7.71 ± 3.59*	9.02 ± 4.73*
HbA1c (%)	5.53 ± 0.24	8.23 ± 1.63*	8.11 ± 2.14*
Total Cholesterol (mmol/L)	4.58 ± 0.77	6.02 ± 1.39*	5.83 ± 2.01*
HDL (mmol/L)	1.37 ± 0.36	1.17 ± 0.33	1.39 ± 0.64
LDL (mmol/L)	2.63 ± 0.72	3.45 ± 0.96*	3.07 ± 1.06
TG (mmol/L)	1.07 ± 0.40	2.63 ± 1.99	2.29 ± 2.91
BUN (mmol/L)	4.05 ± 1.33	5.14 ± 1.58	8.32 ± 3.98*#
Serum Creatinine (μmol/L)	66.27 ± 14.16	71.73 ± 16.59	132.01 ± 49.86*#
eGFR (mL/min 1.73m ²)	94.09 ± 14.13	91.31 ± 21.41	55.52 ± 27.94*#
24-hour Urine Quantitative Albumin (mg) ^{&}	8.50 (4.55, 19.13)	8.45 (4.20, 16.13)	1818 (1102, 3411.5)*#
24-hour Urine Quantitative Protein (mg) ^{&}	53.20 (41.35,76.53)	54.25 (36.98,78.15)	2688.00 (1330.50,4119.00)*#

Data presented as mean ± standard deviation of the means.

[&]Non-normal distribution, Data presented as median (25% percentiles,75% percentiles).

A value of $P < .05$ was considered significant.

*indicate significant difference relative to healthy group.

#indicate significant difference relative to DM group.

concentration to urinary creatinine concentration, are shown in Figure 1. The glypican-5 level was significantly higher in the DN (2.37 ± 1.46 ng/g) than in the DM (1.55 ± 0.46 ng/g) group ($P < .05$) and the healthy controls (1.29 ± 0.39 ng/g, $P < 0.001$). The glypican-5 levels in the DM group (1.55 ± 0.46 ng/g) and the healthy controls (1.29 ± 0.39 ng/g) were not significantly different ($P > .05$).

Association of glypican-5 level, clinical, and laboratory variables of DN patients. As shown in Figure 2, the urinary glypican-5 level was not significantly correlated with either 24-hour urine protein ($r = -0.099$, $P > .05$) or albumin ($r = -0.145$, $P > .05$) excretion in DN patients. Significant correlations of glypican-5 level and Scr ($r = -0.126$,

$P > .05$), eGFR ($r = 0.04$, $P > .05$), SBP ($r = 0.048$, $P > .05$), or HbA1c ($r = 0.002$, $P > .05$) were also not observed.

Urinary glypican-5 level predicted the progression of renal injury in DN patients.

Clinical and laboratory follow-up was collected from 37 DN patients for a period of 52 weeks. The progression of renal injury was monitored by 24-hour urine protein and albumin excretion and eGFR. As shown in Figure 3C-D, Scr significantly increased in DN patients from $132.01 \pm 49.86 \mu\text{mol/l}$ to $162.46 \pm 73.02 \mu\text{mol/l}$, $P < 0.001$ and eGFR significantly declined from 46.13 ± 26.08 to 55.52 ± 27.94 ml/min/1.73m², $P < 0.001$ over the follow-up period. The changes in 24-hour urine protein excretion from 4886.52 ± 4561.20 to 3825.03 ± 3880.02 mg/day ($P = 0.1$, Figure 3A) and 24-hour urine albumin excretion from 3489.66 ± 3212.86 to 2582.63 ± 2588.04 mg/day ($P = 0.083$, Figure 3B) were not significant.

There was a significant negative correlation of change in the glypican-5 level and eGFR (Figure 4A, $r = -0.786$, $P < 0.001$), and a significant positive correlation of change in glypican-5 level and 24 hour urine protein ($r = 0.33$, $P = 0.046$) and albumin ($r = 0.346$, $P = 0.027$) excretion (Figure 4B, C). No significant correlations were observed between changes of 24-hour urine protein ($r = -0.31$, $P = 0.062$) or albumin ($r = -0.304$, $P = 0.067$) excretion and eGFR (Figure 5A, B). There were also no significant correlations between change of SBP ($r = 0.18$, $P = 0.286$) or HbA1c ($r = -0.05$, $P = 0.769$) and eGFR (Figure 5C, D).

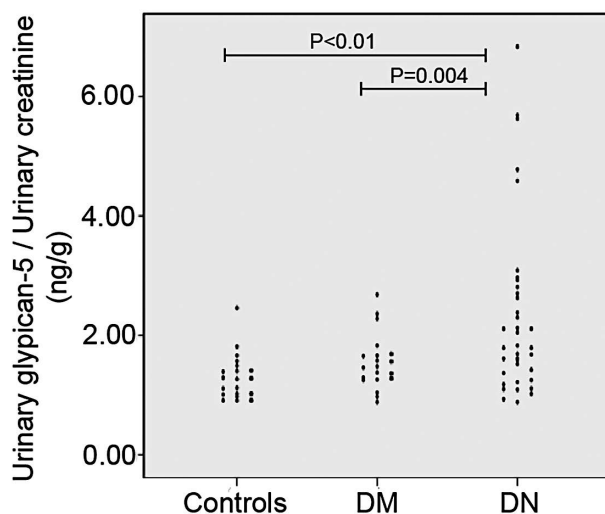


Figure 1. Level of urinary glypican-5 in DN patients, DM patients and healthy controls

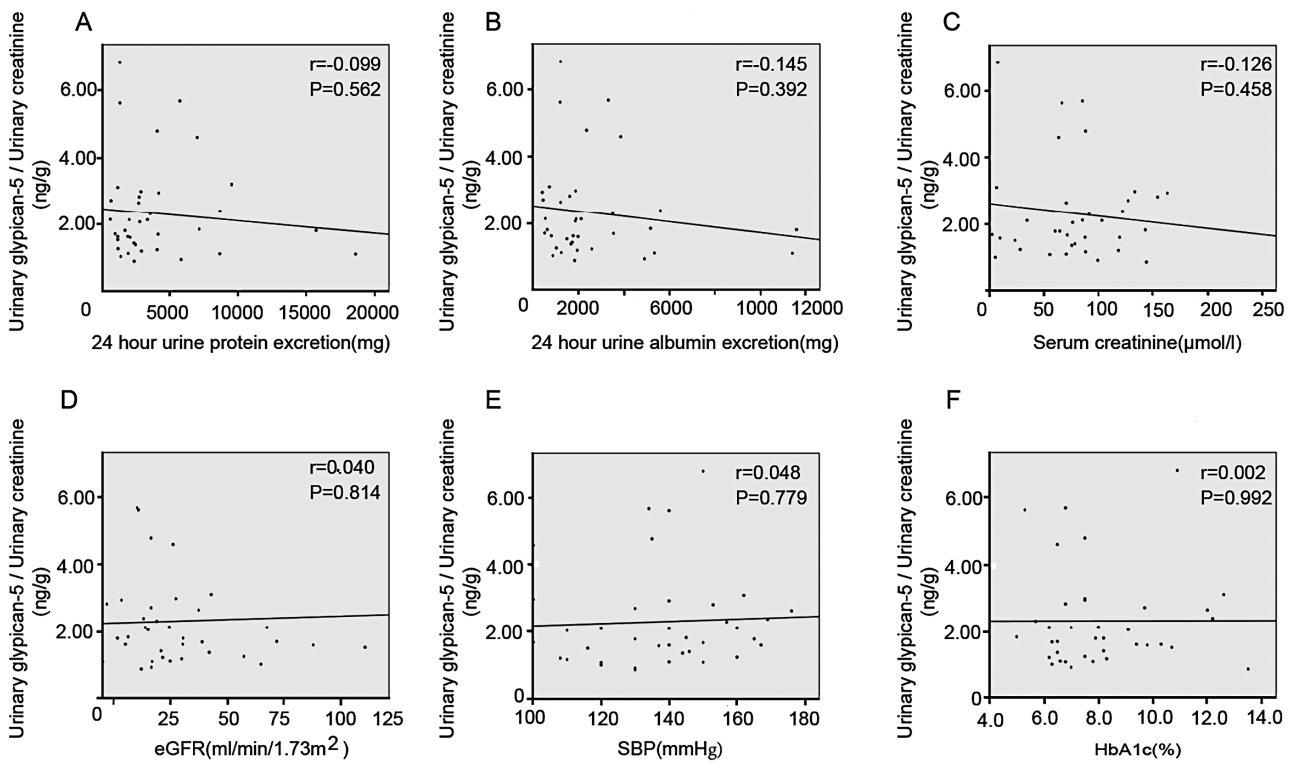


Figure 2. The associations between urinary glypican-5 levels and clinical, laboratory data of DN patients

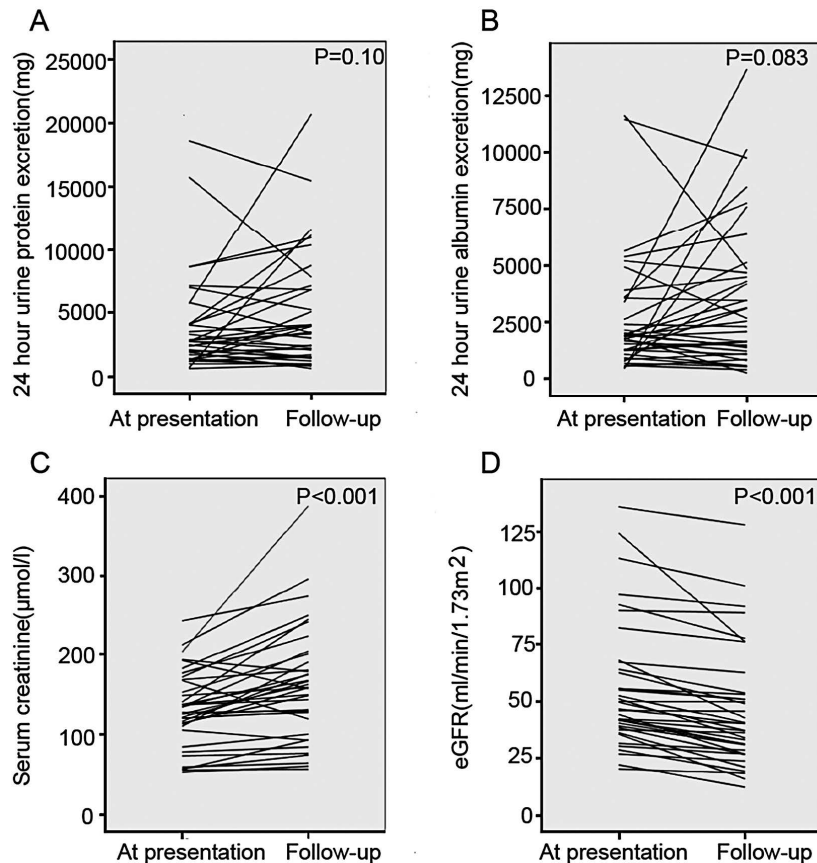


Figure 3. Change of 24 hour urine protein/albumin excretion, serum creatinine and eGFR in DN patients at the 52 weeks follow up compared with that at presentation

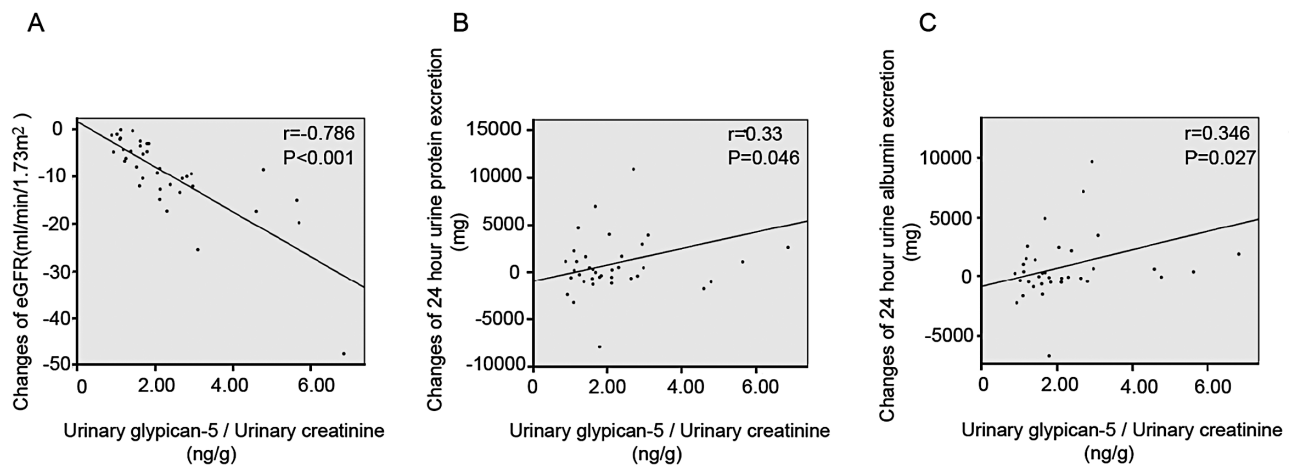


Figure 4. The associations between urinary glypican-5 levels and changes of eGFR, 24-hour urine protein / albumin excretion in DN patients

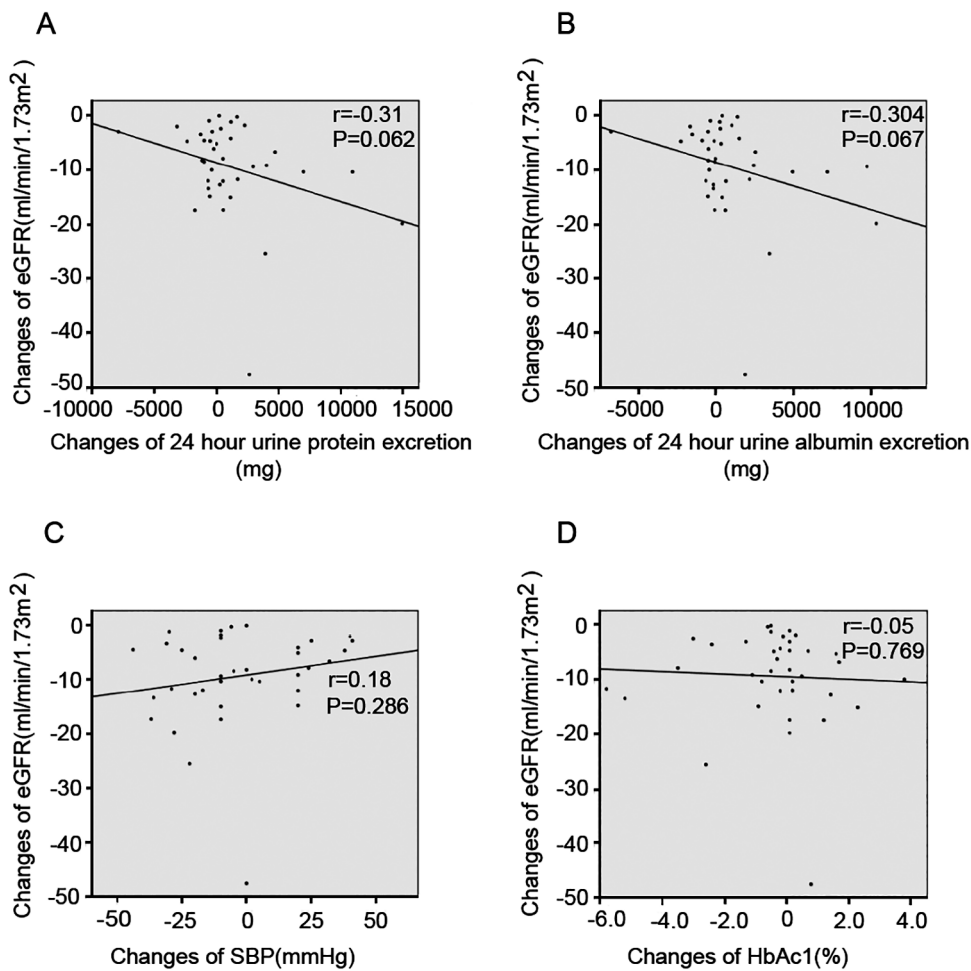


Figure 5. The associations between changes of eGFR and the changes of 24-hour urine protein / albumin excretion, SBP or HbA1c in DN patients

DISCUSSION

In the current study, urinary glypican-5 was assayed in T 2DM patients and healthy controls

and found to be significantly higher in DN patients than in both healthy controls and patients without nephropathy. The glypican-5 level in DN patients

at study enrolment was not correlated with baseline SBP, HbA_{1c}, SCr, 24 hour urine protein or albumin excretion, or eGFR. However, during the follow-up, it was observed that high urinary glypican-5 levels at presentation were significantly correlated with a rapid rise of 24-hour urine protein and albumin excretion and decline of eGFR, two important features of DN. Further analysis shown that the association of urinary glypican-5 and decline in eGFR was independent of changes of SBP, HbA_{1c}, and 24-hour urine protein or albumin excretion. These data indicates that urinary glypican-5 might not only be a biomarker but also probably a pathogenic contributor to the pathogenesis of DN in T2DM.

DN is the leading cause of renal failure worldwide, and despite the use of novel treatments such as ARBs, the risk of ESRD remains high.²² Albuminuria and GFR are recommended as clinical markers of DN^{23,24} because urine albumin excretion increases and GFR declines with progression to ESRD.²⁵ Glomerular injury is central to the pathogenesis of DN,²⁶ and podocyte injury and reduction in podocyte number and density have been linked to increases in urine albumin excretion and a decline in GFR. Loss of podocytes thus appears to play a key role in the progression of glomerular disease and the development of glomerular sclerosis.²⁷ Podocytes can be found not only in the urine of patients with glomerular disease but also in the urine of healthy people. The number of damaged podocytes in the urine has been associated with the development of glomerular sclerosis and loss of renal function,¹² but quantitation is not only difficult, but changes in podocyte number may not accurately reflect loss of renal function.^{14,15} There is evidence that increasing amounts of podocyte-associated synaptopodin, podocalyxin, CD2-AP, a-actinin 4, and podocin mRNA present in urine is associated with the pathogenesis and progression of DN.¹³ Podocyte-associated mRNAs might be useful for monitoring the progression of kidney disease, but do not address the initial causes of podocyte injury, only the existence of damaged podocytes. High glypican-5 expression induces and amplifies podocyte injury.¹⁶ As glypican-5 is expressed predominantly in podocytes, located in the cell surface membrane, and released into the urine depending on intraglomerular conditions,¹⁶ it and some other urinary factors,^{13,14} constitute

a novel class of biomarkers of renal disease. This study evaluated the use of urinary glypican-5 as a noninvasive biomarker to predict the progression of renal function decline in T2DM patients with DN. Higher glypican-5 levels were observed in patients with DN than those without it. Urinary glypican-5 was negatively correlated with changes of eGFR in DN, but positively correlated with the changes of 24-hour urine protein and albumin excretion. Podocyte damage resulting from exposure to hyperglycemic filtrate could account for the appearance of glypican-5 in the urine as well as the loss of podocyte markers, including glypican-5 as previously reported in renal biopsy tissue from DN patients.¹⁶ FGF2 signaling regulates actin dynamics and cell morphology and function in podocytes and it promotes podocyte damage and proteinuria in both experimental and human DN.^{18,28} Glypicans bind FGF2 and are necessary for FGF2 signal transduction,²⁹ and a high level of glypican-5 enhances FGF2 signaling, induces podocyte injury, and causes albuminuria.^{16,18}

Albuminuria is known to be a marker of kidney damage and a predictor of DN progression.^{26,30} Microalbuminuria occurs in 20% to 30% of T2DM patients at the time of diagnosis. Further kidney involvement is indicated by the development of macro albuminuria, which occurs in approximately 3% of T2DM patients with microalbuminuria each year. The change in eGFR that occurs over time is highly dependent on the degree of underlying albuminuria; consequently, patients with macro albuminuria are at risk of an accelerated decline in GFR.^{31,32} In this patient series, high urinary glypican-5 levels at presentation were associated not only with increasing urinary protein and albumin excretion but also with declining eGFR. However, the changes of urinary protein or albumin excretion observed on follow-up were not correlated with the decline in eGFR. The results indicate that urinary glypican-5 levels were an earlier maker of the progression of DN than urinary protein and albumin excretion. Previous reports found that achieving optimal glucose³³ or systolic blood pressure (SBP) control³⁴ were essential steps in successfully delaying the progression of DN. In these patients, there were no significant differences in the HbA_{1c} and SBP values at baseline and after 52 weeks of follow-up, and the decline in eGFR was not correlated with the changes of HbA_{1c}

or SBP. This further suggests that high urine glypican-5 is likely to contribute to an ongoing decrease in eGFR. The study limitations include short-term follow-up that may have weakened the association between eGFR decline and changes of urinary protein and albumin excretion. It was not designed to find a causal link between high urine glypican-5 and decline in eGFR. Finally, MDRD-based eGFR rather than direct measurement of GFR (mGFR) was used. However, mGFR using iothalamate clearance has its own limitations. It is subject to large daily individual variation that limits its value for estimating the slope of renal function decline. mGFR is also not available in many hospitals.

In conclusion, urinary glypican-5 was specifically elevated in type 2 diabetes patients with DN and it was associated with disease progression. Further research are necessary to investigate the direct mechanism of renal function rapid decline with high urinary glypican-5 and develop new therapies to reduce the risk of ESRD in type 2 diabetes patients with DN.

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AUTHORS' CONTRIBUTION

RZ designed the study, contributed to the database, performed the statistical analysis and drafted the manuscript. LZ conceived of the study, interpreted the data and drafted the manuscript. SZ and HY performed the Elisa assay. BY, WD and BZ collected urine and blood samples. YC, SL, XZ and QZ participated in clinical data collection and statistics. WS interpreted the data. XL edited the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare that they have no competing interests.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of ethics committee of Guangdong

General Hospital and with the 1964 Helsinki declaration and its later amendments.

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- Correspondence to:
 Xinling Liang, PhD
 Department of Nephrology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences,
 106 Zhongshan No. 2 Road, Guangzhou, 510080, China
 Email: xinlingliang_ggh@163.com
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