
Urine podocin:nephrin mRNA ratio (PNR) as a podocyte stress biomarker

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Abstract

Background. Proteinuria and/or albuminuria are widely used for noninvasive assessment of kidney diseases. However, proteinuria is a nonspecific marker of diverse forms of kidney injury, physiologic processes and filtration of small proteins of monoclonal and other pathologic processes. The opportunity to develop new glomerular disease biomarkers follows the realization that the degree of podocyte depletion determines the degree of glomerulosclerosis, and if persistent, determines the progression to end-stage kidney disease (ESKD). Podocyte cell lineage-specific mRNAs can be recovered in urine pellets of model systems and in humans. In model systems, progressive glomerular disease is associated with decreased nephrin mRNA steady-state levels compared with podocin mRNA. Thus, the urine podocin:nephrin mRNA ratio (PNR) could serve as a useful progression biomarker. The use of podocyte-specific transcript ratios also circumvents many problems inherent to urine assays.

Methods. To test this hypothesis, the human diphtheria toxin receptor (hDTR) rat model of progression was used to evaluate potentially useful urine mRNA biomarkers. We compared histologic progression parameters (glomerulosclerosis score, interstitial fibrosis score and percent of podocyte depletion) with clinical biomarkers [serum creatinine, systolic blood pressure (BP), 24-h urine volume, 24-h urine protein excretion and the urine protein:creatinine ratio (PCR)] and with the novel urine mRNA biomarkers.

Results. The PNR correlated with histologic outcome as well or better than routine clinical biomarkers and other urine mRNA biomarkers in the model system with high specificity and sensitivity, and a low coefficient of assay variation.

Conclusions. We concluded that the PNR, used in combination with proteinuria, will be worth testing for its clinical diagnostic and decision-making utility.

Keywords: glomerular disease; podocyte; proteinuria; urine biomarker; urine podocin:nephrin mRNA ratio

Introduction

An ideal biomarker for prevention of progression of glomerular diseases will predict who will progress to end-stage kidney disease (ESKD) (unless effective intervention is implemented) and who will not progress (and therefore does not need expensive monitoring or exposure to
potentially toxic medications). Progressive podocyte depletion causes glomerulosclerosis and progression to ESKD, and is the likely driver of most forms of progressive glomerular diseases in man [1–9]. This realization provides the opportunity to develop urine podocyte monitoring strategies that could potentially revolutionize the management of glomerular diseases.

Podocytes can be detected in urine in association with glomerular injury using antibodies to podocyte-specific proteins and by podocyte-specific mRNAs [10–28]. We recently reported that nephrin mRNA expression was relatively down-regulated in relation to podocin mRNA both in kidney cortex and in the urine pellet of rats that were progressing to ESKD [27, 28]. The ratio of two cell-specific markers expressed by the same cell would be particularly attractive as a biomarker because it would reduce assay variation and not be subject to alteration caused by urine concentration, quality of recovered RNA, cell viability and contamination by other cells that often complicate urine assays. We therefore used a rat model of progression to test the hypothesis that urine mRNA biomarkers could provide useful information that might be useful for clinical diagnosis and decision-making.

Subjects and methods

hDTR Fischer 344 rat model of progression

All animal studies were approved by the University of Michigan Committee on Use and Care of Animals. Studies were performed as previously described [27–30]. Heterozygous human diphtheria toxin receptor (hDTR) transgenic rats received an injection of diphtheria toxin (DT) in normal saline containing 0.1 mg/mL rat albumin as a carrier. In the first experiment, podocyte injury was initiated using 25 ng/kg DT injected intravenously into 100 g rats (n = 63). In the receiver operating characteristic (ROC) experiment, the DT doses were injected intraperitoneal (IP) (n = 17). Glomerular and interstitial fibrosis on 3 μm Masson’s trichrome-stained sections was done by a blinded observer. The mean glomerular fibrosis score was estimated from 50 consecutive glomerular cross-sections by estimating the proportion of each glomerulus that was sclerosed. The interstitial fibrosis score was estimated using the Metamorph Imaging program (Metamorph Imaging System, Universal Imaging Corp., Downingtown, PA, USA) to measure the percent of the non-glomerular cortex area that stained blue in five randomly selected ×10 images [28]. Paraformaldehyde-lysine-periodeate-perfused and fixed paraffin-embed for sectioning was used to count the podocyte number and glomerular epithelial protein 1 (PTPro) (GLEPP1)-positive podocyte area as a percentage of tuft area using the Metamorph system as previously described [28, 30, 31].

Urine processing

Urine was collected overnight (average 15 h) and centrifuged at 4°C for urine processing. Urine was re-suspended and the pellet was resuspended in RLT/RNase-Free BR Buffer and then frozen at −80°C for assay according to the RNeasy Qiagen protocol (Germantown, MD).

RNA preparation and qRT-PCR assay

The total urine pellet RNA was purified (RNeasy Mini Kit cat. No. 74106; Qiagen). Quantification of the nephrin, podocin, transforming growth factor β1 (TGFβ1), aquaporin-2 (AQP2) mRNA abundance and cDNA synthesis were performed with the 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix, with sample cDNA in a final volume of 10 μL per reaction. TaqMan Probes (Applied Biosystems) were as follows: Rat: NPHS1 (nephrin) cat. no. Ra00575235_m1, NPHS2 (podocin) cat. no. Ra00709834_m1, TGFβ1 cat. no. Ra00572010_m1, AQP2 (aquaporin-2) cat. no. Ra00563755_m1. All data were from 2 μg sample cDNA measured in triplicate. The standard curves were constructed using these serially diluted standards for rats [28]. The CT values were used to analyze mRNA levels from the standard curve using SDS 2.2.2 software (Applied Biosystems).

Statistical methods

Results are shown as mean ± SEM except where otherwise noted. Differences among the two groups were tested by Student’s t-test and among more than two groups by the Kruskal–Wallis test. When the Kruskal–Wallis test was significant, a Scheffe test was carried out for post hoc analysis. Partial correlation coefficients between two variables A and B while controlling for the effects of a third variable C were assessed by performing separate linear regressions of A on C and B on C, then calculating the Pearson correlation coefficient between the residuals from these two regressions. P < 0.05 was considered to be statistically significant.

Results

hDTR rat model of progression

The hDTR transgenic rat model of progression progresses to ESKD over 8–12 weeks following depletion of 30–40% of the podocytes [27–29]. Histologic parameters of progression are illustrated in Figure 1A and B showing progressive glomerulosclerosis and interstitial fibrosis in proportion to podocyte loss [27–29]. Figure 1C–E demonstrates the high correlation between these parameters over a wide range of injury.

Figure 2 shows longitudinal data for progression to ESKD in a subset of animals with more severe disease to illustrate the time course of events during the progression process. As would be expected, systolic blood pressure (BP) and 24-h urine volume increased progressively as animals progressed towards ESKD (Figure 2A and B). Serum creatinine would also be expected to increase progressively over time, but was measured only once at the end of study in these experiments. For correlation analysis shown in Table 1, the data at or close to ESKD were used (indicated by the open boxes at the top of each panel). As would be expected proteinuria shown as the urine protein:creatinine ratio (PCR, Figure 2C) was increased early during progression and remained abnormal throughout the progression process.

Urine mRNA biomarkers are also shown in Figure 2 and follow the changes previously reported for this model system [27, 28]. Twenty-four hour urine AQP2 mRNA steady-state level (a tubular biomarker) changed relatively little during progression (Figure 2D). In contrast, 24-h urine TGFβ1 mRNA (a biomarker of the pro-fibrotic milieu) increased ∼100-fold by 2 weeks after initiation of injury and remained elevated throughout the progression period (Figure 2E). Two urine podocyte markers (24-h urine podocin and nephrin mRNAs) both increased ∼50 to 100-fold during the initial acute injury phase (days 0–21) (Figure 2F and G). However, during the chronic progression phase of injury, the 24-h urine nephrin mRNA returned towards baseline while the 24-h urine podocin mRNA remained elevated throughout the progression phase as previously reported [27, 28]. The ratio of podocin:nephrin mRNAs therefore increased and remained
stable and >10-fold above baseline throughout the chronic progression phase (Figure 2H). For subsequent correlation analysis, we used the mean values obtained over the whole chronic phase of progression (weeks 3–9) as indicated by the open box above each dataset (see Figure 2).

‘Gold standard’ measures of progression

Function follows structure in progressive glomerular diseases. Therefore ‘gold standard’ parameters for progression used in this analysis were glomerulosclerosis and interstitial fibrosis. Figure 1C shows that these two parameters were highly correlated with each other over a wide range of injury \( (r^2 = 0.77) \). Podocyte depletion as measured by either glomerular tuft podocyte number or GLEPP1-positive area of the tuft (%) was highly correlated with glomerulosclerosis \( (r^2 = 0.75 \text{ and } 0.72, \text{ respectively}) \) (Figure 1D and E). The podocyte number and GLEPP1-positive area were also both strongly correlated with interstitial fibrosis.
However, if glomerulosclerosis was statistically controlled using partial correlation analysis, then the strong relationship between podocyte depletion and interstitial fibrosis was lost ($r^2 = 0.08$). This result is compatible with the concept that podocyte depletion primarily drives glomerulosclerosis and that interstitial fibrosis is a downstream event.
correlation when factored by AQP2. Therefore, podocin urine TGF-β tubular marker (AQP2 mRNA). In contrast, both 24-h correlated with by 24-h urine AQP2 mRNA (a tubular marker) serving in injury during progression over and above that provided provided useful information related to ongoing podocyte rate of podocyte loss from glomeruli) significantly (Figure 3A). The 24-h urine podocin mRNA (reflecting the podocyte stress) which highly correlated with all ‘gold standard’ progression parameters of progression (Table 1 and Figure 3B). The PNR correlation was as good as any standard gold parameters of progression (Table 1 and Figure 3A). The 24-h urine podocin mRNA (reflecting the Glomerulosclerosis score Interstitial fibrosis score

Table 1. Correlation coefficients (r²) values calculated from 63-hDTR Fischer 344 rats

<table>
<thead>
<tr>
<th>% Podocyte depletion</th>
<th>Glomerulosclerosis score</th>
<th>Interstitial fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Podocyte depletion</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>Glomerulosclerosis score</td>
<td>0.75</td>
<td>0.72</td>
</tr>
<tr>
<td>Interstitial fibrosis score</td>
<td>0.60</td>
<td>0.58</td>
</tr>
</tbody>
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The ‘gold standard’ progression parameters are shown in the left column for each dataset and are used for comparison with other ‘gold standard’ parameters (top dataset), clinically used biomarkers (second dataset), timed urine mRNA excretion (third dataset) and mRNA biomarkers expressed as ratios (bottom dataset). The r² values >0.5 are highlighted in grey for emphasis.

Clinical biomarker analysis

Table 1 and Figure 3A show the degree to which each clinical biomarker (serum creatinine, systolic BP, 24-h urine volume, 24-h urine protein excretion and the urine PCR) correlated with each ‘gold standard’ progression parameter. As would be anticipated if clinical biomarkers were useful they all correlated well with the degree of glomerulosclerosis and interstitial fibrosis (at the time of euthanasia of rats) over the wide range of injury analyzed.

Urine mRNA biomarker analysis

A similar approach was used to determine the degree to which urine mRNA biomarkers would correlate with ‘gold standard’ parameters of progression. The most powerful urine mRNA marker tested was the urine podocin:nephrin mRNA ratio (PNR, a measure of podocyte stress) which highly correlated with all ‘gold standard’ parameters of progression (Table 1 and Figure 3B). The PNR correlation was as good as any clinical biomarker at correlating with ‘gold standards’ (Figure 3A). The 24-h urine podocin mRNA (reflecting the rate of podocyte loss from glomeruli) significantly correlated with ‘gold standards’ even when factored by a tubular marker (AQP2 mRNA). In contrast, both 24-h urine TGFβ1 and nephrin mRNA lost their significant correlation when factored by AQP2. Therefore, podocin mRNA in contrast to nephrin and TGFβ1 mRNAs also provided useful information related to ongoing podocyte injury during progression over and above that provided by 24-h urine AQP2 mRNA (a tubular marker) serving as a general measure of nephron loss during progression.

Receiver operating characteristic (ROC) analysis of mRNA urine biomarkers using the hDTR rat model of progression

If the above concepts are correct, then the PNR should be a sensitive, specific and predictive biomarker of progression. To test this hypothesis in an independent dataset, we caused a variable degree of initial podocyte injury in the hDTR rat model and then followed the animals for up to 6 months. If during this time period rats developed ESKD, they were labeled as progressors (n = 9). If during this 6-month period they did not progress to ESKD, then they were labeled as non-progressors (n = 8). We then performed ROC analysis using individual urine samples collected over the first 60 days to determine which urine mRNA markers best predicted whether or not rats would be progressors or non-progressors. In these experiments, the animals go through an acute injury phase lasting up to 21 days followed by a chronic progression phase from day 21 through development of ESKD. Figure 4 shows the results of a ROC analysis for prediction of progression using the urine biomarker at each time point in the study. An area under the curve (AUC) value of 1 means that the data showed perfect sensitivity and specificity for predicting which rats would progress and which would not at that particular time point. A value of 0.5 indicates no predictivity. The PNR (lower panel) was the best predictor of progression during the chronic phase of injury, with an almost perfect AUC value of 1. These data from an independent experiment
therefore support the conclusion that the PNR is a sensitive and specific biomarker of progression in this model system.

Coefficient of variation of biomarker measurements
To provide quantitative information on assay variation, we calculated the coefficient of variation (cv) for randomly selected urine samples during the progression period ($n = 100$ samples from 46 rats each measured in a different assay on a different day) and compared these values with the mean for each animal over the progression period. As shown in Figure 5, the PNR assay had a cv of 36%. In contrast, the 24-h urine mRNA values measured alone had a cv that was 3- to 4-fold higher (120–160%). Factoring by AQP2 as a kidney-specific biomarker improved the cv significantly compared with single timed measurements (down to 70–78%). Therefore, expressing data as a ratio with another marker from the same cell or organ improved the cv.

Discussion
As a first step towards developing urine monitoring tools for glomerular diseases, we used a model system in which we have previously proven that podocyte depletion plays a
Fig. 4. ROC analysis of urine mRNA biomarkers to predict progression to ESKD in the hDTR rat model. ROC analysis was used to determine the potential for a biomarker measured at a point in time to predict which hDTR rats receiving a variable degree of initial injury induced by IP injection of DT progressed to ESKD over a 6-month period \((n = 9)\) and which would not \((n = 8)\). Daily urine samples were collected and assayed for nephrin, podocin, TGFβ1 and AQP2 mRNAs. Injury occurs in two phases. An initial acute injury phase caused by the DT injection occurs before 21 days. In rats that receive a critical degree of injury, this first phase is followed by a progression phase until rats reach ESKD. An AUC of 0.5 is a random result. An AUC of 1 is a perfect prediction. The shaded regions show 95% confidence intervals for the true AUC.
mRNA biomarkers was confirmed by ROC analysis in a separate cohort of rats with variable amounts of injury. We therefore conclude that the PNR has the potential for application as a clinical diagnostic and decision-making marker.

Potential biomarkers for progression can be considered in two groups. ‘Cumulative kidney injury biomarkers’ measure the consequences of cumulative events that have taken place over the lifetime of a kidney. This would include measures of remaining glomerular filtration rate (GFR, e.g. serum creatinine concentration and other estimates of GFR), BP, 24-h urine volume and proteinuria resulting from tubulo-interstitial disease. In contrast ‘dynamic kidney injury biomarkers’ become abnormal early during the injury process and remain abnormal while the progression process is active (e.g. urine protein excretion and urine measures of podocyte loss). Dynamic kidney injury biomarkers therefore have the capacity to be sensitive and specific at early time points and to normalize in response to effective treatment or spontaneous disease remission. This is in contrast to cumulative kidney injury biomarkers which will tend to be insensitive at early phases of injury. ‘Cumulative’ and ‘dynamic’ injury biomarkers therefore provide complementary information. Both are required for effective disease management.

Urine protein excretion is well established to be a powerful marker of progression for glomerular diseases in man, such that there is a debate as to whether the degree of proteinuria per se could serve as a surrogate marker for chronic kidney disease (CKD) outcome [40, 41]. Proteinuria is also thought to play a causative role in promoting interstitial fibrosis and thereby in accelerating the progression process [42–44]. However, we know that massive proteinuria occurs in minimal-change disease without progressive glomerulosclerosis or interstitial fibrosis, so at least in the relatively short term, proteinuria per se is sometimes not associated with the progression of glomerular diseases. Proteinuria is also a non-specific marker of many forms of kidney injury. In contrast, the PNR is a specific marker of podocyte injury/stress that is associated with the progression process.

In cardiology parlance, the PNR could be considered as somewhat analogous to troponin, in that it measures a key proximate event that determines the outcome. Blood troponin levels are a measure of direct damage to muscle fibers of the heart. In the case of the podocyte, it is loss (or phenotype alteration) of the key cell of the glomerulus which determines the glomerular outcome. Validation studies will be required to establish the potential utility of the urine PNR and other podocyte markers in defined clinical settings.

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References


