Urinary exosomal Wilms’ tumor-1 as a potential biomarker for podocyte injury

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Zhou H, Kajiyama H, Tsuji T, Hu X, Leelahavanichkul A, Vento S, Frank R, Kopp JB, Trachtman H, Star RA, Yuen PS. Urinary exosomal Wilms’ tumor-1 as a potential biomarker for podocyte injury. Am J Physiol Renal Physiol 305: F553–F559, 2013. First published June 11, 2013; doi:10.1152/ajprenal.00056.2013.—Renal exosomal Wilms’ tumor-1 (WT-1) staining is used to detect podocyte loss in kidney biopsies. We aimed to determine if urinary exosomal WT-1 could serve as a noninvasive biomarker of podocyte injury. We examined WT-1 by Western blot in a human podocyte-like cell line, a mouse model of podocyte injury, and human subjects with podocyte disorders. WT-1 was detected in exosomal fraction of the conditioned media from podocytes and increased 48 h after hTGF-β1 stimulation. Cellular WT-1 decreased in podocytes following hTGF-β1 incubation. In mice with induced podocyte injury, urinary exosomal WT-1 was detected 1 wk earlier than albuminuria and also tracked the effects of angiotensin receptor blocker (ARB) treatment. In addition, urinary exosomal WT-1 levels at 1 wk post-injury correlated with the severity of glomerular injury at 3 wk later. In human subjects, urinary exosomal WT-1 was significantly increased in focal segmental glomerulosclerosis (FSGS) patients compared with healthy volunteers or steroid-sensitive nephrotic syndrome (SSNS) patients. Urinary exosomal WT-1 was also significantly decreased in patients in remission for either FSGS or SSNS or following steroid treatment in six SSNS subjects. We conclude that urinary exosomal WT-1 is a promising noninvasive biomarker with apparent podocyte specificity that can detect early progression and treatment-induced regression of podocyte injury in FSGS or SSNS. These results warrant longitudinal, prospective studies in a large cohort with a range of podocyte diseases.

exosomes; WT-1; focal segmental glomerulosclerosis; collapsing glomerulopathy; podocyte injury

Glomerular diseases, including hypertension and diabetic nephropathy, account for 90% of end-stage renal disease (ESRD), at a cost of $20 billion per year in the US (1). Podocyte dysfunction, injury, or loss is a common determinant in many glomerular diseases caused by metabolic, genetic, and environmental mechanisms (24). Continuing efforts to classify diverse podocytopathies are based on histopathological criteria (4, 5). Renal biopsy is a powerful tool to diagnose and classify glomerular disease but is limited by sampling error and difficulty in obtaining follow-up renal biopsies to track progression during monitoring of treatment efficacy. Total proteinuria and albuminuria are widely available biomarkers for screening, detecting, and monitoring glomerular disease; however, they are not specific for glomerular injury. Disease progression can be monitored by decreases in kidney function by using equations to estimate glomerular filtration rate (15, 22). Recently, novel biomarkers, including cystatin C and β-trace protein (BTP; prostaglandin D2 synthase) have been combined with traditional biomarkers to assess the extent of glomerular injury (6, 12), but both novel biomarkers are affected by corticosteroids (6), a first-line therapeutic for some glomerular diseases. For a glomerular disease biomarker to improve upon proteinuria, it would ideally be a mediator on the disease pathway, it would allow earlier detection of glomerular specific injury, or it would more accurately predict/monitor efficacy of a treatment so that toxic therapeutic agents can be used sparingly or stopped if they are ineffective.

Podocyte dysfunction is a common feature of primary and secondary glomerular disease. Candidate podocyte biomarkers to measure disease activity such as urinary podocalyxin (9) or numbers of detached podocytes in urine sediment (18) have been hampered by variable results or low number of detectable cells. Urine mRNA concentrations of podocyte-specific genes have been more successful in animal models (8, 21), but validation in human subjects has been limited to four lupus nephritis patients (21). WT-1 is a well-known transcription factor required for normal kidney development, and loss of renal WT-1 is associated with many glomerular diseases (17). Decreases in WT-1 staining have been used to confirm podocyte injury in biopsies (23). Urinary WT-1 mRNA was significantly increased in patients with diabetic nephropathy and chronic glomerulonephritis but was undetectable in normal volunteers (13). Lee et al. (14) reported that urinary exosomal WT-1 was detected in 25 of 40 patients with active childhood nephrotic syndrome, but no difference in exosomal WT-1 was observed between focal segmental glomerulosclerosis (FSGS; n = 7) and “non-FSGS” (n = 18, not all of whom underwent kidney biopsy). These findings indicate that the potential of urinary WT-1 as a noninvasive biomarker for podocyte injury needs further evaluation.

We (25) previously detected WT-1 protein in urinary exosomes in two animal models of podocyte injury and from patients with FSGS. In the present study, we examine whether urinary exosomal WT-1 protein can predict the severity of podocyte injury before treatment and can reflect the response to treatment, whether urinary exosomal WT-1 can distinguish between FSGS and ste-
roid-sensitive nephrotic syndrome (SSNS), and how urinary exosomal WT-1 responds to steroid treatment.

MATERIALS AND METHODS

Human podocyte-like cell line. Conditionally immortalized human podocyte cells (AB8/13; gift from Dr. Moin Saleem) (20) were cultured in RPMI supplemented with 10% FBS, insulin-transferrin-selenium (ITS), and 100 U/ml penicillin and streptomycin (Invitrogen). After incubation for 5 days at 37°C under growth-restricted conditions, medium was replaced with serum-free RPMI with or without 10 ng/ml human TGF-β1 (R&D Systems, Minneapolis, MN). Conditioned medium (9 ml) was collected from a T25 flask, or proteins were harvested from cells cultured on 12-well plates after 48 h of incubation, and samples were stored at −80°C.

Mouse model of podocyte injury. A mouse model of collapsing glomerulopathy (CG) was created with podocin/rTTA X TRE/Vpr double-transgenic mice (n = 9, 25–35 g, 12 wk) that were subjected to uninephrectomy and then given doxycycline (Sigma-Aldrich, St. Louis, MO) in drinking water (2 mg/ml) for 5 days. Doxycycline induction (3); CG plus treatment with olmesartan (n = 4), an angiotensin II receptor blocker (ARB); and a control group without doxycycline induction (n = 4). Olmesartan (Bio-Serv, Frenchtown, NJ) was given at 10 mg/kg body wt in the food (11), and 24-h urine samples were collected in metabolic cages before uninephrectomy, before doxycycline, and 1, 2, 3, and 4 wk after doxycycline administration. Under anesthesia at day 28, blood samples were collected from the abdominal aorta, one-half of the left kidney was removed and immediately fixed in 10% neutral buffered formalin solution for paraffin, and then mice were euthanized. Urine and serum were stored at −80°C for later use. Mouse experiments were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and were approved in advance by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

To confirm that nephrotic syndrome was present, urinary albumin excretion and serum albumin were measured as previously described (25). Paraffin-embedded kidney blocks were cut at 4 μm thickness, deparaffinized, rehydrated, and then stained with periodic acid Schiff (PAS) reagent for histological examination. Cortical thickness was assessed by morphometry of PAS-stained slides from mouse kidney. A line bisecting the inner stripe was drawn to exclude most or all of the medulla/papilla, followed by a radial line that generated two 90° sectors, which were further bisected by two lines that created four 45° sectors. Cortical thickness was determined along the resulting five radial lines (see inset, Fig. 2, D–F). Quantification of renal cortex thickness was measured by Image J software. Glomeruli with segmental or global lesions were counted in each kidney and expressed as a percentage of total glomeruli.

Urine samples from FSGS and SSNS patients. All human subjects gave informed consent or assent under IRB-approved protocols at Cohen Children’s Medical Center or NIDDK. Most urine samples were collected at Cohen Children’s Medical Center from healthy volunteers (n = 5) and patients (n = 25) with FSGS or SSNS. FSGS diagnosis was confirmed by biopsy; SSNS was diagnosed based on the response to corticosteroid, in accord with the prevailing standard of care in pediatric nephrology. Single urine samples were collected from FSGS patients (n = 7, including 5 active proteinuric cases and 2 remission cases) and SSNS patients (n = 12, including 5 active cases and 7 remission cases). Paired urine samples were collected in a separate set of SSNS patients (n = 6) before and after steroid treatment.

All urine samples were processed by centrifugation at 1,000 g for 10 min to remove urinary sediments and then stored at −80°C (25). Four milliliters of each urine sample from Cohen Children’s Medical Center were frozen without protease inhibitors before exosome preparation (25). Urine samples from three biopsy-confirmed FSGS patients at the NIH Clinical Center were used to test whether protease inhibitors (Complete Mini, Roche) were necessary to optimize exosomal WT-1 recovery, and whether freezing had any effect on recovery. Laboratory personnel were blinded to the clinical status until completion of Western blotting analysis of urinary exosomal WT-1.

Exosome isolation from cell culture media or urine samples. Exosome isolation was performed as previously described (26). Both media and urine samples were extensively vortexed during thawing with ice remaining. Eight milliliters of cell culture medium or 4–8 ml of urine samples was used for exosome isolation by further differential centrifugation (17,000 g for 15 min and 200,000 g for 1 h). Exosomal pellets were suspended by equal volume of isolation solution and SDS Laemmli buffer with DTT (60 mg/ml), denatured at 60°C for 10 min, and then stored at −80°C until analysis by Western blotting.

Immunoblotting. Gel loading of urinary exosome-associated proteins was normalized by volume for cell culture media, by duration of collection for urine samples from animals, or by urine creatinine for spot urine samples from human subjects, as previously described (25, 26). Protein samples were separated by 1-D SDS/PAGE electrophoresis, and then gels were transferred to PVDF membranes. After blocking with 5% milk (1 g

Fig. 1. Wilms’ tumor-1 (WT-1) expression in a human podocyte-derived cell line and exosomes from conditioned media. A: WT-1 decreased in podocyte lysates 48 h after treatment with transforming growth factor (TGF)-β1 compared with untreated podocytes, and WT-1 density was normalized by β-actin. B: exosomal WT-1 secreted into media increased 48 h after TGF-β1 stimulation compared with untreated podocytes, and TSG101 was unchanged in media exosomes. Media exosomal WT-1:TSG101 ratio increased 48 h post-TGF-β1 treatment. TSG101 Western blot density (×10^5 units) was unchanged by TGF-β1 treatment: 26.1 ± 1.5 (untreated) vs. 24.3 ± 1.2 (TGF-β1, P = 0.9). *P < 0.05 vs. untreated podocytes.
h), membranes were probed overnight at 4°C with rabbit polyclonal antibody to WT-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with peroxidase-conjugated, affinity-purified donkey anti-rabbit IgG (1:100,000; Jackson Immunolabs, West Grove, PA) for 90 min at room temperature. The antibody-antigen reactions were visualized using an ECL Plus Western blotting detection system (GE Healthcare). The densities of WT-1 bands were quantitated by Kodak Image Station 2000 MM.

Statistical analysis. All data are expressed as means ± SE. Differences between groups were analyzed for statistical significance by t-test or one-way ANOVA. The receiver operating characteristics (ROC) curve was analyzed by Graphpad Prism. A P value < 0.05 was accepted as statistically significant.

RESULTS

WT-1 secreted by cultured human podocytes. We confirmed that WT-1 was expressed in cultured human podocytes and that cellular content decreased after TGF-β1 stimulation (Fig. 1A). Then we examined WT-1 in exosomes from the conditioned media of cultured podocytes. WT-1 was detected in conditioned media-derived exosomes and also increased after TGF-β1 stimulation (Fig. 1B). The increase in exosomal WT-1 content is unlikely to have arisen from an increase in exosome release, as levels of exosomal TSG101, a pan-exosomal marker, were unchanged by TGF-β1 (Fig. 1B).

Urine exosomal WT-1 as a biomarker for ARB-attenuated podocyte injury in mice. Rodent collapsing glomerulopathy (CG) was induced by the combination of uninephrectomy and administration of doxycycline to activate the transgene in Vpr transgenic mice (11). Mice developed symptoms of nephrotic syndrome including substantial albuminuria, which was significantly increased above baseline at 2 wk (Fig. 2A) and hypoalbuminemia (Fig. 2B; from control 3.18 ± 0.03 to CG 2.32 ± 0.09 g/dl, P < 0.05). Furthermore, in kidneys from the...
Urinary exosomal WT-1 outperforms albuminuria for early prediction of glomerular injury in mice. We next examined whether urinary exosomal WT-1 could predict the severity of glomerular damage in a mouse CG model of podocyte injury. We previously demonstrated that urinary exosomal WT-1, detectable 1 wk before albuminuria, was significantly above baseline, and then exosomal WT-1 increased along with the progression of glomerular injury by albuminuria and peaked at 4 wk when glomerulosclerosis was evident histologically (25). ARB treatment decreased the excretion of urinary exosomal WT-1 (Fig. 3A). We compared urinary exosomal WT-1 to the urine albumin:creatinine ratio (ACR) at the first week, before glomerulosclerosis would be detectable. There was some overlap between vehicle and treatment groups in urinary ACR (Fig. 3B), because there was a highly variable extent of glomerular damage in the vehicle group. When individual mice were compared within each group, albuminuria was not variable at 1 wk, but levels of exosomal WT-1 at 1 wk clearly had more variability (Fig. 3C), which accurately reflected the variability in glomerular damage seen histologically at 4 wk (Fig. 3E). By contrast, ACR did not predict renal histological change at 4 wk (Fig. 3D). Even those vehicle mice that had slightly higher glomerulosclerosis compared with ARB-treated mice had a small but clear distinction in urinary exosomal WT-1 levels (Fig. 3, C and E).

Urinary exosomal WT-1 differentiates FSGS from SSNS in patients. We extended our urinary exosomal WT-1 findings from animal models by testing a small cross-sectional cohort that included subjects with FSGS or SSNS; both groups included subjects with active nephrotic syndrome as well as patients in remission. Urinary exosomal WT-1 was strikingly increased in FSGS subjects with active nephrotic syndrome (vs. control subjects) and significantly decreased in SSNS subjects. The area under the curve (AUC) was 0.96, and ROC curve for urinary exosomal WT-1 in active FSGS and active SSNS patients (Fig. 2, C, E, and F) and the glomerular lesions (Fig. 2, H and I).

The major findings in the present study are the following. 1) WT-1 is secreted in the exosome fraction of conditioned medium from cultured podocytes. TGF-β1 increased exosomal WT-1 and decreased intracellular WT-1 in cultured podocytes; 2) urinary exosomal WT-1 predicted the onset of disease earlier than proteinuria in a mouse model of collapsing glomerulopathy. WT-1 at 1 wk is better than proteinuria to predict renal histological change at 4 wk (Fig. 3E). By contrast, ACR did not predict renal histological change at 4 wk (Fig. 3D). Even those vehicle mice that had slightly higher glomerulosclerosis compared with ARB-treated mice had a small but clear distinction in urinary exosomal WT-1 levels (Fig. 3, C and E).
ARB treatment effect 3 wk later; and 3) urinary exosomal WT-1 could distinguish active FSGS from active SSNS, although the number of patients was small.

Exosomes from urine have the potential to diagnose kidney diseases because exosomes can come from any epithelial cell lining the nephron. However, exosomes do not inherently have specificity for exactly that reason. One way to overcome this limitation of exosomal-derived biomarkers is to focus on cell type-specific proteins. WT-1 is constitutively expressed on podocytes in healthy adult kidneys (16), and WT-1 expression decreased in kidney biopsies from primary FSGS (3). These findings suggest that WT-1 may be important for the maintenance of normal podocyte function. We found that, in vitro, WT-1 was detectable in human podocytes and changed with TGF-β1 stimulation, suggesting that WT-1 in exosomes may reflect podocyte-specific changes in function.

However, the application of urinary exosomal WT-1 in clinical settings remains unclear. There is a large unmet clinical need to adequately monitor the progression of FSGS and response to therapy. If disease progression or response to therapy could be predicted earlier, clinical decision-making could be enhanced. For example, the first-line therapy of corticosteroids can have several undesirable off-target effects, which could be minimized if this therapy were discontinued earlier in nonresponsive patients. Other experimental and future therapeutics may also have toxicities that would benefit from an earlier readout on efficacy. Proteinuria and albuminuria are used as surrogate markers for glomerular function, but they do not necessarily reflect podocyte damage, as other factors, such as tubular reuptake of albumin through megalin/cubulin, may affect net urinary albumin excretion. An exosomal biomarker such as WT-1 can serve as an alternative biomarker or could complement proteinuria/albuminuria. When we compared the time course of exosomal WT-1 and albuminuria in individual mice, there was substantial variability in the severity of FSGS as measured by glomerulosclerosis at 4 wk after induction of podocyte-derived Vpr protein. This variation was accurately predicted by exosomal WT-1 levels at 1 wk, clearly outperforming albuminuria. There may be sampling bias introduced by sectioning due to different sizes of sclerotic and patent glomeruli. Because the transgenic Vpr mouse model induces a severe form of FSGS, collapsing glomerulosclerosis, our in vivo results may need to be further validated in other animal models with less podocyte injury.

Next, we examined whether urinary exosomal WT-1 could distinguish between FSGS and SSNS, as predicted in the animal models. We found that urinary exosomal WT-1 not only distinguishes between active FSGS and active SSNS but also between active SSNS patients and SSNS patients in remission. Although the ROC AUC was high at 0.96, these results are preliminary because of the small number of patients. In a small longitudinal study, urinary exosomal WT-1 also
significantly decreased in SSNS after steroid treatment compared with pretreatment.

If these results from human subjects can be validated in a larger cohort of patients with podocyte injury by various causes, it may be possible to monitor patients more frequently during progression and treatment of FSGS and SSNS in order to accelerate clinical decision-making, such as limiting exposure to toxic therapeutics. Furthermore, larger cohorts may reveal differences between FSGS, SSNS, and other complex podocyte-associated glomerular diseases such as diabetic nephropathy or lupus nephritis, but additional biomarkers may be needed to complement WT-1 to increase the specificity of diagnosis.

However, there are several technical challenges in expanding our studies on exosomal WT-1, as Lee et al. (14) may have encountered. They recently reported that urinary exosomal WT-1 was detected in 25 of 40 patients with childhood active nephrotic syndrome, and, contrary to our findings, they found no difference between FSGS and non-FSGS subjects, although most of the latter group did not undergo kidney biopsy and so the diagnosis is unknown. Also, in contrast to our findings, they could not detect WT-1 in 15 of 40 subjects. One possible reason for these discrepancies between their results and ours may be sensitivity of detection. Interlaboratory differences in Western blot threshold of detection are difficult to compare without a defined purified standard and/or a more readily quantifiable assay such as ELISA. Unfortunately, exosomal WT-1 is not amenable to a conventional ELISA; the exosomal membrane would need to be solubilized by detergent, but that would likely interfere with the antibody-WT-1 interactions. Another possible reason for the discrepancy could be the trapping of exosomes in the 17,000-g pellet during storage at −80°C, which can be mitigated by vigorous vortexing (26) and/or treatment with dithiothreitol (7). We examined whether freezing or addition of protease inhibitors could affect detection of exosomal WT-1, as had been previously shown for NKCC (26), but we found that neither freezing nor protease inhibitors alone could explain the discrepancy. Finally, there may be other factors that affect the level of urine exosomal WT-1 in patients, such as genetics, nutrition, or other environmental factors. An alternative method to detect urinary WT-1 does not require exosome isolation; as reported by Kubo et al. (13), WT-1 mRNA was detected in urinary sediments from patients with various glomerular diseases, but the variability was apparently higher, and AUC analysis was not performed. Other noninvasive methods to evaluate podocyte-specific injury include counting numbers of podocytes in urine sediment by podocalyxin immunofluorescent staining (10), measuring the podocin/AQP2 mRNA ratio in urinary sediments (21), or assessing direct podocalyxin protein in whole urine with a self-developed ELISA kit (9). Each method has inherent limitations, so a panel of podocyte biomarkers might increase diagnostic accuracy for specific podocyte injury.

All urinary biomarkers, including albuminuria/proteinuria, benefit from normalization to properly set thresholds for kidney disease diagnosis. Whereas creatinine is used to normalize albumin excretion, exosomal biomarkers may be improved by normalizing for a biomarker that is constant for exosome excretion. TSG101 may adequately normalize exosomal biomarkers from cultured cells but would need to be validated in vivo; for example, TSG101 is overrepresented in cell-mediated rejection patients compared with all transplant patients, but the number of patients is small (19). TSG101 and other pan-exosomal biomarkers have been validated (7) but only for normal humans. One conceptual limitation is that if podocyte-derived exosomes are a small percentage of the total number of urinary exosomes (the percentage is unknown), then changes in overall podocyte exosome excretion that confound the excretion rate of exosomal WT-1 levels would not be adequately corrected by a marker such as TSG101 that is found in exosomes from all cell types. Normalizing by urine creatinine may be the best method currently available, but it remains to be determined what normalization method will most robustly separate the two groups (active SSNS from FSGS) in larger cohorts.

Our data suggest that urinary exosomal WT-1 could be a useful noninvasive, specific biomarker for early detection of podocyte injury, monitoring progression, and predicting response to therapy, to improve care for FSGS and SSNS patients. Our findings need to be replicated and extended to larger study groups with diverse podocyte diseases with longitudinal follow-up.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


