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ORIGINAL

Urinary Exosomal mRNA of WT1 as Diagnostic and Prognostic Biomarker for Diabetic Nephropathy

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Abstract : Diabetic nephropathy (DN) is the major cause of end-stage renal failure and is associated with increased morbidity and mortality as compared to other causes of renal disease. Albuminuria is often the first clinical indicator of the presence of DN. However, albuminuria or proteinuria is a common symptom in patients with various renal disorders. Therefore, specific biomarkers for the diagnosis of DN are required. A primary hallmark of DN is the progressive damage and death of glomerular podocytes, resulting in the leaking of proteins into the urine. Urinary exosomes released by podocytes are microvesicles containing information of the originated cells. Podocyte-derived signal transduction factors (PDSTFs) are good candidates to assess podocyte injuries. The profile of PDSTFs in urinary exosomes from patients with DN is different from that from patients with minimal change nephrotic syndrome. In addition, PDSTFs molecules in exosomes were derived from primary murine podocytes under high glucose conditions. Among PDSTFs in urinary exosomes, Wilms tumor 1 (WT1) levels reflected damage of diabetic glomeruli in the patients. Urinary exosomal WT1 can predict the decline in eGFR for the following several years. In conclusion, urinary exosomal WT1 is a useful biomarker to improve risk stratification in patients with DN. J. Med. Invest. 65 : 208-215, August, 2018

Keywords **:** *diabetic nephropathy, exosome, biomarker, WT1, PDSTF*

INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of chronic kidney failure. About 30-40% of patients with type 1 or type 2 diabetes develop evidence of nephropathy, but recent reports indicate that patients with type 2 diabetes can commonly progress to a significant degree of renal impairment while remaining normoalbuminuric state (1, 2). In contrast, several biopsy studies have shown that albuminuric patients with type 2 diabetes frequently suffer from nondiabetic kidney disease (3-5), but the exact prevalence is not known. Among the characteristic findings of DN, podocytes are involved in the development of glomerular hypertrophy, podocytopenia, glomerulosclerosis, and foot process effacement (6). Together with glomerular endothelial cells and glomerular basement membrane, podocytes form the glomerular filtration barrier in the kidney. Podocyturia may be an expression of glomerular disease and is evaluated using urinary podocyte-specific molecules. Given their dysregulation in kidney disease, podocytes and their specific proteins and mRNA pose as attractive candidates as either diagnostic or predictor biomarkers of disease (7). Jim *et al.* investigated the morphologic alterations of podocyte-specific proteins in diabetic nephropathy biopsies from patients with Type 2 diabetes mellitus and found significant downregulation of synaptopodin, podocin and nephrin expression in the diabetic group as compared to controls (8). Thus, determination of which markers are truly important for podocyte injuries due to diabetes mellitus in terms of diagnosis, prognosis and therapy represents a critical issue.

Although microalbuminuria remains the gold standard for early detection of DN, it is not a sufficiently accurate predictor of DN risk. In addition, the gold standard procedure for diagnosis is a kidney biopsy, but this procedure incurs a risk of bleeding complications of variable severity, from transitory hematuria or asymptomatic hematoma to life-threatening hemorrhage. From such reasons, the diagnosis can be made in most cases without a kidney biopsy. Exosomes are 40-100 nm membrane vesicles secreted into the extracellular space by numerous cell types. These structures can be isolated from body fluids including urine and plasma. Exosomes were first discovered over 30 years ago and were considered to be little more than a means of cellular garbage disposal (9). The most important biological function of exosomes is their possible use as biomarkers in clinical diagnosis. Urinary exosomes are an appealing source for biomarker discovery as they contain molecular constituents of their cell of origin, including proteins and genetic materials, and they can be isolated in a non-invasive manner. Urinary exosomes are secreted into the urine when a multivesicular body fuses with the membrane of cells from all nephron segments. One of the first publications describing vesicles in the urine dates back to 1987 when Wiggins *et al.* described the presence of exosomes in urine from nephrotoxic nephritis rabbit (10). For DN, considerable advances have been achieved with biomarkers derived from urinary exosomes. Accordingly, Wilm's tumor-1 (WT1) protein expression appears to increase with the decline of the renal function in DN (11). Interestingly, urinary WT1, most likely derived from the podocyte, may thus qualify as a simple marker of podocyte injury rather then a specific marker of DN (12, 13). However, the detection of WT1 protein in western blot in

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these reports was ambiguous, because large amount of albumin in loaded samples distorted the bands and there were no adequate controls to exclude non-specific bands. Moreover, the direct useful-ness of measuring WT1 proteins as biomarkers is hampered by diffi-culty in reproducibility in terms of quantification. Interest in uri-nary exosomes intensified after the discovery that they contain not only protein but also mRNA and microRNA (miRNA) markers of renal dysfunction and structural injury.

The Wilms' tumor suppressor protein WT1 functions as a tran-scriptional regulator of genes controlling growth, apoptosis, and differentiation. WT1 has been shown to play crucial roles during embryogenesis, in particular during kidney development. The per-sistent expression of WT1 throughout life suggests that WT1 plays a role in the homeostasis of the mature podocyte (14). WT1 regulates the expression of many genes indispensable for podo-cyte's development or homeostasis, such as podocalyxin (15), nephrin (16, 17), BMP7 and so on (18). WT1 mRNA expression was reported in a temporal pattern that is restricted from embryo to short after birth (19). WT1 null mice lack kidneys (20), and, when engineered to express a human WT1 transgene, the animals sur-vive but develop mesangial sclerosis (21), depending on the WT1 expression level. In this study, we evaluated the significance of urinary exosomal WT1 as a non-invasive diagnostic and predictive biomarker in patients with DN.

MATERIALS AND METHODS

Study design–Twenty patients with overt or heavy proteinuria due to DN or minimal change nehrotic syndrome (MCNS), and 5 healthy subjects were enrolled in this study. DN and MCNS were proven by renal biopsy. Type 2 diabetes mellitus was diagnosed according to the Japan Diabetes Society criteria (22). All of the procedures were performed in accordance with the guidelines of the Helsinki Declaration on Human Experimentation and the Ethical Guidelines on Clinical Research published by the Japanese Health, Labour and Welfare Ministry. This study was approved by the Ethics Committee of Tokushima University, and informed written consent was obtained from all patients and healthy sub-jects.

Laboratory examination–Creatinine in serum was measured by a enzymatic method. Twenty four-hour urine protein in g/day was determined to multiply the 24-hour urine volume by the urine protein concentration measured using a pyrocatechol violet molyb-date dye-binding method. Body mass index (BMI) was calculated by dividing weight (in kg) by height squared (in m^2).

Urine preparation and Isolation of Urinary Exosome–Urine sam-ples (50-150 mL) were collected from healthy subjects and pa-tients, (before biopsy in the case of patients) in sterile containers and centrifuged at 3000 rpm fixed angle rotor for 10 min at room temperature (within 1 hour of collection) to remove any particulate matter including cells and cell debris. The clarified urine was stored at -80°C before further analysis. The supernatants were ul-tracentrifuged for 1 h at 70,000 xg using an angle rotor (RP-65 ; Hitachi Koki Co, Ltd.). The pellets were resuspended in 5 ml of 0.25 M sucrose, 20 mM HEPES-NaOH (pH 7.2) and pour carefully on top of 30 ml sucrose density gradients (2-0.25 M sucrose, 20 mM HEPES-NaOH (pH 7.2)) in 40 PA centrifuge tube (329607A ; Hitachi Koki Co, Ltd.). That sample were ultracentrifuged for 20 h at 100,000 xg using a swing rotor (RPS-28SA, Hitachi Koki Co, Ltd.) and fractionated to 2 ml x 12 samples in 5 PA centrifuge tube (332245A ; Hitachi Koki Co, Ltd.) by collecting from the bottom of the tube. The fractions were diluted with 3 ml PBS, and ultra-centrifuged for 1hr at 200,000 xg using a swing rotor (RPS-50-2 ;

Hitachi Koki Co, Ltd.). The pellets were resuspended in 50 µl PBS. The recovery efficiency and purity were analyzed by silver staining and western blot.

Western blot–Urinary proteins were resolved by SDS-PAGE ac-cording to Laemmli method. Cultured cells were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonindet P-40, 0.25% SDS, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsul-fonyl fluoride, 10 mg/ml of aprotinin), and incubated for 1 h at 4°C. After centrifugation, the supernatants were used as total cell lysates. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Hybond-ECL ; GE Healthcare) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at a constant voltage of 10 volts for 30 min. Membrane were probed with the following antibodies : mouse monoclonal anti-WT1 antibody, Clone 6FH2 (Millipore, Billerica, MA) at a dilution of 1 : 500 ; mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich) at 1 : 5000 ; rabbit polyclonal anti-WT1 antibody (Santa Cruz Biotech-nology, CA) at 1 : 500 ; mouse polyclonal anti-CD63 antibody (Santa Cruz Biotechnology, CA) at 1 : 1000 ; rabbit polyclonal anti-CD81 antibody (Cosmobio, Japan) at 1 : 1500 as a primary antibody, re-spectively. ECL Western blotting detection system (GE Healthcare). On the one hand, the gel was stained using the silver staining method (Bio-Rad Silver Stain).

RT-PCR and Quantitative RT-PCR analysis

Purified human fetal kidney and adult kidney total RNA (25 µg) were purchased from Agilent Technologies Inc. (Santa Clara, CA) and diluted to 250 ng/µl. Total RNA was extracted from collected exosomes and cultured podocytes using the TRIzol reagent (Invitrogen). The RNA was reverse-transcribed with oligo(dT) primer using a SuperScript first-strand synthesis kit (Invitrogen) to generate the first-strand cDNA, followed by PCR to detect the expression of podocyte-specific genes and GAPDH. The sequences of the PCR primers were as follows : 5'-GGACATAGTCTGC-ACTGTCGAT-3' and 5'-GGCAAATCTGACAACAAGACG-3' for Nephrin ; 5'-GTACGAGAGCGATAACCACACA-3' and 5'-GGC-TTTTCACCTGTATGAGTCC-3' for WT1 ; 5'-AAAACCAAAGAA-ACCACCACCT-3' and 5'-GGCATCTTTGGTCTATTTGCAG-3' for CD2AP ; 5'-GCTACTACCGAATGGAAAATGC-3' and 5'-AGTTC-TGTTGCTGGGAGAAGAC-3' for Podocin ; 5'-TGCACAAAATC-AACAATGTGAA-3' and 5'-TTCTCGTAGTCCTCCATCAGGT-3' for Actn4 and 5'-TGCATGGCCGTTCTTAGTTGGT-3' and 5'-CGCTACACTGACTGGCTCAGCG-3' for Rn18s. The reaction products were separated on 1.5% agarose gel and stained with ethidium bromide. In quantitative RT-PCR analysis, amplification was conducted in MiniOpticon real-time PCR detection system (Bio Rad) using TaqMan gene expression assays (Applied Biosys-tems, CA). The cycling parameters were 10 minutes at 95°C, followed by 50 cycles of 15 sec at 95°C and 60 sec at 60°C. Normalization was performed using Rn18s as internal standards.

Histological Studies–Histopathological studies were performed on human tissues. Kidney specimens (n = 20) were obtained from renal biopsies. Kidney tissue blocks for light microscopy examina-tion were fixed with Dubosq-Brazil's solution and embedded in paraffin. Cryopreserved kidney tissues were cut in 4-µm-thick sections and fixed in acetone for 5 min. Multiple sections were prepared and stained with periodic acid silver methenamine (PAM) and periodic acid-Schiff's reagent (PAS). Kidney sections were treated as previously described (23, 24). For immunofulorescence, cells were grown on type I collagen coated glass coverslips and fixed using 4% paraformaldehyde in PB for 10 min. Subsequently, cells were permeabilized with 0.1% Triton X. Sections were incu-bated with the anti-WT1 (Santa Cruz, CA), and anti-Nephrin (IBL) antibodies followed by incubation with the appropriate fluorescent

secondary antibodies. All procedures were performed at room temperature. Specimens were viewed with a confocal laser scanning microscopy (LEICA, Wetzlar, Germany).

Cell culture–Conditionally immortalized murine podocytes were purchased from Cell Lines Service (CLS, Eppelheim, Germany) (25). Podocytes were grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 4 mM glutamine, 5% exosome-free fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin. Podocytes between passage 5 and 10 were cultured on the plates. The cells were passaged routinely at 80% confluence by using Accutase (Millipore, Billerica, MA). Conditionally immortalized podocytes were propagated under permissive condition at 33°C as previously described (25). To induce differentiation, cells were switched to 37°C (nonpermissive condition). The subconfluent cell monolayers were cultured in serum-deprived media containing 5 mM D-glucose and 0.5% FBS for 24 h and then divided into three experimental groups according to glucose concentration. The normal glucose (NG) group comprised confluent cell monolayers cultured with 5 mM D-glucose, the high glucose (HG) group was cultured in 30 mM D-glucose and the osmotic control (OC) group was cultured in 30 mM D-mannitol. Confluent cells were taken at 24 h after stimulation and used for the extraction of protein. Cell nuclear and cytoplasmic fractions were prepared using a nuclear/cytosol fractionation kit of Biovision Inc. (Mountain View, CA, USA) according to the manufacture's direction.

Statistical analysis

For analysis of differences between patients with DN and MCNS, the Mann Whitney U test for non-normally distributed data. The area beneath the receiver operating characteristic (ROC) curves was used to show the power of a biomarker in diagnosis of DN. Specificity, sensitivity were calculated. Statistical significance was accepted as p < 0.001 in all tests.

RESULTS

Exosomes Are Present in Human Urine

Isolation of exosomes from cell culture media and body fluids is presently a tedious, non-specific, and difficult process. The widely used approach is based on ultracentrifugation in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes away from other vesicles and particles (26). Exosomes settle at the density of 1.13 to 1.19 g/ml as can be seen in Figure 1A that shows a silver staining of fractions following sucrose density gradient centrifugation of exosomes. Samples were next analyzed by Western blots with antibodies specific to CD63 and CD81 (Fig. 1B, C), well characterized exosomal markers (26, 27). We picked fractions 5 and 6 that correspond to the density of exosomes and also showed highest enrichment of proteins and the surface molecules CD63 and CD81.

Podocyte-derived signal transduction factors Are Present in Urine from Patients with CKD

There is a database of urinary exosome proteins is based on published protein mass spectrometry data from the NHLBI Epithelial Systems Biology Laboratory (ESBL). The set of identified podocyte vesicle proteins was compared to protein sets extracted from ExoCarta (http : //exocarta.ludwig.edu.au/, April 2011 version), a database specially dedicated to exosomes from various species and their components (28). According to the ExoCarta database, 6 podocyte-derived signal transduction factors (PDSTFs) have been identified in human exosomes isolated from human urine. All data are from urinary exosomes isolated from patients
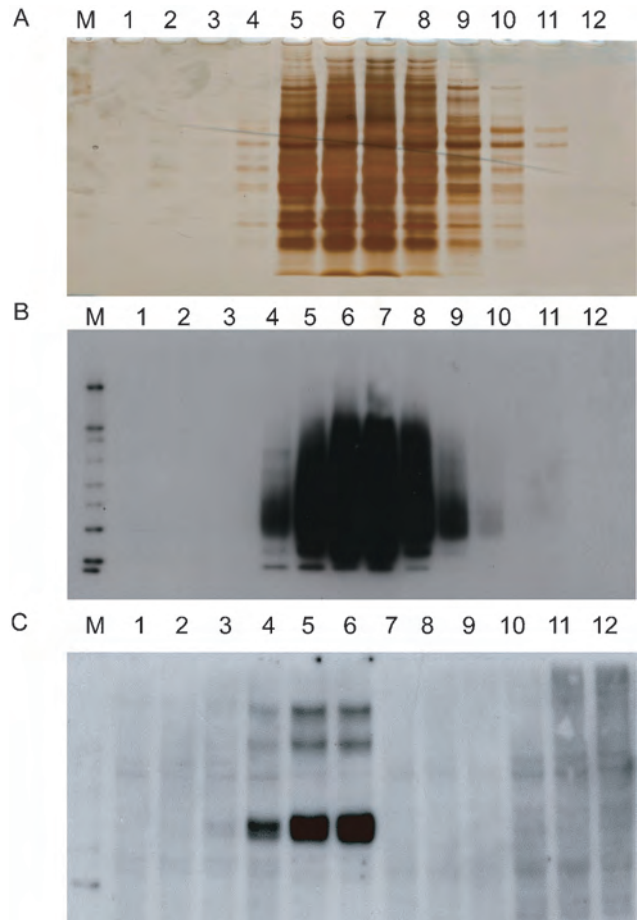

Figure 1. Isolation and fractionation of urinary exosome on a sucrose gradient density.
Exosome pretreated with lysis buffer was loaded on a sucrose density gradient prepared by layering 12 sucrose solutions of concentrations. The gradients were removed from the top and the density of each fraction (lane 1 to 12) was calculated by weighing a fixed volume of each fraction. The fractions were analyzed by silver staining (**A**), western blot analysis for CD63 (**B**), and CD81 (**C**) in exosome. The results are representative of at least five independent experiments. M ; molecular size marker.

with DN or MCNS and healthy human volunteers (29, 30). First, we examined whether PDSTFs and aquaporin-2 (AQP2) is expressed in the exosomes from 3 groups : patients with DN, patients with MCNS and healthy subjects. The water channel AQP2 is one biomarker that can be readily measured in urine (31). Excretion of AQP2 in urinary exosome was reported to be detected from healthy subjects who had no evidence of recent kidney or urinary tract disease (32). Among PDSTFs in exosome, CD2AP was expressed in all groups as well as AQP2 (Fig. 2A). Next, we investigated whether PDSTFs show significant difference between patients with DN and MCNS. Out of 6 molecules of PDSTF, the WT1 mRNA in urinary exosomes was positive by RT-PCR method only in the patients with DN (Fig. 2B).

Glomerular immunoreactivity for WT1 in patients with DN or MCNS–We investigated the association between glomerular expression of WT1 and podocyte injuries in patients with DN and patients with MCNS. Reduction of glomerular immunoreactivity for WT1 was correlated with the severity of sclerotic lesions in diabetic renal glomeruli, as previously reported (33) (Fig. 3). On the
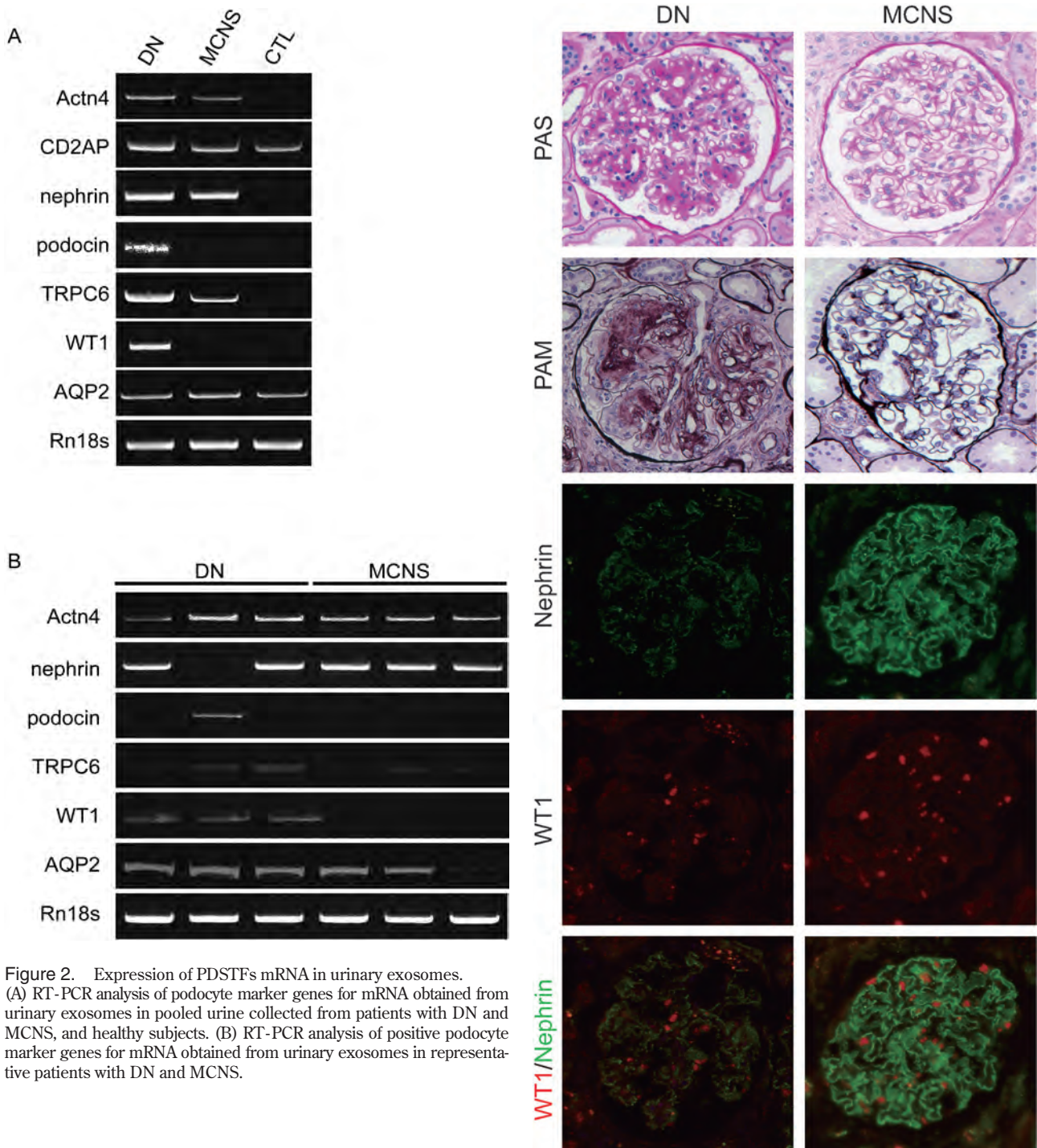
Figure 2.   Expression of PDSTFs mRNA in urinary exosomes.
(A) RT-PCR analysis of podocyte marker genes for mRNA obtained from urinary exosomes in pooled urine collected from patients with DN and MCNS, and healthy subjects. (B) RT-PCR analysis of positive podocyte marker genes for mRNA obtained from urinary exosomes in representative patients with DN and MCNS.

Figure 3.   Light Micrograph and Glomerular expression of WT1 in patients with DN and MCNS.
Representative photomicrographs of periodic acid-Schiff methenamine (PASM), periodic acid-Schiff staining (PAS), and immunohistochemical staining of WT1 and Nephrin are shown (original magnification x 400). The left panels are data from patients with DN, and the right panels are from patients with MCNS.

other hand, the reduction in number of WT1-positive podocytes was not observed in patients with MCNS. Moreover, because nephrin gene expression is transcriptionally activated by WT1 (34), glomerular Nephrin expression was reduced in parallel with WT1 expression in patients with DN (Fig. 3). These histological observations suggest that the expression levels of WT1 is not associated with the severity of proteinuria.

Cultured Podocyte-derived WT1 in exosome in high glucose stimulation

To elucidate the mechanisms of exosome secretion from podocyte, we examined the expression of WT1 in cultured podocytes with high glucose stimulation. The expression of WT1 was decreased in podocytes treated with high glucose. Similarly, mannitol stimulation as an osmotic control presented a slightly decreased

expression of WT1 protein (Fig. 4A). In the steady state, WT1 regulates nephrin transcription as a nuclear transcriptional factor through binding to the promotor region of nephrin (35). Next, we examined the distribution of WT1 protein in podocytes treated with

high glucose. High glucose stimulation significantly induced cytoplasmic translocation of WT1. This phenomenon was not observed in podocytes treated with mannitol (Fig. 4B). Cytoplasmic translocation of WT1 may be followed by release extracellularly. Actually we confirmed that conditioned media from cultured podocytes exposed high glucose release exosomes (Fig. 4C). However, we could not detect WT1 protein in the exosomes obtained from cultured media. This is probably due to the relatively low amount of exosomal WT1 protein and due to the threshold of western blot. To cope with this problem, we performed RT-PCR analyses, indicating WT1 mRNA was contained the exosomes from cultured media (Fig. 4D). Thus, high glucose stimulation significantly affected homeostatic balance in podocyte, resulting in cytoplasmic translocation of WT1 and release exosome containing both WT1 protein and mRNA.
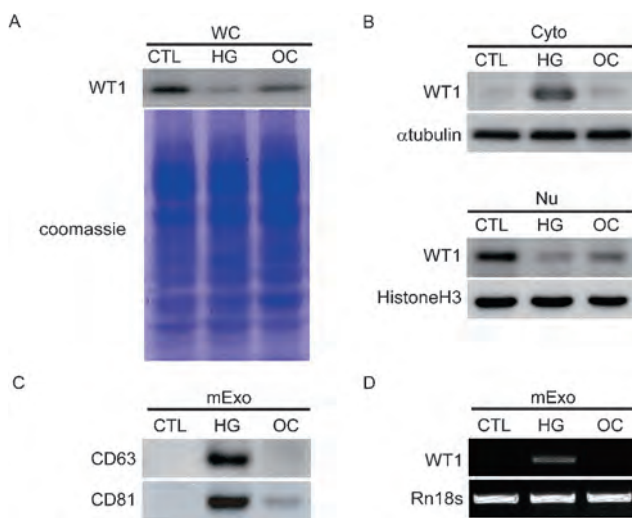
Table 1. Demographic and clinical characteristics of patients with DN or MCNS

	DN (n = 10)	MCNS (n = 10)	p value
Age, y	57.0 ± 6.2	32.8 ± 15.1	< 0.001
Weight, kg	64.9 ± 8.0	65.0 ± 14.3	0.837
Height, cm	166.2 ± 8.8	162.9 ± 9.4	0.681
BMI, kg m⁻²	23.8 ± 3.6	23.6 ± 4.1	0.931
SBP, mmHg	143.0 ± 16.7	112.2 ± 10.0	< 0.001
DBP, mmHg	80.4 ± 5.8	65.4 ± 7.1	< 0.001
Cr, mg/dL	1.4 ± 0.8	0.8 ± 0.2	0.099
eGFR	52.8 ± 27.3	92.7 ± 20.1	0.006
BUN, mg/dL	20.6 ± 10.3	11.4 ± 4	0.059
UA, mg/dL	5.6 ± 1.4	6.5 ± 1.8	0.714
u-prot, mg/dL	249.6 ± 183.0	736.1 ± 793.5	0.340
u-Cr, mg/dL	56.9 ± 22.8	161.6 ± 108.8	0.001
PCR	5.0 ± 3.5	4.2 ± 2.6	0.667

All values given as mean (standard deviation) ; BMI = body mass index ; SBP = systolic blood pressure ; DBP = diastolic blood pressure ; PCR = protein-creatinine ratio ; n = sample size

Figure 4. WT1 in exosome in media from cultured podocytes exposured with high glucose or mannitol.
(A) WT1 protein in mouse podocytes cultured in exosome-free medium containing normal glucose (CTL, 5.5 mM), high glucose (HG, 30 mM) or D-mannitol (30 mM) (OC) for 24 h was monitored by western blot. Counter stained with coomassie blue was shown in order to visualize total protein and thus confirm equal sample loading. WC ; whole cell lysate. (B) Western blot analysis for WT1 of nuclear (Nu) and cytoplasmic (Cyto) fraction and the loading controls histone H3 and α-tubulin. (C) Western blot analysis for CD63 and CD81 as exosome markers for exosomes obtained from conditioned media (mExo) for cultured podocytes. (D) RT-PCR analysis for WT1 mRNA obtained from exosome in cultured media. Rn18 gene was used as internal control. The results are representative of at least three independent experiments.

Patients' demographic and clinical characteristics and evaluation for CKD patients

Baseline characteristics of patients with MCNS or DN are shown in Table 1. Average age at biopsy was 44.8 ± 16.6 years, and mean follow-up time after renal biopsy was 57.2 months (range : 7-98 months). Although patients with DN were older, had higher SBP and DBP, There was no significant difference in proteinuria levels and renal function, that is, serum creatinine levels or eGFR values between the two groups. Urinary exosomal WT1 mRNA were significantly increased in the patients with DN compared with patients with MCNS and control subjects (Fig. 5A). Next, receiver

operating characteristic (ROC) curve analysis was performed to examine whether urinary exosomal WT1 mRNA could be used as a diagnostic biomarker for patient with DN. It showed an area under the ROC curve (AUC) of 0.705 (Fig. 5B). The result indicated that urinary exosomal WT1 mRNA might be a good candidate biomarker to diagnose DN. Next, the elevated value of urinary exosomal WT1 mRNA on rapid decline in kidney function was examined in our patients. During the follow-up period, 15.0% (3/20) patients with DN developed end-stage renal disease (ESRD) (Fig. 5C). On the other hand, no patients with MCNS developed ESRD. The rate of progression to ESRD was lower in the patients with low urinary exosomal WT1 mRNA (0% vs. 15%, P = 0.049). In conclusion, patients with DN whose WT1 mRNA values high developed rapid decline in eGFR.

DISCUSSION

Diabetes mellitus is a chronic disease that affects 366 million people worldwide (6.4% of the adult population) and is expected to rise to 552 million by 2030 (36). This is a clinical syndrome characterized by persistent albuminuria (> 300 mg/24 h, or > 300 mg/g creatinine), a relentless decline in glomerular filtration rate (GFR), raised arterial blood pressure, and enhanced cardiovascular morbidity and mortality. Although microalbuminuria is a widely used indicator for DN, its diagnostic accuracy is limited by the fact that structural damage might precede albumin excretion (37). In addition, microalbuminuria is not specific for the presence of DN alone as non-diabetic patients with progressive chronic kidney disease may also develop microalbuminuria (38). At present, the most reliable diagnostic method is the renal biopsy to diagnose DN, but it is impossible to perform biopsies for all cases due to its invasiveness. Therefore, sensitive and specific biomarkers that can predict patient's susceptibility to DN are needed (39). Increasing evidence suggests that these glomerulopathies are frequently caused by primary lesions in the renal podocytes. Among the characteristic findings of diabetic nephropathy, podocytes are involved in the development of glomerular hypertrophy, podocytopenia, glomerulosclerosis, and foot process effacement (40). Podocyturia is well described in many glomerular diseases including DN (41, 42). However, the number of podocyte in urine is a result of podocyte
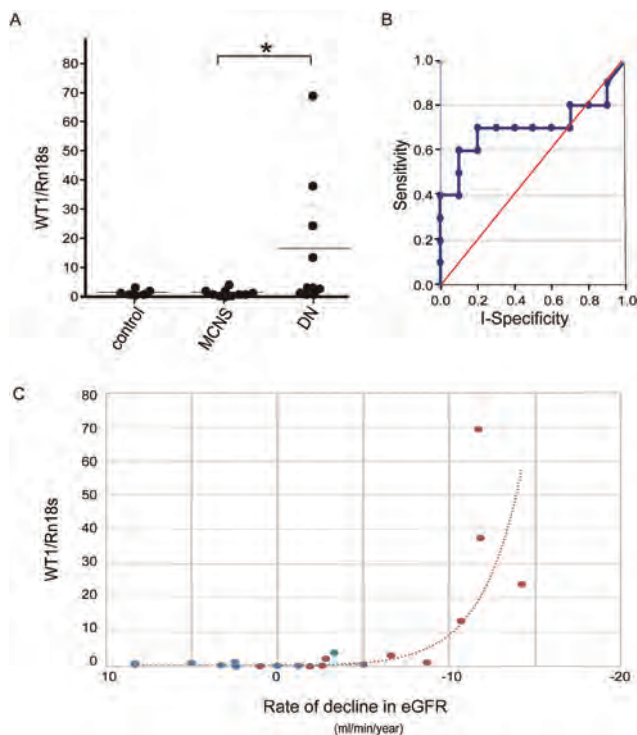
Figure 5.   Expression levels of WT1 and changes in eGFR after the measurement of WT1.
(A) Expression of urinary exosomal WT1 mRNA from patients with DN or MCNS and healthy subjects determined by qPCR. (B) ROC curve analyses of urinary exosomal WT1 mRNA for diagnosis of DN. (C) Rate of change in eGFR after the measurement of urinary exosomal WT1 mRNA levels in the patients with DN (red circle) and MCNS (blue circle).

depletion and do not show the states of podocyte injuries prior to podocyte loss. Therefore, it is strongly anticipated that some urinary biomarkers can provide us information on kidney disease status.

Exosomes are small vesicles containing unique RNA and protein cargo, secreted by all cell types in culture. Urine is emerging as a biological fluid suitable to perform liquid biopsy in a minimally invasive manner. It is believed that urine is an ideal specimen for finding potential biomarkers of kidney diseases due to easy accessibility and can directly reflect the real-time status of the kidney inflammation and tissue damage. The difficulty, however, associated with urine is that their exosomal concentration and overall composition varies largely depending on the urine volume and food uptake while the blood volume remain constant. Quite a lot of differentially expressed proteins identified by mass spectrometry in the exosomal proteome could not be verified by western blot analysis (43). This might be due to the relatively low amount of exosomal protein in the urine while the detection threshold of western blotting might not be sensitive enough for the identification of individual proteins. Given these issues, measurement the amount of mRNA in exosome form urine sample is useful in terms of quantification. In addition, exosomes isolated by ultracentrifugation alone contain both exosomes and aggregated protein and cell free nucleic acid contaminants. Therefore, we carried out sucrose gradient centrifugation in this study. As a result, we could effectively separates exosomes and other extracellular vesicles from these contaminants.

In this study, we characterized the association between urinary exosomal biomarkers and kidney function in patients with DN or

MCNS. Although five biomarkers, WT1, podocin, Actn4, CD2AP, and Nephrin, which are all correlated with podocyte homeostasis, only urinary exosomal WT1 mRNA levels could differentiate DN from MCNS in RT-PCR assay. Subsequent analysis using qPCR revealed that not only patients with MCNS but control subjects exhibits a slight amount of urinary exosomal WT1 mRNA in their urine. From these facts, it is considered that podocytes may secrete exosomal WT1 mRNA even in the physiological steady state. More importantly, prolonged hyperglycemia will gradually cause significant damage on podocytes in the patients with diabetes mellitus, resulting in podocyte loss. Compared with patients with MCNS, even though the level of albuminuria was about the same, patients with DN exhibited eminent exosomal WT1 excretion in urine. In this process, high amount of urinary exosomal WT1 mRNA was observed and associated with future decline in eGFR. So far extensive evidence has accumulated to suggest that WT1 acts as a complex multifunctional nuclear protein involved in at least two levels of gene expression control : transcription and RNA processing (44, 45). Thus, the relation between WT1 and eGFR decline goes beyond reflecting overall worse clinical status and CVD outcomes.

In vitro, western blot analyses revealed that WT1 protein partially translocated to cytoplasm from nucleus under high glucose stimulation. Moreover, exosomes extracted from media of cultured podocyte with treated high glucose exhibited WT1 mRNA expression. Exosomes are homogenous small particles ranging from 40 to 100 nm in size and are derived from the endocytic recycling pathway. In endocytosis, endosomes where intraluminal vesicles bud off into an intra-cytoplasmic lumen directly fuse with the plasma membrane and release exosomes into the extracellular space (46), instead of fusing with the lysosome. This cytoplasmic translocation of WT1 may be followed by the secretion of exosome from podocytes.

In conclusion, our study has shown that urinary exosomal WT mRNA, especially in patients with DN, can be a non-invasive biomarker predicting future decline in renal function. More valuable is that high level of urinary exosomal WT mRNA separates patients with DN from those with MCNS. Due to the limitation of patients from one institute, further validation in prospective cohorts from areas and races is needed.

CONCLUSIONS

Urinary exosomes are microvesicles released by podocytes containing information of the originated cells. PDSTFs are good candidates to assess podocyte injuries. The profile of PDSTFs in urinary exosomes from patients with DN is different from that from patients with MCNS. Among PDSTFs in urinary exosomes, WT1 mRNA levels reflected damage of diabetic glomeruli in the patients. Urinary exosomal WT1 mRNA can predict the decline in eGFR for the following several years in patients with DN.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

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