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CLINICAL SIGNIFICANCE OF URINARY MESSENGER RNA EXPRESSION OF ALPHA ACTIN, SYNAPTOPODIN, PODOCIN, AND PODOCALYXIN IN PROTEINURIC PATIENTS

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Abstract

Podocyte biology is a science promising a greater understanding of the mechanistic nature of proteinuria-related diseases. Injury to the podocytes and subsequent urinary excretion has a key role in glomerular disease pathogenesis and progression.

Aim: To obtain an insight into the clinical significance of urinary mRNA profile of podocytes in proteinuric patients, we evaluated urinary messenger RNA (mRNA) expression of four podocyte proteins: alpha actin, synaptopodin, podocin, and podocalyxin, in glomerular insults of different aetiologies: diabetes, HCV and immune disorders and correlated them with proteinuria and renal function.

Methods: The study enrolled 80 proteinuric and non proteinuric Egyptian patients and 20 apparently healthy controls. Proteinuric patients (n=60) were divided into 3 groups according to etiology: HCV (n=20), nondiabetic (n=20) and diabetic (n=20) groups. In addition to a diabetic nonproteinuric group (n=20). Quantification of mRNA of synaptopodin, podocin, alpha actin and podocalyxin, in urinary sediment was performed by Real-Time PCR.

Results: In the proteinuric HCV group the urinary mRNA levels of synaptopodin were significantly increased as compared to other diseased groups, Yet, no correlation was found between the urinary mRNA expression of the 4 podocyte proteins and HCV viral load.

In the proteinuric nondiabetic group: Age and serum creatinine and synaptopodin mRNA expression were significantly lower than group in HCV proteinuric group. Alpha actin mRNA was significantly higher as compared to proteinuric diabetic patients. Yet Podocin mRNA was significantly lower than the proteinuric diabetic patients. Podocalyxin mRNA inversely correlated to alpha actin mRNA.

In the proteinuric diabetic group: Proteinuria was significantly lower than both proteinuric HCV and proteinuric non- diabetic groups, and did not correlate to renal function or podocyte proteins.

Urinary mRNA expression of alpha actin was significantly decreased as compared to control, proteinuric non diabetic and non- proteinuric diabetic groups. Also, the Podocin mRNA gene expression was significantly decreased when compared to proteinuric HCV and non-proteinuric diabetic.

In the non proteinuric diabetics, alpha actin mRNA correlated with creatinine clearance and Synaptopodin mRNA correlated with serum creatinine.
Podocalyxin mRNA showed no significant difference between any of the studied groups.
Conclusion: Our study found increased excretion of mRNA podocyte proteins in non proteinuric diabetes, and decrease with development of proteinuria which confirms the podocyte depletion hypothesis. HCV viral load has no effect on the excretion pattern. Urinary Synaptopodin mRN|A was increased in kidney disease regardless of urinary protein excretion. Podocalyxin mRNA showed no significant difference from controls.

Key words: proteinuria, podocyte, podocyte proteins, HCV

INTRODUCTION

Podocytes are highly specialized cells that play a part in the glomerular filtration barrier function **(Shankland, 2006)**. Glomerular tuft injury may possibly change the architecture and function of podocyte, leading in podocyte effacement and podocytopenia, then progressive glomerulosclerosis **(Kriz, 2002)**.

In keeping a healthy glomerular filtration barrier, podocyte-associated proteins are important. Slit diaphragm nephrin and podocin have been related to alpha actinin-4 and Synaptopodin cytoskeletal proteins that facilitate dynamic podocyte morphology rearrangements. Podocalyxin is a luminal membrane sialoglycoprotein which restricts the albumin passage to the space of Bowman. Disarrangements of these proteins by different pathways, such as immune, infectious, ischemic or toxic, lead in the filtration barrier and proteinuria being damaged **(Barisoni et al, 2009)**.

Regardless of the insult, the outcome depends on the number of depleted podocytes. If more than 40% (a critical percentage), the exposed glomerular basement membrane (GBM) begins adherence to capsule of Bowman, beginning with a sclerotic process which progresses to end stage renal disease(ESRD) **(Wharram et al, 2005)**.

In this study, we investigated the amount of urinary mRNA expression of four podocyte proteins: Synaptopodin, Podocin, Alpha actin and Podocalyxin in glomerular insults of different aetiologies as occurs in diabetes, immune disorders and HCV infection, and correlate them with proteinuria and renal function

METHODS

Material and Methods:

Patients and methods:

For the current study, 80 Patients were recruited from Theodor Bilharz Research Institute (TBRI) internal medicine and nephrology outpatient clinics, and 20 controls from healthy staff working at TBRI.

All subjects were divided into the following 5 groups:
Group I (n=20): The control group included twenty apparently healthy subjects.
GroupII (n=20): This group included 20 HCV Antibody positive proteinuric patients.

Group III (n=20): This group included 20 non diabetic proteinuric patients.
(Renal biopsy diagnosed the etiologies of group II and III as follows: 4 patients had focal proliferative, 14 had membranous, 15 had membranoproliferative, 2 amyloid and 5 systemic lupus erythematosus).
Group IV (n=20): This group included 20 diabetic proteinuric patients.
Group V (n=20): This group included 20 diabetic non-proteinuric patients.

Routine laboratory investigations including fasting, 2 hour postprandial blood glucose, urea, creatinine, total bilirubin, alanine transaminase, urinary creatinine and urinary protein were assayed by Beckman Coulter AU480 analyzer (Beckman Coulter, Inc., Brea, California). Using the Cockcroft and Gault formula, creatinine clearance has been calculated:
Creatinine Clearance) ml/min = [[140 - age (year)]*weight (kg)]/[72*serum Cr(mg/dL)] (* 0.85 for women)

The formula was applicable to all subjects as the serum creatinine was stable

In compliance with the 1975 Helsinki Declaration and as amended in 2012, the study protocol was approved by the Theodor Bilharz Research Institute institutional review board prior to the beginning of the enrolment of participants.

All patients and controls are subjected to:
*Full history and clinical examination.
*Routine laboratory tests including blood urea, serum creatinine, test for albuminuria with dipstick, 24-hour urinary proteins and creatinine clearance estimation. * Specific laboratory tests:
-Quantification of podocyte mRNA (Synaptopodin, Podocalyxin, α actin-4 and Podocin) in urinary sediment by **Real-Time** PCR.

Alpha actin, Synaptopodin, Podocin and Podocalyxcin genotype **Real-Time** PCR assays:

1. Methods of Real-Time PCR:

1.1 Collection of urine samples and total RNA extraction:
Shortly after collection, at 3000 rpm for 30 min at 4^{o}C, the urine was centrifuged. The supernatant was removed and the rest of the cell pellet was stored until use at -80 ^{o}C.
In compliance with the manufacturer's protocol, total RNA was extracted (RNeasy Mini Kit, Qiagen, Germany)

Using the relative absorption ratio at 260/280 on a nanodrop 2000, the RNA concentration and purity have been verified using NanoDrop spectrophotometer (Thermo, Wilmington, USA). For RT-PCR, RNA specimens having a ratio greater than 1.8 were used.

1.2 Reverse transcription

Reverse transcription in compliance with the manufacturer's protocol RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific, USA) 2 µL total RNA was mixed with 1 µL Oligo (dT) 18 primer, 4 µL (5X) Reaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2

µL (10 mM) dNTP Mix and 1 µL RevertAid M-MuLV RT (200 U/µL) solution and completed with nuclease-free water to a volume of 20 µL.
The reverse transcription was carried out for 60 min at 42 ° C, followed by inactivation step for 5 min at 70 ° C. Until utilization, the resulting cDNA was stored at -20 °C.

1.3 Real-time PCR
The relative abundance of synaptopodin, podocalyxin and α-actin4, podocin mRNA, was measured using the StepOne™ Real-Time PCR System (Applied Biosystems, California, USA). As the reference housekeeping gene, human β-actin was used. The following sequences of the oligonucleotide primer were used:
Synaptopodin: forward 5'-CTTACGGCGGTGACATCTC, reverse 5'-GGTCCTGAGCCTCGATCC;
Podocalyxin: forward 5' CTTGAGACACAGACACAGAG, reverse 5'-CCGTATGCCGCACTTATC;
α-actin4: 5'- GATGGTCTTGCCTTCAATG, reverse 5'- TGTTCACGATGTCCTCTG;
Podocin: forward 5' TGGCTGTGGAGGCTGAAG, reverse 5'-TGAAGGGTGTGGAGGTATCG;
β-actin: forward 5'- TGGCACCCAGCACAATGAA, reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA
Real-Time PCR was proceeded as follow: 2 µL cDNA, 10 µL SYBR Green/ROX qPCR Master Mix (2X Maxima SYBR Green/ROX qPCR Master Mix (2X), 0.4 ml forward primer (10 mM), 0.4 µL reverse primer (10 mM), 0.4 µL ROX Reference dye and to obtain a 20 µL reaction volume, 6.8 µL nuclease free water was mixed. All specimens were executed in duplicate.

A two-step process was used to perform the PCR Reaction: 95 °C for 10 min, 40 cycles for 15 s at 95 °C and 60°C for 30 s. The dissociation curves (DC) and melting temperatures (Tm) were determined.

The target gene abundance/housekeeping gene abundance equation has been used to assess each gene's level of expression. Negative controls containing ddH2O in all runs.

Statistical analysis
The results were presented as average, standard error, median (minimum-maximum) or number (%). Categorical data [n(percent)] comparison was carried out using Chi square test or Fisher exact test whenever it was appropriate. Comparison between parameters in different groups was done using Kruskal-Wallis test followed by Mann-Whitney test as post-hoc test if significant findings were obtained. Spearman correlation coefficient was used to correlate between various studied parameters in each group. The Computer Software Statistical Package for Social Sciences (SPSS) (version 19 windows) was used for data analysis. P value ≤ 0.05 was considered significant.

RESULTS

Demographic features and kidney function tests in all studied groups were presented in (Table 1): In groups II, IV and V, age was substantially increased as compared to group I. Yet, group III was significantly lower than group II.

Serum creatinine showed a significant increase as compared to controls in all groups. Creatinine clearance was significantly decreased in diseased groups as compared to controls. Protein in urine levels showed a statistically significant decrease in IV as compared to both II and III.

Table 1: Demographic features and kidney function tests of all the studied groups.

	Group I	Group II	Group III	Group IV	Group V	p value
	Control (n= 20)	**Proteinuric HCV (n= 20)**	**Proteinuric nondiabetic (n= 20)**	**Proteinuric diabetic (n= 20)**	**Non proteinuric diabetic (n= 20)**	
Age (yrs.)	40.0 (18.0-56.0)	56.0 (19.0-70.0) [a]	40.0 (21.0-68.0) [b]	56.5 (40.0-70.0) [a]	49.0 (39.0-63.0) [a]	0.001*
Gender (F/M)	10/10	3/17	7/13	16/4	9/11	0.001*
S. creatinine(mg/dl)	0.70 (0.50-1.00)	2.50 (0.90-7.00) [a]	1.05 (0.50-5.60) [ab]	1.40 (0.60-4.00) [ab]	1.0 (1.0-1.1) [abd]	0.001*
Creatinine clearance(ml/min)	104.5 (90.0-115.0)	46.0 (13.0-90.0) [a]	71.0 (14.0-120.0) [ab]	55.0 (10.0-100.0) [a]	89.5 (80.0-98.0) [abcd]	0.001*
Protein in urine (g/day)	----	1.83 (0.12-13.50)	3.40 (0.40-11.40)	0.40 (0.05-2.30) [bc]	----	0.001*

Data are expressed as median (minimum-maximum). p> 0.05= not significant.
a = p< 0.05 compared with group I. b = p< 0.05 compared with group II, c = p< 0.05 compared with group III.
d = p< 0.05 compared with group IV.

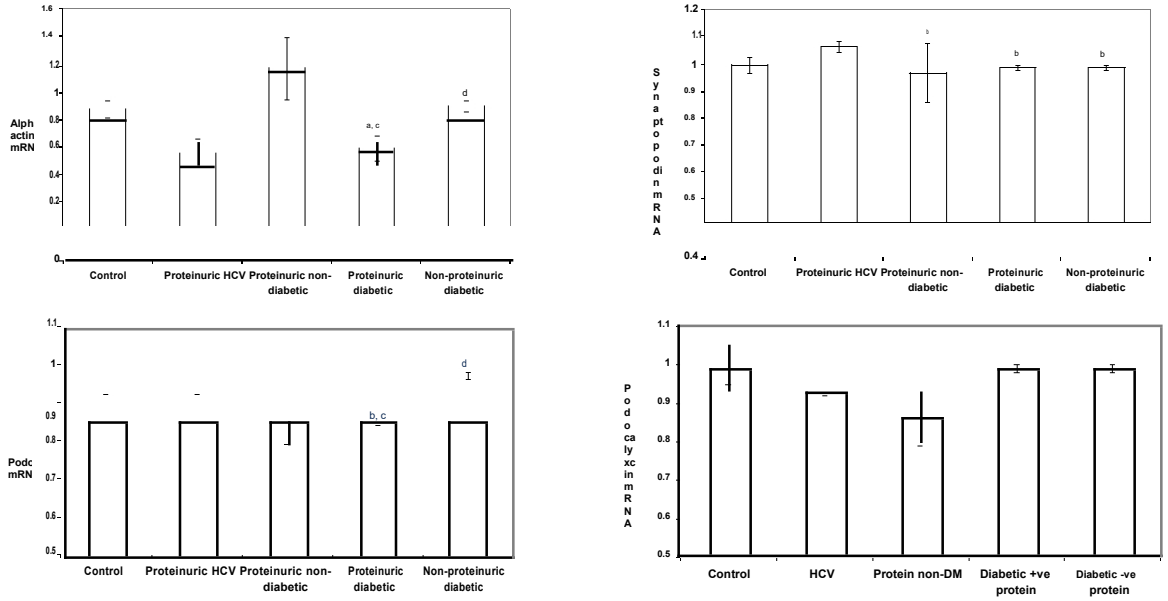

Fig.1 : Mean values (± SE) of urinary protein mRNA expression of podocyte proteins in different studied groups.
a= p< 0.05 compared with control group. b=p< 0.05 compared with proteinuric HCV group.
c=p< 0.05 compared with proteinuric non diabetic group. d=p< 0.05 compared with proteinuric diabetic group.

A- Urinary mRNA levels of **alpha actin** were significantly decreased in group IV as compared with group I, III and V (p< 0.050)

B- Urinary mRNA levels of s**ynaptopodin** were significantly increased group II relative to group III, IV and V (p<0.050)

C- Urinary mRNA levels of **podocin** were significantly decreased in group IV when compared with groups II and V and increased as compared to group III (p< 0.050).

D- Urinary mRNA levels of **podocalyxin** showed no statistical significant difference between different studied groups.

Correlations between expressed genes and different studied Parameters :
In group I: Serum creatinine is positively correlated to age (r=0.502; p=0.024). Creatinine clearance is negatively correlated to podocin mRNA (r=0.460; p=0.041). Positive correlation between podocalyxin mRNA and both synaptopodin (r=0.691; p=0.001) and podocin mRNA (r=0.712; p=0.001) was found.

In group II: gene expression of alpha actin mRNA was positively correlated with synaptopodin (r= 0.545; p= 0.013) and podocin mRNA (r= 0.901; r= 0.001). Also synaptopodin mRNA was positively associated with podocin mRNA (r= 0.557; p= 0.011). No correlation was found between the HCV viral load and the podocyte proteins mRNA.

In group III: gene expression of alpha actin mRNA was negatively correlated with podocalyxcin mRNA (r= -0.493; p= 0.027).

In group IV: gene expression of alpha actin mRNA was negatively correlated with age (r= -0.453; p= 0.045) and positively correlated with synaptopodin (r= 0.588; p= 0.006) and podocin mRNA (r= 0.703; r= 0.001). Also synaptopodin mRNA was positively correlated with podocin mRNA(r= 0.623; p= 0.003).

In group V: gene expression of alpha actin mRNA was positively correlated with creatinine clearance (r= 0.695; p= 0.001) and synaptopodin mRNA (r= 0.580; p= 0.007). Synaptopodin mRNA was positively correlated with both creatinine level (r= 0.464; p= 0.039) and creatinine clearance (r= 0.484; p= 0.030). Also, gene expression of podocin mRNA was negatively correlated with age (r= -0.666; p= 0.001).

DISCUSSION:

Podocytes have been highly specialized epithelial cells, which have a role in regulating glomerular function, covering the outer aspect of the Glomerular Basement Membrane. Podocytopathies have been reported in both foot process effacement and reduction in the number of podocytes **(Steffed et al, 2001)**.

The detection of urinary mRNA of podocyte by **Real-time** PCR can measure low abundance genes from even one single cell, and provides information for the progression of the glomerular disease **(Liss 2002)**. **Yu et al, 2005**, stated that a more specific indicator of disease activity than proteinuria is the detection of podocyte products in urine. We investigated whether podocyte proteins represent the degree and type of glomerular damage in the urine and correlated their amount in urine with renal function and proteinuria.

In our study, in diabetes, the mRNA levels of actin and podocin were found to be significantly increased in non proteinuric diabetics as compared to proteinuric diabetics. However, **Zheng et al, 2011**, confirmed the increase of podocyte protein mRNA in urine in diabetic nephropathy patients.

In diabetics, age correlated inversely with podocin mRNA in group V, and negatively with alpha actin mRNA in group IV. Podocin mRNA positively correlates with alpha actin in the

same group. This may be due to glomerular aging and loss of their podocytes as the disease progresses **(Wiggins et al, 2005)**. Aging is related to a decline in the number of podocytes, and risk of end stage renal disease increases with age **(Toneli and Riella,2014)**.**Sato et al,2009**, found urinary podocin mRNA to correlate with progression and chronicity in diabetes.

Alpha actin mRNA also correlated to creatinine clearance in group V, and age correlated inversely with podocin mRNA. These 2 correlations may indicate that podocyturia begins early in the normoalbuminuric stage of diabetes and decreases gradually as podocytopenia ensues. **Hara et al 2012**, demonstrated this on studying podocalyxin mRNA excretion in diabetics. They found also podocalyxin mRNA correlating with HgbA1C, denoting the effect of hyperglycemia on the glomerular capillary barrier, and with urinary proteins: albumin and tubular proteins, denoting tubular injury together with glomerular injury in early diabetes, but not correlating with serum creatinine nor with GFR.

Synaptopodin mRNA was seen to correlate with serum creatinine, creatinine clearance and with alpha actin in group V. This goes with **Kwon et al, 2016,** study on diabetics, who found that synaptopodin mRNA excretion correlated with serum creatinine, as impaired renal function, podocyte loss is possibly reflected, meaning irreversible glomerular damage in advanced kidney disease. Proteinuria in their study was not correlated with any podocyte proteins nor with renal function and there were no statistical difference in proteinuria between the diabetics and nondiabetics. In our study, Group IV proteinuria was substantially lower than group III and group II, and also not correlated to renal function or podocyte proteins.

Direct injury to the epithelial cells has been described in HCV disease **(Fabrizi et al, 2002)**. Damage to a few of the podocytes appears to spread via the domino effect **(Tchikawa et al, 2005)**. This occurs through the CD81 and Toll like Receptors which allow HCV binding to the renal cells and the production of an immune response **(Andre et al, 2005)**. A systemic immune response to HCV allows production of mixed cryoglobulinemia **(Cocquerel et al, 2006)**. Renal signs include proteinuria and microscopic hematuria and mild to moderate renal impairment **(Johnson et al, 1993)**.

In Group II, the age, serum creatinine has been substantially increased, creatinine clearance has been substantially reduced in relation to the other groups i.e. the patients were older and kidney function was the worst among the other groups. Proteinuria was less than that of proteinuric non diabetics (Group III), but non-significant (proteinuria is not correlated with renal function). However, no statistical significance was found between the expression of the mRNA of the 4 genes and renal function, no correlation with age except with podocin mRNA, suggesting chronicity of the renal affection **(Sato et al,2009)**, nor with the HCV viral load , denoting that the HCV status per se had no effect on the expression of the mRNA genes.

In Group III, age and serum creatinine were significantly lower than the Group II, and significantly higher creatinine clearance and higher insignificant proteinuria.

Synaptopodin mRNA was significantly lower than the HCV group, but not correlating with serum creatinine as seen in diabetics studied by **Kwon and his colleagues in 2016**.

Podocalyxin mRNA, inversely correlated to alpha actin. **Rodriguez et al, 2014**, in their study on non-diabetic proliferative and nonproliferative glomerulopathies, found no specific pattern of podocyte mRNA expression, but there was a positive correlation between proteinuria and podocyte proteins in the acute phase of the proliferative forms, which decreased overtime after treatment. Synaptopodin mRNA was not associated with histological disease, proteinuria or renal function, as this molecule is considered a housekeeping gene, and its expression in the podocyte cytoskeleton is relatively constant. It may not impact the glomerular permeability of proteins in disease processes **(Mundel et al, 1997)**. **Szeto et al, 2005** did not find a correlation between synaptopodin mRNA and the rate of decline of renal function.

Another study (in press, personal communication), on the same mRNA podocyte proteins but in urine of nephrotic non diabetic patients (mainly Membranous and Focal segmental Glomerulosclerosis) and Lupus nephritis, found increased mRNA of alpha actin, decrease that of synaptopodin and podocalyxin, and nonsignificant difference in podocin mRNA. In Lupus nephritis patients, there was significant increase in mRNA expression of alpha actin, synaptopodin and podocin. Podocalyxin mRNA was significantly decreased. In active Lupus, the mRNA expression of podocin was increased. The other 3 proteins showed no significant difference between active and inactive Lupus nephritis.

CONCLUSION

This study showed increase excretion of podocyte mRNA in non proteinuric diabetics, that decreased with development of diabetic nephropathy .This can confirm the podocyte depletion hypothesis which states that an autonomous process occurs, regardless of the initial mechanism of podocyte injury, leading to progress by further podocyte loss till reaching end stage renal disease. Second, in advanced kidney disease, urine synaptopodin mRNA was raised independent of urine protein excretion (significantly correlated with serum creatinine). Third, HCV status per se, had no effect on urinary podocyte mRNA excretion pattern. Fourth, podocalyxin mRNA showed no significant difference observed between studied groups.
Understanding the glomerular biology can open the way to new potential applications for clinical targeting, as the podocyte proteins measure disease activity better than proteinuria

COMPETING INTERESTS

The authors declared no competing interest.

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