The Podocyte Count Detected by an Improved Immunocytochemical Method has Higher Diagnostic Efficiency than Enzyme-Linked Immunosorbent Assay and Serum Cystatin C to Evaluate the Early Stage Damage of Glomerular

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Background: In renal diseases, earlier injury to glomerular may lead to the abscission of podocyte. The number of podocyte in urine may reflect the severity of glomerular damage. Podocalyxin (PCX) was considered as a podocyte marker. Many methods were used to detect podocyte. Applications of these methods were limited by tricky, expensive, and low accuracy. Here, we improved an immunocytochemical method to count the number of podocyte in urine. **Methods:** In this study, we counted the numbers of podocyte in urine by our improved method and detected the PCX levels in urine by enzyme-linked immunosorbent assay (ELISA) in glomerulopathy patients and healthy controls. The serum levels of cystatin C (CysC), blood

urea nitrogen (BUN), creatinine (CR), uric acid (URIC), and β 2-Microglobulin (β 2-MG) in all subjects were detected. Correlation analysis and diagnostic efficiencies comparisons among immuocytochemical method, ELISA, and the biochemistry index were also performed. **Results:** The podocyte counts in patients were significantly higher than controls. Podocytes counts positively correlated with PCX concentrations and the serum CysC. The podocyte count had higher diagnostic efficiency than PCX concentrations detected by ELISA and serum CysC. **Conclusions:** The podocyte count detected by our improved method had higher diagnostic efficiency than ELISA and serum CysC. J. Clin. Lab. Anal. 31:e22029, 2017. © 2016 Wiley Periodicals, Inc.

Key words: cystatin C; diagnostic efficiency; enzyme-linked immunosorbent assay; glomerular damage; immuocytochemical stain; podocyte count

INTRODUCTION

Glomerular filtration barrier has a unique structure: the fenestrated capillary endothelium, glomerular basement membrane (GBM), and the slit diaphragm between podocyte foot. The podocyte covers the outer aspect of GBM to limit the leakage of proteins (1, 2). In renal diseases, earlier damage to the barrier may lead to the abscission of podocyte. In this case, podocyte appears in the urine. The number of podocyte in urine may reflect the severity of glomerular damage at early stage (3).

Podocalyxin (PCX) is a member of the sialomucin protein family. It was originally identified on podocyte

and was considered as a podocyte marker in many test methods. (2, 4, 5). A number of methods were used to detect podocyte, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA), Western blot, and flow cytometry (6). The application of

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immunofluorescence was limited because of the fluorescence quenching. Western blot could be time consuming and tricky. Absolute count by flow cytometry maybe high cost. ELISA, which detect the concentration of podocalyxin (PCX) in urinary sediment was a mature method but with low accuracy.

Serum cystatin C (CysC), blood urea nitrogen (BUN), creatinine (CR), uric acid (URIC), and β 2-Microglobulin (β 2-MG) are important parameters to evaluate the kidney function in clinical work. The CysC was considered to be the most sensitive biochemistry indicator of renal function (7).

Here, in our study, we improved an immunocytochemical method to count the number of podocyte in urine. To evaluate the diagnostic efficiency of our new method, we counted the numbers of podocyte in urine by our improved method and detected the PCX levels in urine by ELISA in glomerulopathy patients and healthy controls. We also detected the serum levels of CysC, BUN, CR, URIC, and β 2-MG in all subjects. Correlation analysis and receiver operating characteristic (ROC) curve were performed. The diagnostic efficiencies of our improved immunocytochemical method, ELISA, and the biochemistry index were compared.

MATERIALS AND METHODS

Subjects

Patients

A total of 45 patients (24 men and 21 women) with biopsy-proved glomerulopathy were recruited from

Provincial Hospital Affiliated to Shandong University from June 2015 to September 2015. The recruitments of patients were based on the results of renal biopsy before the immunosuppressive therapy. However, before definitive diagnosis, urinary tract infection, renal calculus, renal cyst, kidney neoplasm, and other systemic disease (such as hypertension, diabetes mellitus, and cardiopathy) must be excluded. Patients were subdivided into two groups as previously described (2): (a) 17 patients with proliferative forms of glomerulopathy (IgA nephropathy (IgA N)-11, lupus nephritis class IV (LN-IV)-6) and (b) 28 patients with nonproliferative forms glomerulopathy (membranous nephropathy (MN)-19, minimal change disease (MCD)-6, focal segmental glomerulosclerosis (FSGS)-2, lupus nephritis class V (LN-V) -1).

Controls

We recruited 20 healthy volunteers (10 men and 10 women) randomly from the Health Examination Center of Provincial Hospital Affiliated to Shandong University as a control group. None of the controls had any renal diseases or other systemic diseases.

Clinical features of all participants were shown in Table 1. This study was approved by the Ethical Committee of Shandong Provincial Hospital Affiliated to Shandong University. Informed consents were obtained from each participant to collect their urines and blood samples.

Reagents

Mouse anti-Human podocalyxin monoclonal antibody was obtained from R&D system. Mouse SP

TABLE 1. The Clinical Features of Patient and Control Groups

Clinical features	Control group	Patient group (<i>n</i> = 45)		<i>P</i> value ^b
		Proliferative group	Nonproliferative group	
<i>N</i>	20	17	28	
Gender (male/female)	(10/10)	(10/7)	(14/14)	
<i>P</i> value ^a		0.419 ^a	0.615 ^a	0.396 ^b
Age (years)	31.90 ± 6.41	28.12 ± 8.70	30.11 ± 6.420	
<i>P</i> value ^a		0.085 ^a	0.253 ^a	0.376 ^b
CysC (mg/l)	0.74 ± 0.09	1.63 ± 0.56	1.11 ± 0.45	
<i>P</i> value ^a		<0.001 ^a	0.001 ^a	0.001 ^b
β 2-MG (mg/l)	1.34 ± 0.25	2.99 ± 2.05	1.95 ± 0.86	
<i>P</i> value ^a		0.001 ^a	0.004 ^a	0.024 ^b
URIC (μ mol/l)	276.70 ± 79.46	398.31 ± 81.10	363.67 ± 78.22	
<i>P</i> value ^a		<0.001 ^a	0.001 ^a	0.174 ^b
BUN (mmol/l)	4.37 ± 0.60	6.40 ± 3.60	5.59 ± 2.84	
<i>P</i> value ^a		0.019 ^a	0.067 ^a	0.417 ^b
CR (μ mol/l)	79.64 ± 9.96	86.24 ± 21.04	72.90 ± 33.22	
<i>P</i> value ^a		0.222 ^a	0.386 ^a	0.157 ^b

CysC, cystatin C; β 2-MG, β 2-Microglobulin; BUN, blood urea nitrogen; CR, creatinine; URIC, uric acid.

^aData compared with control group.

^bComparison between proliferative and nonproliferative groups.

Immunohistochemistry kit and DAB kit were obtained from ZSGB Bio (Beijing, China). Human podocalyxin ELISA kit was purchased from Solarbio (China, Shanghai). Phosphate buffer solution (PBS) was from MXB (China, Fuzhou).

Urine Collection and Preparation

Clean midstream morning urine (about 100 ml) was obtained from the participants. Fifty-milliliter urine was centrifuged at $960 \times g$ for 5 min. The urine supernatant were collected and stored at -20°C until used. The sediment was mixed with the remaining 50 ml urine. The mixture was also centrifuged at $960 \times g$ for 5 min. After centrifugation, the sediment was used to prepare smears by a smear centrifuge at $300 \times g$ for 4 min. When the smears were dry, they were fixed with acetone at 4°C for 15 min. All the operations should be performed within 2 hr after the sample collection.

Immunocytochemical Staining

The smears of urine sediment were rehydrated with PBS for 3 min and permeabilized with 0.1% Triton X-100 for 5 min. Then washed smears with PBS three times for 3 min. Smears were treated with 3% hydrogen peroxide (H_2O_2) for 10 min to inactivate the endogenous peroxidases. Rinsed smears with distilled water (dH_2O) and dipped them in PBS for 5 min. Incubated smears with goat serum at room temperature for 15 min to block nonspecific binding. Discarded the blocking serum and incubated smears with diluted mouse anti-human PCX monoclonal antibody at the final concentration of $15 \mu\text{g/ml}$ at 37°C for 120 min. Negative controls were performed at the same time. Then washed smears three times in PBS for 5 min. Incubated smears with $200 \mu\text{l}$ biotin-labeled goat anti-mouse IgG antibody at 37°C for 15 min. Washed smears in PBS three times for 5 min. Incubated smears with horseradish peroxidase-labeled streptavidin for 15 min at room temperature. Washed smears in PBS three times for 5 min. Smears were incubated with DAB 10 min for coloration under the microscope. Then rinsed smears with PBS for 5 min. After that, stained smears with hematoxylin for 2 min and washed. Processed smears with 1% hydrochloric acid in ethylalcohol for 30 sec and 1% ammonia water for 30 sec. Then, smears were dehydrated with gradient alcohol and vitrified with dimethylbenzene. Finally, use neutral gum to seal the smears.

Podocyte counting was performed under the microscope according to the following principles: (a) five areas on each smear were chosen: upper right, upper

left, center, lower right, and lower left; (b) four high-power fields (10×40) in every area were examined; (c) only the holonomic podocyte was counted; and (d) the counting results were indicated as "X/20HP".

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of PCX in urine supernatant was detected by ELISA according to the manufacturer's protocol. Briefly, each well of ELISA plate was treated with $40 \mu\text{l}$ dilution and $10 \mu\text{l}$ urine supernatant or standard solution at 37°C for 30 min. After wash with washing buffer (0.1% Tween20, 0.9% NaCl), the wells were treated with conjugate solution (containing anti-mouse-kappa-POD and anti-mouse-lambda-POD from sheep) at room temperature for 2 hr. After that, at room temperature, wells were washed and treated with $50 \mu\text{l}$ substrate solution (1 mg/ml ABTS) for 1 hr. At the end of incubation, the absorbance at 405 nm was evaluated immediately.

Measurements of Serum

Blood urea nitrogen (BUN), creatinine (CR), uric acid (URIC), cystatin C (CysC), and $\beta 2$ -Microglobulin ($\beta 2$ -MG) in serum were detected. Measurements were performed on a Beckman AU5400 instrument (Beckman Coulter, Shang Hai, China) with dedicated reagents according to the instructions of the manufacturer.

Statistical Analysis

Statistical analysis was performed using SPSS (version 16.0, SPSS Inc., Chicago, IL). Data were expressed as mean $\pm$ SD. Statistical significance was analyzed by Student's *t*-test. Statistical significance was analyzed by Chi-square test. Correlation analysis was carried out by Pearson's correlation. Receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic efficiency. The differences of the area under the curve (AUC) were compared with Z-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

The Urine Podocytes Numbers of Patients, Detected by Immunocytochemical Staining, were Higher than that of Healthy Controls

Podocytes were found in all patients. Podocytes in urine under the microscope were shown in Figure 1. However, podocytes were not found in any of the controls. The positive rate of urine podocyte in patient

Fig. 1. The podocyte under the microscope detected by immunocytochemical stain. The podocyte under the microscope detected by improved immunocytochemical stain. Hematoxylin staining was used to display the podocytes. Original magnification $\times 400$. (A) Podocyte with single nucleus. (B) Podocyte with double nuclei.

was significantly higher than control ($P < 0.01$). Patients in proliferative group had higher counts of podocyte compared with patients in nonproliferative group ($P < 0.01$). The data were shown in Table 2.

The Concentrations of PCX in Urine of Patients were Higher than Controls

We detect the PCX concentrations in urine of patients and controls by ELISA. We found that the patients had a higher concentration of PCX than controls ($P < 0.05$). Otherwise, patients in proliferative group had higher concentrations of PCX than that of patients in nonproliferative group ($P < 0.01$). The data were shown in Table 2.

Analysis of the Clinical Features of All Participants

The main clinical features of all participants were shown in Table 1. The gender and age of patients and controls were matched without statistical differences. The levels of serum CysC, $\beta 2$ -MG, and URIC of patients were significant increased than those of controls. The concentrations of serum BUN in proliferative group were higher than controls. Moreover,

patients in proliferative group had increased levels of CysC and $\beta 2$ -MG compared to patients in nonproliferative group ($P < 0.05$).

Correlation Analysis

Urine podocyte counts detected by immunocytochemical staining displayed high-positive correlations with PCX concentrations detected by ELISA ($r = 0.899$, $P < 0.001$), serum levels of CysC ($r = 0.878$, $P < 0.001$), and $\beta 2$ -MG ($r = 0.266$, $P = 0.035$). Otherwise, the PCX levels detected by ELISA merely correlated with the serum concentrations of CysC ($r = 0.848$, $P < 0.001$). The data were shown in Table 3.

Nonparametric ROC Analysis of Urinary Podocyte Counts, PCX Levels, and Serum CysC in Patients

Nonparametric ROC analysis was performed to assess the diagnostic efficiency of urine podocytes counts, PCX levels, and serum CysC. The area under the curve (AUC) was compared. Data were shown in Table 4 and Figure 2. The AUCs of the three markers were 0.977, 0.821, and 0.890, respectively. All the three markers can effectively discriminate patients and

TABLE 2. Comparisons of Podocyte Counts and PCX Concentrations between Patient and Control Groups

	Control group	Patient group ($n = 45$)		<i>P</i> value
		Proliferative group	Nonproliferative group	
<i>N</i>	20	17	28	
Podocyte counts (/20HP)	0	20.35 $\pm$ 17.47	8.39 $\pm$ 1.92	
<i>P</i> value		<0.001 ^a	<0.001 ^a	0.006 ^b
PCX concentration (ng/ml)	5.99 $\pm$ 0.98	8.39 $\pm$ 1.92	6.81 $\pm$ 1.34	
<i>P</i> value		<0.001 ^a	0.025 ^a	0.002 ^b

^aData compared with control group.
^bComparison between proliferative and nonproliferative groups.

TABLE 3. Correlation between the Clinical Features and the Podocyte Counts and PCX Concentrations

		Podocyte counts	PCX concentration	CysC	β 2-M	URIC	BUN	CR
Podocyte counts	Pearson correlation		0.899	0.878	0.266	0.150	0.029	-0.133
	<i>P</i> value		<0.001	<0.001	0.035	0.240	0.823	0.299
PCX concentration	Pearson correlation	0.899		0.848	0.184	0.197	-0.007	-0.080
	<i>P</i> value	<0.001		<0.001	0.149	0.122	0.957	0.531

PCX, podocalyxin; CysC, cystatin C; β 2-MG, β 2-Microglobulin; BUN, blood urea nitrogen; CR, creatinine; URIC, uric acid.

TABLE 4. Diagnostic Efficiency Analysis of Urine Podocytes Counts, PCX Levels, and Serum CysC.

	AUC	95%CI	<i>P</i> value
Podocyte counts	0.978	0.906–0.999	<0.001
PCX concentration	0.821	0.706–0.905	<0.001
CysC	0.890	0.788–0.954	<0.001

PCX, podocalyxin; CysC, cystatin C; AUC, area under the curve; 95%CI, 95% confidence interval.

controls. But urine podocyte count measured by immunocytochemical method had significantly higher diagnostic efficiency than PCX concentration detected by ELISA ($P = 0.001$) and serum CysC ($P < 0.001$).

DISCUSSION

In our research, glomerulopathy patients were subdivided into two groups according to the renal biopsy, proliferative group, and nonproliferative group. We detected the podocytes counts by our improved immunocytochemical staining and PCX concentrations

by ELISA in urine of glomerulopathy patients and healthy controls. The serum levels of CysC, BUN, CR, URIC, and β 2-MG were also detected. The diagnostic efficiency comparison among podocytes counts, PCX concentrations, and CysC was also performed. Our data suggested that the podocytes counts in patients were significantly higher than controls. Podocytes counts positively correlated with PCX concentration and the serum CysC. The podocytes counts detected by our improved method have higher diagnostic efficiency than PCX concentrations detection by ELISA and serum CysC.

Podocytes detection usually carried out by Immunofluorescence (8). However, the application of immunofluorescence was limited because of the fluorescence quenching. Other methods, such as Western blot, absolute count by flow cytometry, and ELISA, could be time consuming, tricky, expensive, and low accuracy (9, 10). In our study, we improved an immunocytochemical staining to count the podocyte in urine. Improvements were as follows: (a) More urine: about 100 ml clean midstream morning urine was used. (b)

Fig. 2. Nonparametric receiver operating characteristic curves to assess the diagnostic efficiency of podocyte count, ELISA, and cystatin C.

More centrifuge: the urine was centrifuged twice and the smears were prepared by a smear centrifuge. (c) The smears were permeablized by 0.1% Triton X-100 to avoid the flake of cells. (d) Hematoxylin staining made the podocytes easy to recognize. (e) Negative control and positive control were set up. These improvements greatly improved the sensitivity and specificity of the immunocytochemical methods.

Podocytes counts by our improved immunocytochemical staining can effectively evaluate the early glomerular injury (11). It was also positively correlated with the level of serum CysC. Podocytes counts, as a noninvasive method, combined with serum CysC can be used for the diagnosis of early glomerular injury.

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