CLINICAL STUDY

Urinary messenger RNA expression of podocyte-associated molecules in patients with diabetic nephropathy treated by angiotensin-converting enzyme inhibitor and angiotensin receptor blocker

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Abstract

Background: Podocyte injury and its subsequent loss in urine play an important role in the pathogenesis of diabetic nephropathy; blockade of the renin–angiotensin system may ameliorate the damage.

Methods: In a non-randomized setting, we studied 71 patients with diabetic nephropathy on a stable dose of angiotensin-converting enzyme inhibitor (ACEI). In 37 patients, angiotensin receptor blocker (ARB) was added (the combination group); ACEI alone was continued in the other 34 (the control group). The mRNA expressions of nephrin, podocin, and synaptopodin in urinary sediment were measured at 0 and 12 weeks.

Results: Baseline glomerular filtration rate (GFR) correlated with the urinary expression of nephrin ($r=0.320$, $P=0.007$), podocin ($r=0.336$, $P=0.004$), and synaptopodin ($r=0.350$, $P=0.003$). After adjusting for the baseline expression, the combination group had a significantly lower urinary synaptopodin expression (7.49 (95% confidence interval CI, 0.62–115.29) vs 14.83 (95% CI, 1.03–241.43), $P=0.026$) than the control group after 12 weeks of treatment. The percentage change in urinary podocin expression over 12 weeks of treatment had a modest correlation with the rate of GFR decline in 1 year ($r=-0.243$, $P=0.041$).

Conclusion: In patients with diabetic nephropathy, urinary mRNA expression of podocyte markers correlated with baseline renal function. Urinary expression of synaptopodin was lower after 12 weeks of ACEI and ARB combination therapy. Our result suggests that serial measurement of urinary podocyte markers may have a value for the monitoring of therapeutic response.

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Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease (1). It is characterized by persistent proteinuria, unremitting renal function decline, and increased cardiovascular morbidity and mortality (2). The renin–angiotensin system (RAS), especially local renal RAS activation, has been recognized to play a key role in the pathogenesis of diabetic nephropathy (3). Blockade of the RAS is currently the standard therapy in the treatment of diabetic nephropathy (4). Dual blockade of RAS by angiotensin-converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) offers additional renoprotective effect when compared with monotherapy by either agent alone (5).

Blood pressure, proteinuria, serum creatinine, and glomerular filtration rate (GFR) are commonly used for the clinical monitoring of diabetic nephropathy and its therapeutic response to RAS blockade. However, none of

them is entirely satisfactory. Recent studies showed that podocyte injury, an important pathogenetic process of diabetic nephropathy (6), might be a more sensitive means to assess the degree of glomerular damage (7) as well as the response to blockade of RAS (8–10). However, clinical application of this strategy is limited because renal biopsy and immunohistological techniques are required. In the last few years, with the development of reliable RNA extraction techniques from urinary sediment and RT real-time quantitative PCR (RT-QPCR), measurement of mRNA expression in urinary sediment has become an emerging tool for the study of kidney diseases (11). Our previous cross-sectional study has showed that the mRNA expression of podocyte markers, such as nephrin, podocin, and synaptopodin, in urinary sediment is increased in patients with diabetic nephropathy (12). The objective of the present study was to examine the change in gene expression of podocyte-associated molecules in the

urinary sediment of patients with diabetic nephropathy after treatment with ACEI and ARB.

Patients and methods

Patient selection and follow-up

From 2004 to 2005, we studied 37 patients with diabetic nephropathy in the Li Ka-Shing Specialty Clinic, Prince of Wales Hospital, Hong Kong. All patients had type 2 diabetes according to the 1997 World Health Organization criteria (13) and were receiving ACEI therapy for diabetic nephropathy that was diagnosed by a long history of diabetes, proteinuria over 0.5 g/day, and the absence of clinical or laboratory evidence of other kidney disease (14). They were treated with an addition of ARB (irbersatan 300 mg/day) to ACEI (combination group). We studied another 34 patients with diabetic nephropathy, who were continued with ACEI (control group). The treatment group assignment was not randomized but decided by each individual clinician. This study adheres to the Declaration of Helsinki and informed consents were obtained from all subjects.

All patients were followed every 8 weeks for 56 weeks. Pre-existing comorbid conditions were recorded. The definition of coronary heart disease included a history of angina or previous myocardial infarction. Cerebrovascular disease was defined as a history of stroke or transient ischemic attack. Hypertension was defined according to the Joint National Committee VII criteria (15). The modified Charlson comorbidity index was used to calculate a comorbidity score (16). Clinical data including serum creatinine, urea, electrolyte, albumin, liver enzymes, and 24-h urine protein were measured at each visit. The GFR was estimated by a standard equation (17). The rate of GFR decline was calculated by the least-square regression method. All physicians were blinded from the results of urinary gene expression. Dosage of antihypertensive medication remained unchanged during the study period.

Urinary gene expression

At 0 and 12th week, a whole-stream early morning urine specimen was collected for gene expression study. Urinary mRNA extraction was performed according to method described previously (11). In brief, urine samples were centrifuged at 3000 *g* for 30 min and at 13 000 *g* for 5 min at 4 °C shortly after collection. Supernatant was then discarded and the urinary cell pellet was lysed by RNA lysis buffer (Qiagen Inc). Specimens were stored in –70 °C until use. RNeasy mini kits (Qiagen Inc.) were used to extract total RNA according to manufacturer's protocol. DNase was used to wipe off probable genomic DNA. We confirmed the purity of RNA by the relative absorbance at

260/280 nm ratio using a spectrometer. Our previous data have shown that the integrity of RNA isolated from urinary sediment by this method is adequate for RT-QPCR (18).

For RT, 5 µl total RNA were mixed with 1 µl random primers (150 ng), 1 µl dNTP mix (10 mM each), 4 µl 5× first-strand buffer, 2 µl dithiothreitol (0.1 M), 1 µl Superscript II RNase H Reverse Transcriptase (all from Invitrogen), and make up to 20 µl with H₂O. RT was performed at 65 °C for 5 min, 25 °C for 10 min, 42 °C for 50 min, and then inactivate reaction at 70 °C for 10 min. The resulting cDNA was stored in –70 °C until use.

In this study, we quantified urinary expression of nephrin, podocin, and synaptopodin using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Commercially available Taqman primers and probes, including two unlabeled PCR primers and one FAM dye-labeled TaqMan MGB probe were used for all the target genes (all from Applied Biosystems). The primer and probe set was deliberately designed across the intron–exon boundary so as not to detect possible genomic DNA. For RT-QPCR, 10 µl universal master mix, 1 µl primer and probe set, 2 µl cDNA, and 7 µl H₂O were mixed to make a 20 µl reaction volume. Each sample was run in triplicate. RT-QPCR were performed at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. To normalize the mRNA expression level of each target gene, 18S rRNA (Applied Biosystems) was used as the house-keeping gene. Results were analyzed with Sequence Detection Software version 1.7 (Applied Biosystems). In order to calculate the differences of expression level for each of the target genes among samples, the $\Delta\Delta C_T$ method for relative quantification is used as described previously (19) and validated in our previous study (20).

Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 11.0 (SPSS Inc., Chicago, IL, USA). All the results were presented in mean $\pm$ s.d. for data normally distributed and median (lower and upper quartiles) for the others. Since data of gene expression levels were highly skewed, log transformation and non-parametric statistical methods were used. We used Mann–Whitney *U* test to compare gene expression levels between groups, Wilcoxon signed-rank test to compare gene expression levels before and after treatment, and Spearman's rank-order correlations to test associations between gene expression levels and clinical parameters. To compare the gene expression level after treatment with adjustment for the baseline values, we used the log-transformed expression level for general linear model, with treatment group as the grouping variable and baseline expression as covariate. Data of this part are expressed as geometric mean and

95% confidence interval (CI). A P value <0.05 was considered statistically significant. All probabilities were two tailed.

Results

Demographic and baseline clinical information of the study subjects are displayed in Table 1. Baseline and follow-up data on renal function, proteinuria, and gene expression level in urinary sediment are summarized in Table 2. There was no significant difference in age, sex, baseline proteinuria, serum creatinine, baseline urinary podocin, or synaptopodin expression between the groups, while estimated GFR and baseline urinary nephrin expression were slightly higher in control group (see Table 2). Baseline urinary gene expression of nephrin, podocin, and synaptopodin were significantly higher in male than female patients (Mann–Whitney U test, $P=0.010$, $P=0.001$, and $P<0.001$ respectively). There was no significant correlation between age and the expression of target genes. After 3 months of treatment, proteinuria improved significantly in the combination group but remained static in the control group. On the other hand, serum creatinine increased and GFR decreased significantly in both groups.

Baseline urinary gene expression and clinical parameters

The relationship between urinary gene expression and baseline clinical parameters is summarized in Table 3.

Table 1 Demographic and baseline clinical information.

	Control group	Combination group
Number of patients	34	37
Age (year)	56.88 $\pm$ 8.87	61.50 $\pm$ 8.37
Sex (M:F)	27:7	25:12
Body height (cm)	164.74 $\pm$ 9.70	163.48 $\pm$ 9.63
Body weight (kg)	60.05 $\pm$ 11.91	57.86 $\pm$ 10.89
Body mass index (kg/m ²)	22.02 $\pm$ 3.29	21.75 $\pm$ 4.50
Pre-existing comorbidity, no. of cases (%)		
Hypertension	79.41	81.08
Cerebrovascular disease	14.71	16.22
Coronary heart disease	29.73	29.41
Diabetic retinopathy, no. of cases (%)	21 (61.8%)	20 (54.1%)
Charlson comorbidity index	3.91 $\pm$ 1.30	4.87 $\pm$ 2.53
ACEI, no. of patient (dosage (mg/day)) ^a		
Lisinopril	25 (10 (2.5–20))	22 (10 (2.5–20))
Ramipril	6 (2.5 (1.25–10))	12 (3.75 (1.25–10))
Captopril	2 (62.5)	1 (50)
Perindopril	1 (4)	2 (8)
On diuretics, no. of cases (%)	12 (35.3%)	7 (18.9%)

ACEI, angiotensin-converting enzyme inhibitor.

^aDosage in median (range).

Significant correlations were found between estimated GFR and the urinary expression of nephrin ($r=0.320$, $P=0.007$), podocin ($r=0.336$, $P=0.004$), and synaptopodin ($r=0.350$, $P=0.003$). There was no relation between urinary expression of nephrin, podocin or synaptopodin, and baseline proteinuria.

Change in urinary expression of target genes

Urinary expression of podocin and synaptopodin significantly increased from 0 to 12 weeks in the control group, while the increase in nephrin expression just fell short of statistical significance ($P=0.074$). On the other hand, urinary expression of nephrin, podocin, and synaptopodin increased significantly in the combination group. After adjusting for the baseline expression, the combination group had a significantly lower urinary synaptopodin expression (7.49 (95% CI, 0.62–115.29) vs 14.83 (95% CI, 1.03–241.43), $P=0.026$), and marginally lower nephrin (6.20 (95% CI, 0.52–99.09) vs 12.95 (95% CI, 0.71–273.75), $P=0.1$) and podocin expression (6.27 (95% CI, 0.37–141.76) vs 12.89 (95% CI, 0.45–433.18), $P=0.14$) than the control group after 12 weeks of treatment, although the later two comparisons did not reach statistical significance. There was no correlation between the percentage reduction in proteinuria and the change in urinary expression of any target gene (details not shown).

Urinary gene expression and renal function decline

All patients completed the study. There was no significant difference in blood pressure between the groups throughout the study period (Table 2). Serum creatinine increased and GFR decreased significantly after 12 months in both the groups (details not shown). However, the GFR decline was significantly slower in the combination group than the control group (0.31 ± 0.41 vs 0.79 ± 0.67 ml/min per 1.73 m^2 /month, $P=\text{relation between change in proteinuria and GFR decline}$ ($r=0.129$, $P=0.3$). Neither the baseline urinary expression of any target gene nor the expression at 12 weeks had any significant correlation with the rate of renal function decline in 1 year. The percentage change of urinary expression of podocin, but not nephrin or synaptopodin, after treatment for 12 weeks had a modest but significant correlation with the GFR decline ($r=-0.243$, $P=0.041$; Fig. 1).

Discussion

Podocyte injury in diabetic nephropathy, including a diminution of podocyte density and abnormal podocyte-specific protein expression pattern, has been extensively investigated (21–23). Recent studies have linked

Table 2 Baseline and follow-up data on renal function, proteinuria, and urinary gene expression.

	Control group		Combination group	
	0 week	12 weeks	0 week	12 weeks
Blood pressure (mmHg)				
Systolic	148.24 ± 13.54	143.9 ± 12.6	144.43 ± 19.85	144.4 ± 20.9
Diastolic	80.09 ± 9.83	76.0 ± 10.1	76.49 ± 8.86	75.1 ± 9.9
Renal function				
Proteinuria (g/day)	1.59 ± 1.36	1.70 ± 1.28	1.45 ± 1.27	1.02 ± 0.87*
Serum creatinine (μmol/l)	150.24 ± 32.59	159.09 ± 34.42*	165.67 ± 31.71	174.31 ± 38.94*
Estimated GFR (ml/min per 1.73 m ²)	44.04 ± 8.68	40.86 ± 8.13*	37.93 ± 9.68 [†]	36.14 ± 10.30*
Urinary gene expression, median (interquartile range) ^a				
Nephrin	4.86 (1.63–37.56)	13.86 (3.63–55.23)	1.30 (0.79–10.59) [‡]	6.11 (1.85–15.15)*
Podocin	5.44 (0.25–28.35)	16.53 (2.36–44.33)*	1.31 (0.08–7.37)	6.08 (0.51–24.37)*
Synaptopodin	5.86 (1.28–24.32)	18.46 (2.70–51.58)*	3.05 (0.57–11.66)	9.91 (1.29–23.30)* [§]

GFR, glomerular filtration rate; **P* < 0.05 vs 0 week, [†]*P* = 0.004, [‡]*P* = 0.012 versus control group; [§]*P* = 0.026 versus control group after adjusting for the baseline expression.
^aLevels are displayed as folds to healthy control.

podocyte damage to increased renal angiotensin II (ANG II) level in diabetic nephropathy (24). Although the exact molecular mechanisms of podocyte injury remain illusive, both types of the ANG II receptors have been detected in podocytes after injury, implying a possible direct effect of ANG II (25). Furthermore, numerous reports suggest that blockade of the RAS can protect these cells (9, 26, 27). In the present study, we examined the urinary mRNA expression of podocyte-associated molecule, namely nephrin, podocin, and synaptopodin, in diabetic nephropathy patients treated with ACEI and ARB.

We have previously demonstrated an increase of nephrin, podocin, and synaptopodin mRNA expression in the urinary sediment of patients with chronic kidney diseases and diabetic nephropathy (12). In the present study, we found that the urinary expression of podocyte-associated molecules correlated with baseline renal function. The result is distinctly different from our previous study, which examined a group of patients with early diabetic nephropathy (12). It is possible that at an early stage of disease, the quantity of urinary podocyte reflects ongoing glomerular damage and is expectedly inversely related to the renal function. When diabetic nephropathy becomes advanced, as in our present study, glomerular podocyte becomes depleted, and its quantity in urine is therefore proportional to the number within the kidney and therefore the renal function. More importantly, the change in urinary expression of podocin probably correlated with the renal prognosis (see Fig. 1). Taken together, urinary expression of podocyte-associated molecules may have

important roles in risk stratification of patients with diabetic nephropathy.

In the present study, we observed a progressive increase in urinary expression of podocyte-associated molecules in both groups, suggesting an increasing loss of podocyte in the urine despite ACEI and/or ARB treatment. Although not confirmed in the present study, the increase in urinary nephrin expression with time is unlikely to be intra-individual variation. In fact, our previous study on lupus patients showed the urine expression of many genes varied little with time in stable patients (28). Interestingly, proteinuria and GFR decline rate improved in the combination group when compared with that in the control group, but the change in urinary expression of podocyte-associated molecules was less dramatic. Our result suggests that dual therapy with ACEI and ARB does not completely abolish urinary podocyte loss and the renal protecting effect is largely contributed by other mechanisms (e.g., effect on intra-glomerular hemodynamics). Our result could be explained by following reasons. (1) Diabetic nephropathy is a disease characterized by unremitting renal injury in spite of treatment. Podocyte detachment may continue with therapy. In fact, during the study, GFR deteriorated in both groups, indicating persistent progression of renal damage. (2) For RAS blocking therapy, 3 months is a short period to take any measurable effect on podocytes, and dosages of ACEI and ARB in this study are relatively low. (3) The density of podocyte-associated molecules per cell might have increased due to therapy, resulting in a raised urinary level despite a similar, or even reduced, number of

Table 3 Correlations between urinary gene expression and baseline clinical parameters.

	Proteinuria	Serum creatinine	Estimated GFR
Nephrin	<i>r</i> = 0.165, <i>P</i> = 0.176	<i>r</i> = −0.134, <i>P</i> = 0.264	<i>r</i> = 0.320, <i>P</i> = 0.007*
Podocin	<i>r</i> = −0.032, <i>P</i> = 0.792	<i>r</i> = −0.107, <i>P</i> = 0.375	<i>r</i> = 0.336, <i>P</i> = 0.004*
Synaptopodin	<i>r</i> = 0.019, <i>P</i> = 0.874	<i>r</i> = −0.100, <i>P</i> = 0.408	<i>r</i> = 0.350, <i>P</i> = 0.003*

GFR, glomerular filtration rate.

Figure 1 Relation between GFR decline and percentage change in urinary expression of podocin. Data are compared by Spearman's rank correlation coefficient.

urinary podocyte loss. Nonetheless, our finding indicates that monitoring of urinary mRNA expression of podocyte-associated molecule has little value for the monitoring of therapeutic response to ACEI or ARB in patients with diabetic nephropathy.

One shortcoming of our study is that the dosage of ACE inhibitor was low. It remains uncertain whether there is any effect – on proteinuria or urinary podocyte marker – by simply increasing the dosage of ACE inhibitor, although recent studies suggest that combination of two drugs at low dose is more effective than giving one at maximal dose (29, 30). In addition, very few patients of our study were treated with diuretics, which would possibly have beneficial effects without increasing blockade of the RAS. It is also important to note that treatment allocation was not randomized in the present study. According to our local health care policy, the government does not cover the drug expense of ARB when a patient was already treated with ACE inhibitor. As a result, patients would receive combination therapy only if they agreed to pay for the ARB. Such a policy may, at least theoretically, pose selection bias between the combination and control groups, although we observe no significant difference in any baseline demographic or clinical parameter between the combination and control groups, except a slightly lower estimated GFR in the former (Tables 1 and 2).

Although we found a statistically significant correlation between percentage change of urinary podocin expression and rate of renal function decline, it is possible that the association is merely a type 1 error due to multiple statistical comparisons. Furthermore, the correlation coefficient was weak and could therefore only explain a small part of the GFR decline. Our result is more for generating hypothesis; further studies are needed to confirm the finding. Although gene expression study remains an expensive and time-consuming process at this

moment, high throughput technology is underway and quantification of the urinary podocyte marker has the potential of widespread clinical utility if proved valid.

During our *post hoc* analysis, we found that urinary expression levels of podocyte-associated molecules were significantly higher in male patients when compared with that of female patients. The observation is similar to that by Orlandi *et al.* who studied circulating nephrin expression (31). The actual reason of this gender effect is unknown. Estrogen seems unlikely to be the determinant because most of the female patients in this study are postmenopausal. It remains possible that contamination of urine sample by genital epithelium in female patients may affect the quality of RNA, but our previous study in lupus nephritis showed that the influence was minimal (32).

We did not perform immunostaining for the urine sediment in this study and, as a result, could not ascertain the cellular origin of mRNA. However, to the best of our knowledge, cells other than podocytes in the urine sediment do not express nephrin, podocin, or synaptopodin. Since the microenvironment of urinary podocytes is quite different from that of renal tissue, the correlation between podocyte-associated molecules mRNA expression in the urine sediment and that of glomeruli needs further exploration. Further studies are also needed to compare urine mRNA of the podocyte-associated molecules and their urine level by western blot (22).

In conclusion, we found that urinary mRNA expression of nephrin, podocin, and synaptopodin correlated with baseline renal function in patients with diabetic nephropathy, and the expression tends to increase with disease progression. Urinary expression of podocyte-associated molecules is largely unaffected by ACEI and ARB therapy. Our result indicates that serial measurement of urinary expression of podocyte-associated molecules may have an additional value for the monitoring of therapeutic response to ACEI or ARB in patients with diabetic nephropathy.

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