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Urinary Podocalyxin; a potential new marker for early diabetic nephropathy in type 2 diabetes mellitus

KEYWORDS: Diabetic nephropathy – Podocytes - Urinary podocalyxin

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ABSTRACT ***Aim:*** *Our aim was to study urinary podocalyxin (u-PCX), a podocytes specific protein, as an early marker for diabetic nephropathy.*

Methods: *A cross-sectional study included 116 patients with type 2 diabetes mellitus (T2DM)which were divided into three groups; group A, (normoalbuminuria), group B, (microalbuminuria) and group C, (macroalbuminuria) and 45 apparently healthy control subjects were included. Estimation of u-PCX/creatinine ratio, urinaryalbumin/creatinine ratio and urinary N-acetyl-β-D-glucosaminidase / creatinine ratio (u-NCR). Serum creatinine, HbA1c & estimated glomerular filtration rate (eGFR) were done.*

Results:*Diabetic patients had significant higher levels of u-PCX, albuminuria, and u-NCR (345.27±253.47 Vs 31.28 ±9.54 ng/mmol, 243.82± 373.67 Vs 8.42± 2.41mg/gm, 3.08±2.79 Vs 0.18±0.09U/mmol, P<0.001 respectively).In patient groups, u-PCX showed significant positive correlation with both albuminuria & u- NCR (r=0.782, P< 0.001 & r=0.865, P< 0.001, respectively) and showed significant negative correlation with eGFR (r= -0.291, P< 0.038). By binary logistic regression analysis, u-PCX was a risk factor for microalbuminuria and early DN.*

Conclusion: *U-PCX can be considered as one among early markers for diabetic nephropathy.*

Introduction:

Microalbuminuria is an early clinical marker of diabetic kidney disease (DKD) resulting from damage to the glomerular filtration barrier, which comprises endothelial cells, glomerular basement membrane (GBM) and Podocytes [Mogensen,1987]. Although microalbuminuria in diabetic patients is considered to be the best predictor of progression to end-stage renal disease [Zeeuw, Ramjet and Zhang, 2006] and cardiovascular events [Ninomiya, Perkovic, and Galan, 2009]; earlier, more sensitive and specific markers of kidney damage might help diagnose and treat diabetic nephropathy (DN) at an earlier stage to prevent the progression to renal failure [Abraham and Gautham, 2012]. The tubular damage in DN appears to be independent of glomerular injury. Early renal dysfunction may be predicted by the early rise in urinary N-acetyl-β-D-glucosaminidase (NAG) in diabetic as the majority of patients may show glomerular hyperfiltration [Fu, Xiong and Fang, 2011]. It is accepted that podocytes' injuries play an essential role in the progression of DKD [Weil , Lemley and Yee , 2011]. Monitoring urine podocytes and podocytes-specific proteins can reveal potentially interesting urinary markers for the early diagnosis of DKD [Dalla, Masiero , and Roiter , 2003]. Podocalyxin is an integral membrane protein on the foot processes of kidney podocytes [Vitureira , Andres , Perez , Martinez , Bribian , Blasi , Chelliah , Domenech , Castro, and Burgaya , 2010]. It is a sialoprotein contributing to the negative charge of glomerular membrane [Doyonnas , Kershaw , Duhme , Merkens , Chelliah , Graf and Mc Nagny , 2001]. In addition, it is found in all three germ layers during embryogenesis, as well as hematopoietic progenitors, megakaryocytes and platelets, vascular endothelia, mesothelial cells lining organs, and a subset of neurons [Vitureira , McNagny , Soriano , and Burgaya , *2005*]. Because of the proximity of the apical region of Podocytes to the urinary space, pathological events occurring in this region are expected to be more easily detectable in urine than those occurring in the basal or slit diaphragm regions of Podocytes [Hara , Yanagihara ,and Kihara , 2005]. Podocalyxin(PCX) is now known to play a role in podocytes morphogenesis and maintenance of structural integrity [Doyonnas , *2001*]. In this study, we investigated urinary-PCX(u-PCX), as an early urinary marker for identifying Podocytes injury in the early phase of DN and the degree of association with microalbuminuria and u-NCR.

Methods

Subjects and samples collection

A cross-sectional study was carried out in Riyadh National hospital- Riyadh, KSA, between Jan., 2013 to Sep., 2014. One hundred & sixteen patients with type2 DM(T2DM), 67 males (57.76%) & 49 females (42.24%) included and they were divided into three groups; group A, 51 patient with normoalbuminuria, group B,37 patients with microalbuminuria and group C, 28 patients with macroalbuminu-

Volume : 6 | Issue : 1 | JANUARY 2016 | ISSN - 2249-555X

ria. DN was defined urinary albumin excretion ≥ 30mg/gm creatinine. 45 healthy individuals were served as a control group. They were age, sex and BMI matched with the patients groups (tab.1). Inclusion criteria were patients with T2DM while exclusion criteria were any patients with known kidney disease whatever the cause, uncontrolled hypertension, fever, urinary tract infection (UTI), congestive heart failure, severely uncontrolled DM) HbA1c > 9%) as well as menstruating and pregnant females. **First morning voided samples (FMV)** were obtained from all subjects. Urine samples were stored at −70°C within 2 h of collection until quantification by ELISA for u-PCX, albuminuria and u-NAG and calculation of u-NCR and u-PCX. Blood samples were collected for laboratory tests. The clinical characteristics of the patients and healthy controls are shown in table 1. All were subjected to brief history taking, clinical examination, serum creatinine & calculation of eGFR(ml/mim/1.73m^2) by using the modification of diet in renal disease (DMRD) [Levey , Bosch , Lewis , Greene , Rogers , and Roth ,*1999*],HbA1c, lipid profile, BMI, and blood pressure measurements(systolic & diastolic blood pressure; SBP & DBP respectively). The protocol met the requirement of the local institutional ethics board and written consents from all participants were taken prior to the study.

Detection of albuminuria

Albuminuria was estimated by immunoturbidimetry method using Boerhinger reagents (Germany). Normoalbuminuria is defined as urinary albumin < 30 mg/gm creatinine. Microalbuminuria is defined as urinary albumin 30-300 mg/gm creatinine Macroalbuminuria is defined as urinary albumin >300 mg/gm creatinine.

Detection of urinary N-acetyl-β-D-glucosaminidase NAG

Urinary enzyme NAG was estimated kinetically by using FAR NAG (FAR srl, Pescantina, VERONA, ITALY) reagents [Bazzi, Petrini, and Rizza,2002]. The results were expressed in units/liter (U/L). U-NAG was expressed as the enzyme activity per mmol of urine creatinine (u-NCR U/mmol). U-NCR was positive if ≥**1.9 U/mmol** (for men) or ≥***2.25 U/mmol*** (for women).

Detection of urinary podocalyxin (u-PCX)

Human Podocalyxin (PCX) ELISA Kit (**Wuhan Hi-tech Medical Devices Park, Wuhan, Hubei Province 430206, and P. R. China**) was used for the quantitative determination of human PCX concentrations in urine. Urine collection: Use a sterile container to collect urine samples (early morning samples). Remove any particulates by centrifugation for 15 minutes at 1000xg, 2-8°Cand aliquot and store samples at -20°C or -80°C. Centrifuge again before assaying to remove any additional precipitates that may appear after storage. This assay employs the quantitative sandwich enzyme immunoassay technique **using purified human PCX antibody.** The minimum detectable dose of human podocalyxin is typically less than **0.09 ng/ml**. This assay has high specificity for detection of human podocalyxin. No significant cross-reactivity or interference between human podocalyxin and analogues was observed. The positive value ***for u-PCX was > 65 ng/mmol.***

Western blot analysis for Urinary podocalyxin

The presence of u-PCX in urine samples (obtained after centrifugation) was confirmed by western blot analysis. The proteins in the sample were separated on 5–20% (wt/vol.) SDS-PAGE, and then transferred (under reducing conditions) into a PVDF membrane. The membrane was incubated for 1 h at room temperature with monoclonal antibodies against u-PCX. The membrane was incubated with anti-mouse IgG labelled with HRP (**Wuhan Hi-tech Medical Devices Park, Wuhan, Hubei Province 430206 P. R. China**) and finally visualized using diaminobenzidine. Specificity this assay has high specificity with less than 1% crosses reactivity with recombinant mouse Podocalyxin and recombinant human Endoglycan.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL). Data are expressed as mean value ± SD. Differences between four groups in clinical data were compared by one way ANOVA test for normally distributed values. Correlations between u-PCR and albuminuria, u-NCR, eGFR and s.creatinine were estimated by the Spearman rank-order correlation. The diagnostic efficiency of u-PCX, u- NCR and eGFR for the detection of early diabetic nephropathy was compared by receiver operating characteristic (ROC) curve. The differences of the area under the curve (AUC) between markers were performed by Z-test. Multiple regression analysis using the u-PCR as the dependent variables to analyze the significant predictors for u-PCR excretion. Binary logistic regression analysis was done to assess u-PCX as a risk for early diabetic nephropathy with microalbuminuria. Differences between groups were considered to be statistically significant when P< 0.05.

Results

Compared to controls as expected, diabetic patients presented with significantly higher u-PCR (P <0.001) (***figure.1***). Albuminuria and u-NCR were significantly higher in the patient groups (P <0.001). Furthermore; total cholesterol, triglycerides, HbA1c, and SBP were significantly higher in the patient group (p < 0.001) while age, gender, BMI, DBP, s.creatinine and eGFR showed no statistically significant differences between both groups (P> 0.05)(***table.1***).

Among patients' groups;

In group A: It showed that u-PCR and u-NCR levels were significantly higher than the controls (tab.1), moreover 26 patients (50.98%) had positive u-PCR values and 14 patients (27.45%) had positive u-NCR values. It also showed a significant lower levels of albuminuria, SBP, and HbA1C than group B and C (P<0.001) and no statistically significant differences as regard, gender, age, BMI, DBP, lipid profile, s. creatinine and eGFR. **In groups B & C:** they showed that, 32 patients (86.49%) in group B and 26 patients (92.86. %) in group C had positive u-PCR. Group C had significant higher levels of urinary markers than group B. As regard s.creatinine; it was insignificantly higher levels in group C than group B (P>0.05) while age, gender, lipid profile, eGFR, DBP and BMI showed insignificant differences between both groups (P> 0.05) (***table.1***).

Correlations

In the patients' groups, There was significant positive correlation between u-PCR and both albuminuria (***figure.2***) & u-NCR (r=0.782, P<0.001 & r=0.865, P<0.001, respectively) & also with HbA1C(r=0.735 P <0.001) while showed significant negative correlation with eGFR (r= -0.190 P< 0.041).). **In group A**; There was significant positive correlation between u-PCR and both albuminuria and u-NCR(r= 0.578, P<0.001& r=0.451, P=0.001 respectively) while there was significant negative correlation between u-PCR and eGFR (r= -0291, P=0.038). In patients' groups; there was no significant correlation between u-PCR and s.creatinine, lipid profile, age, BMI & gender (P > 0.05).

Volume : 6 | Issue : 1 | JANUARY 2016 | ISSN - 2249-555X

Independent predictors of early diabetic nephropathy

By **multiple regression analysis** using the u- PCR, as the dependent variables to analyze the significant predictors. Albuminuria, u-NCR and eGFR, were used as independent variables. Albuminuria and u-NCR were independently and significantly predicted u-PCR (***table.2***). Finally, **binary logistic regression analysis (forward stepwise)** was demonstrated u-PCX as a risk factor for microalbuminuria and early diabetic nephropathy with the odds ratio 1.006 (95% confidence intervals) of 0.989 to 0.996).

Non-parametric ROC analysis of u-PCR in early DN

A nonparametric ROC curve, performed to quantify how **u-PCR** definitely distinguishes between diabetic patients with and without early DN compared to other markers, showed an AUC of 0.852 with a CI 95% of 0.782 to 0.923 (P< 0.001)(***Figure 3***). According to the Youden indices, the cutoff value of 126.13 ng/mmol for u-PCR resulted in the maximum indices, and gave a sensitivity of 89.2% and a specificity of 51%.

In western blot analysis

U-PCX was found as granular structures in the urine precipitates following ultracentrifugation at 435,000 *g* of urine samples from patients with diabetes. The presence of PCX was confirmed by western blot analysis. The precipitate obtained contained a typical PCX band at 165–170 kDa. No band was observed with the control antibody (monoclonal antibody against norovirus [NV]) and PBS.

4-Discussion

In our study; u-PCR levels were significantly elevated in diabetic patient and within group A compared with the healthy controls suggesting that podocytes injury occurred before the appearance of microalbuminuria in diabetic patients (in 52.94% of normoalbuminuric patients). Our results were consistent with Hara et al, study which was the first to find that u-PCR is elevated in diabetic patients with normoalbuminuria [Hara , Yamagata , Tomino *et al*,2012]. However, there was a conflicted finding in Nakamura study who demonstrated that urinary podocytes were absent in diabetic patients with normoalbuminuria, but detected in 53% of microalbuminuric and in 80% of macroalbuminuric patients by immunofluorescence [Nakamura , Ushiyama , Suzuki , Hara , Shimada , Sekizuka et al 2000] which is matched in part with our results as regard micro & macroalbuminuric groups. The present study significantly detected that u-PCR was also present before the appearance of rising of u-NCR in diabetic patients in group A. U-PCR levels were also elevated in 86.49% of microalbuminuric and in 92.86% of macroalbuminuric patients. Another study found that urinary mRNA profiles of synaptopodin, podocalyxin, α-actin-4, and podocin were increased with the progression of DKD, which suggested that quantification of Podocytes-associated molecules in urine will be a useful biomarker of DKD [Zheng , Lv, Ni et al 2011].Some have suggested that podocytes loss occurs in early diabetes and this would contribute to the filtration barrier defect [Pagtalunan , Miller , Jumping-Eagle et al 1997] and others have argued that significant early Podocytes loss does not occur [White , Bilous , Marshall et al 2002]. The pattern of podocytes injuries in diabetics include cellular hypertrophy, foot-process effacement, detachment from the GBM (microvillus transformations & vesiculation of microvilli which shed into the urine) and apoptosis [Kershaw , Beck , Wharram et al 1997]. High levels of u-PCR can thus be considered to reflect marked microvillus transformation and vesicular shedding [Hara , Yanagihara , Hirayama et al 2010]. Our study showed, as Hara et al, insignificant levels of u-PCR in the normal controls which could be explained by a normal physiological turnover, as microvillus transformation is found occasionally on the normal glomerulus [Hara, 2012]. We reported that the levels of u-PCR increased with the extent of albuminuria, and this result consisted with previous finding which reported that podocytes loss contributes to the development of albuminuria [Dalla Vestra, M, et al,2003]. Furthermore, in present study, we also detected that the level of u-NCR was significantly elevated in the Diabetic patients compared with health controls which supported by others who concluded that tubular damage occurs in the early stages of DN [Nielsen SE, Sugaya T, Tarnow L et al 2009]. We reported that u-PCR, significantly and positively, correlated with albuminuria and u-NCR and negatively with eGFR & by regression analysis, albuminuria and u-NCR were independently and significantly predicted u-PCR and by binary logistic regression analysis; u-PCR was demonstrated as a risk factor for microalbuminuria and early DN. We observed a significant correlation between u-PCR levels & HbA1c in the early stage of DN, even in the presence of normoalbuminuria which could point to the role of the uncontrolled diabetes in glomerular capillary-barrier damage. In contrast to serum creatinine levels; eGFR(even with high and/or normal range) was negatively correlate with u-PCR levels in patients groups & this can be explained by the fact that changes in serum creatinine levels clearly indicate renal dysfunction only in the advanced stages of DN in contrast to eGFR which can be affected early before serum creatinine did. In type 2 diabetes, elevated blood pressure has previously been demonstrated to be an independent risk factor for development and progression of DN [Parving,1998]. Some studies showed that systolic but not diastolic blood pressure was associated with increased progression of renal disease [Kasper , Christensen, Hovind, Tarnow, Rossing and Parving, 2004]. Online with these results; our study reported a significant positive correlation between u-PCR levels and SBP which pointed to the important role of blood pressure control in preventing & treating early DN. Finally, by performing the nonparametric ROC plots, we found that the diagnosis efficiency of u-PCR is superior to that of eGFR for assessment of early DN. One of limitations in the present study is that it was not based on longitudinal investigation but was conducted with a cross-sectional study. Thus, further prospective study is needed to assess whether diabetic patients with elevated u-PCR are more susceptible to albuminuria and/or deterioration of renal function. In conclusion: Urinary podocalyxin may be a useful biomarker for early detection of DN.

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Table.1: The demographic, clinical and laboratory data of the patients and control groups.

Parameters	Control group	Normoalbuminuria group	Microalbuminuria group	Macroalbuminuria group	P value
Gender(male/female)	25/20	28/23	20/17	16/12	NS
Age(years)	38.48±4.83	37.20±10.07	40.00±9.12	40.54±10.94	NS
BMI	29.45±4.94	29.06±3.16	30.05±3.30	29.42±3.02	NS

Volume : 6 | Issue : 1 | JANUARY 2016 | ISSN - 2249-555X

Disease duration(year)	---------	3.55±2.68	4.01±3.22	4.94±3.18	NS
SBP(mmHg)	115.37±5.47	117.37±2.28	129.45±4.82	133.2±5.23	0.011*
DBP(mmHg)	72.52±3.27	71.42±1.64	72.85±3.72	73.45±2.65	NS
HbA1c (%)	5.08±0.73	7.05±0.69	7.5±0.68	8.41±0.42	<0.001
Total cholesterol(mmol/L)	4.11±0.13	5.78±0.88	5.65±1.03	5.91±0.58	<0.001
Triglycerides(mmol/l)	0.92±0.05	1.59±0.12	1.62±0.66	1.60±0.48	<0.001
Serum creatinine(µmol/L)	79.63±12.62	82.55±17.39	85.47±17.84	90.25±29.64	NS
eGFR (ml/min)	102.31±10.81	100.34±22.49	94.36± 18.39	93.76±17.28	NS
Albuminuria (mg/gm)	6.42±2.41	7.70±4.42	111.45±54.86	777.48±350.68	<0.001
u-NCR (U/mmol)	0.18±0.09	0.91±1.39	3.31±1. 30	6.42±2.27	<0.001
u-PCX (ng/mmol)	31.28±9.54	187.17± 187.91	372.32 ±170.16	597.49± 235.49	<0.001

U= urinary, mmHg = millimeter mercury, µmol = micromole, L = liter, ml= mille, min = minute, mg = milligram, U = unit, mmol = mill mol & ng = nanogram, gm =gram, ml=milliliter. (*) P value is significant if < 0.05.

Tab.2: Multiple linear regression analysis using u- PCR as dependent variables in the patients with type 2 diabetes

Independent variables	Unstandardized Coefficients		Standardized Coefficients	t value	P value	Adjusted R^2
	B	Std. Error	Beta			
eGFR	-1.156	.859	-.092	-1.346	.181	**0.441**
u-NCR	33.818	10.895	.372	3.104	.002	
Microalbuminuria	.267	.092	.344	2.892	.005	

Fig. 1: u-PCX levels in studied groups

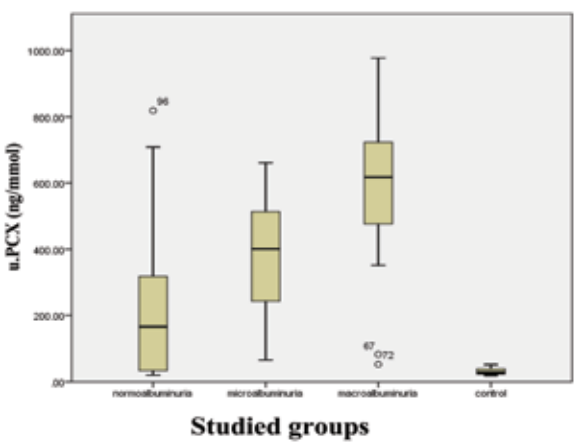


Fig.2: Correlation between u-PCX and albuminuria in patients' groups

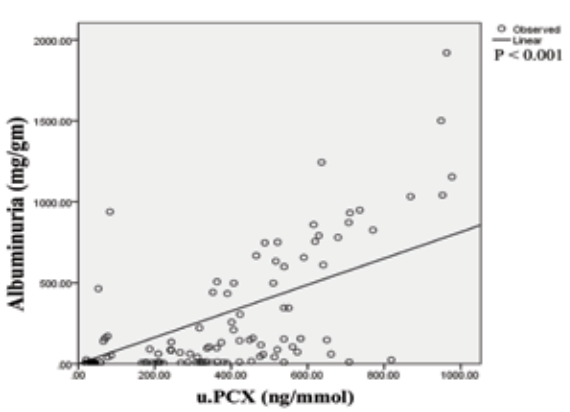
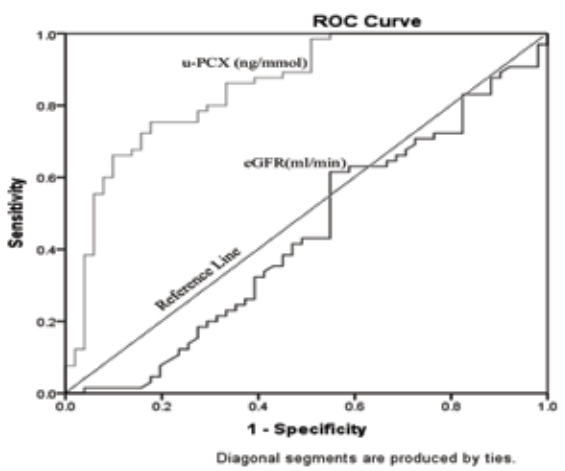


Fig.3: Non-parametric ROC analysis of u-PCR, u-NCR and eGFR



Volume : 6 | Issue : 1 | JANUARY 2016 | ISSN - 2249-555X

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