Urinary Podocyte Count as a Potential Routine Laboratory Test for Glomerular Disease: A Novel Method Using Liquid-Based Cytology and Immunoenzyme Staining

Junichi Sakane\textsuperscript{a}   Hirotsugu Kitayama\textsuperscript{b}   Takashi Inoue\textsuperscript{a}   Akihiro Nakamura\textsuperscript{c}
Masayoshi Yamada\textsuperscript{b}   Yudai Miyama\textsuperscript{b}   Hideki Kawamura\textsuperscript{b}   Hideto Iwafuchi\textsuperscript{d}
Shingo Kamoshida\textsuperscript{e}   Hiroyuki Ohsaki\textsuperscript{e}

\textsuperscript{a}Department of Clinical Laboratory, Shizuoka Children’s Hospital, Shizuoka, Japan; \textsuperscript{b}Department of Nephrology, Shizuoka Children’s Hospital, Shizuoka, Japan; \textsuperscript{c}Department of Clinical Laboratory Science, Faculty of Health Care, Tenri Health Care University, Tenri, Japan; \textsuperscript{d}Department of Pathology, Shizuoka Children’s Hospital, Shizuoka, Japan; \textsuperscript{e}Department of Medical Biophysics, Kobe University Graduate School of Health Sciences, Kobe, Japan

Keywords
Podocyte · Liquid-based cytology · Immunocytochemistry · Urine cytology · Glomerular disease

Abstract

Introduction: This study investigated whether our urinary podocyte detection method using podocalyxin (PDX) and Wilms tumor 1 (WT1) immunoenzyme staining combined with liquid-based cytology can serve as a noninvasive routine laboratory test for glomerular disease. Methods: The presence of PDX- and WT1-positive cells was investigated in 79 patients with glomerular disease and 51 patients with nonglomerular disease. Results: The frequencies and numbers of PDX- and WT1-positive cells were significantly higher in the glomerular disease group than in the nonglomerular disease group. The best cutoffs for PDX- and WT1-positive cell counts for identifying patients with glomerular disease were 3.5 (sensitivity = 67.1% and specificity = 100%) and 1.2 cells/10 mL (sensitivity = 43.0% and specificity = 100%), respectively. Conclusion: Because our urinary podocyte detection method using PDX immunoenzyme staining can be standardized and it detected glomerular disease with high accuracy, it can likely serve as a noninvasive routine laboratory test for various glomerular diseases.

Introduction

Podocytes are highly specialized epithelial cells that play an important role as filters that prevent the leakage of high-molecular-weight proteins, glomerular basement membrane (GBM) components, and endothelial cells [1, 2]. Podocytes are injured by various insults (genetic, mechanical, immunologic, and toxic). Upon podocyte injury, localized changes occur in the slit membrane, followed by foot process effacement [3]. This phenomenon causes a decrease in glomerular filtration function and clinically appears as proteinuria. Other podocyte injury mechanisms include hypertrophy, detachment, apoptosis, and epithelial-to-mesenchymal transition [4–7]. In these cases, podocytes detach from the GBM frequently. Podocytes are terminally differentiated cells that are generally...
Urinary Podocyte Detection in Glomerular Disease

This study assessed whether our urinary podocyte detection method using PDX immunoenzymatic staining can represent a noninvasive routine laboratory test for glomerular disease. Therefore, we investigated the frequencies and numbers of PDX- and WT1-positive cells in patients with and without glomerular disease. Meanwhile, we examined the correlation between the number of positive cells and renal function markers. This is the first report investigating glomerular disease using a urinary podocyte detection method combining SurePath and PDX immunoenzymatic staining.

Materials and Methods

Patients, Urine Samples, and LBC (SurePath) Slides
This study used data of 79 patients (46 males and 33 females, mean age = 10.6 ± 4.1 years) with glomerular disease who underwent renal biopsy and 51 patients (32 males and 19 females, mean age = 6.0 ± 4.8 years) with nonglomerular disease (underwent orthopedic or plastic surgery in Shizuoka Children’s Hospital). The distribution of each glomerular disease is summarized in Table 1.

All samples were residual voided urine after a routine test (glomerular disease: median = 30 mL [5–68 mL], nonglomerular disease: median = 23 mL [5–84 mL]), and catheterized urine and bladder washing urine were not included. In the glomerular disease group, voided urine was collected immediately before renal biopsy. The same urine sample was divided into two aliquots and used to prepare two LBC slides. Then, each slide was stained with PDX and WT1 antibodies. LBC slides were prepared using the SurePath (Rector, Dickinson, Franklin Lakes, NJ, USA) modified manual protocols [13].

Immunoenzyme Staining
LBC slides were fixed in 95% ethanol for 30 min and labeled with primary antibodies of PDX (dilution 1:16,000, clone EPR9518; Abcam, Cambridge, UK) and WT1 (dilution 1:1,000, clone 6F-H2; DakoCytomation, Glostrup, Denmark) using the BOND-MAX automatic immunostaining system (Leica Microsystems, Wetzlar, Germany).

Counting of PDX- and WT1-Positive Urinary Cells
The total numbers of PDX- and WT1-positive cells on each LBC slide were counted on the entire smear area under a light mi-
croscopé (×10 and ×40 magnification) by 2 authors (J.S. and T.I.) blinded to the patients’ diagnoses. Some patients were too young to provide constant amounts of samples. Therefore, to eliminate the effects of urine volume, the number of podocytes in 10 mL of urine was calculated for each patient.

First, we compared the frequencies and numbers of urinary PDX- and WT1-positive cells between the glomerular disease and nonglomerular disease groups. Second, we compared the numbers of PDX- and WT1-positive cells among 3 groups (i.e., IgA nephropathy, Henoch-Schönlein purpura nephritis [HSPN], and minor glomerular abnormalities [MGAs] groups), which are the top three glomerular diseases in this study. Third, we examined the correlations of the numbers of positive cells with renal function markers such as serum creatinine, urine protein-to-creatinine ratio, and occult blood in the urine in patients with glomerular disease.

### Laboratory Test Data

Serum and urine creatinine contents were measured by the enzymatic method (Cygnus Auto CRE; Shino-Test Corp., Tokyo, Japan) using a BioMajesty JCA-BM9130 automatic biochemical analyzer (JEOL Ltd., Tokyo, Japan). Urine protein levels were measured by the pyrogallol red method (Micro TP-AR 2; Wako Pure Chemical Industries, Osaka, Japan) using a BioMajesty JCA-BM9130 automatic biochemical analyzer (JEOL Ltd.). Occult blood in urine scores of 1+ or more on dipstick testing (Uropaper α||| Eiken; Eiken Chemical, Tokyo, Japan) as read by an automated dipstick reader (US-3500; Eiken Chemical, Tokyo, Japan) was defined as positive.

### Statistical Analysis

The Mann-Whitney U test and Kruskal-Wallis test were used where appropriate. p < 0.05 indicated statistical significance. All analyses were performed using StatFlex software (version 7.0; Artec Inc., Osaka, Japan).

### Results

#### Comparisons of PDX- and WT1-Positive Cell Counts between the Groups

PDX- and WT1-positive cells were strongly positive in the cytoplasm. These positive cells appeared singly; they were 20–40 μm in maximum length and round or oval in shape (shown in Fig. 1). In addition, these positive cells were occasionally encased by a cast.

The numbers of PDX- and WT1-positive cells were significantly higher in the glomerular disease group.

---

**Table 1.** The distribution of glomerular disease

| Histologic diagnosis                        | n  |
|---------------------------------------------|----|
| IgA nephropathy                             | 25 |
| HSPN (IgA vasculitis)                       | 19 |
| MGAs                                        | 9  |
| Membranoproliferative glomerulonephritis    | 6  |
| Focal segmental glomerulosclerosis          | 4  |
| Lupus nephritis                             | 3  |
| Membranous nephropathy                      | 3  |
| Alport syndrome                             | 2  |
| Non-IgA mesangial proliferative glomerulonephritis | 1 |
| Mitochondrial nephropathy                   | 1  |
| Acute tubular injury                        | 1  |
| Autosomal dominant tubulointerstitial kidney disease | 1 |
| Poststreptococcal acute glomerulonephritis  | 1  |
| Tubulointerstitial nephritis                | 1  |
| Glomerulonephritis                          | 1  |
| Mixed connective tissue disease             | 1  |
| **Total**                                   | **79** |

---

**Fig. 1.** Images of immunoenzyme staining with PDX and WT1 antibodies showing urinary podocytes. PDX- and WT1-positive cells are scattered between leukocytes (×1,000 magnification).
than in the nonglomerular disease group (shown in Fig. 2). In addition, there were no significant differences in PDX- and WT1-positive cell numbers within the nonglomerular disease group. However, in the glomerular disease group, the number of PDX-positive cells was significantly higher than that of WT1-positive cells.

The best cutoff for urinary PDX-positive cells to differentiate glomerular disease from nonglomerular disease was 3.5 cells/10 mL (sensitivity = 67.1%, specificity = 100%, positive predictive value = 100%, negative predictive value = 66.2%), and the cutoff for WT1-positive cells was 1.2 cells/10 mL (sensitivity = 43.0%, specificity = 100%, positive predictive value = 100%, negative predictive value = 53.1%). These cutoffs produced area under the curves of 0.835 and 0.705 (shown in Fig. 3), respectively, and revealed that glomerular disease could be detected with moderate accuracy (Table 2).

Comparison of PDX- and WT1-Positive Cell Counts among the Three Glomerular Disease Groups

The numbers of PDX- and WT1-positive cells were significantly higher in the IgA nephropathy and HSPN groups than those in the MGAs group (shown in Fig. 4). On the other hand, there were no significant differences

---

**Fig. 2.** Number of PDX- and WT1-positive cells in the glomerular disease and nonglomerular disease groups.

**Fig. 3.** Receiver operating characteristic curve for the cutoff value of urinary PDX- and WT1-positive cells.

**Fig. 4.** Number of PDX- and WT1-positive cells in the three glomerular disease groups. IgA, IgA nephropathy; HSPN, Henoch-Schönlein purpura nephritis; MGA, Minor glomerular abnormalities.
in PDX- and WT1-positive cell numbers between the IgA nephropathy and HSPN groups.

Correlations of PDX- and WT1-Positive Cell Counts with Renal Function Markers in Glomerular Disease

In the glomerular disease group, the numbers of PDX- and WT1-positive cells were not correlated with patient sex, height, weight, age, serum creatinine levels, urine protein-to-creatinine ratio, and occult blood in urine.

Discussion

PDX- and WT1-positive cells exhibited cytoplasmic expression in this study. Researchers accustomed to immunofluorescence staining may raise concerns regarding the WT1 immunoenzyme staining results (cytoplasmic positivity of WT1). Previous studies have reported that WT1 is involved in transcriptional regulation within the nucleus and RNA metabolism and translational regulation in the cytoplasm [16, 17]. Likewise, Western blotting of nuclear and cytoplasmic fractions using WT1 antibody revealed that WT1 predominantly inhabited the nucleus, although cytoplasmic fractions were also positive for WT1 [18]. For these reasons, in this study, WT1-positive cells displayed cytoplasmic expression.

The PDX- and WT1-positive cells of various sizes and shapes were observed in this study. These morphologic features are consistent with previous reports on PDX immunofluorescence staining [10, 11, 19]. One hypothesis explaining the various shapes and sizes of podocytes is miniaturization attributable to apoptosis [8]. Conversely, hypertrophy may be induced to allow the remaining podocytes to cover the GBM in denuded areas following the detachment of other podocytes [20]. For these reasons, in this study, WT1-positive cells displayed cytoplasmic expression.

Table 2. Sensitivity and specificity of PDX- and WT1-positive cells using the best cutoff value

|                      | Nonglomerular disease (n = 51), n (%) | Glomerular disease (n = 79), n (%) | p value |
|----------------------|--------------------------------------|----------------------------------|---------|
| PDX-positive (≥3.5 cells/10 mL) | 0 (0)                               | 53 (67.1)                        | <0.001  |
| PDX-negative (<3.5 cells/10 mL) | 51 (100)                            | 26 (32.9)                        |         |
| WT1-positive (≥1.2 cells/10 mL) | 0 (0)                               | 34 (43.0)                        | <0.001  |
| WT1-negative (<1.2 cells/10 mL) | 51 (100)                            | 45 (57.0)                        |         |

Wang et al. [25] reported that in a pediatric study, PDX-positive cells were detected in 53.8% (35/65) of patients in the glomerular disease group, and the number of PDX-positive cells was higher in this group than in the healthy control group. Their differences were smaller than our findings, although the data cannot be easily compared because the urine volume, cytologic smear preparation method, and immunocytochemistry method were different. One possible explanation for the increased frequency of PDX-positive cells in our study is the improvement of cell recovery using the SurePath method [26–28]. Meanwhile, another study found that PDX immunofluorescence staining of urine sediments was positive in 92.5% (62/65) of children with glomerular disease [10]. However, this discrepancy is likely caused by the inclusion of positive casts and granules in addition to PDX-positive cells in their study. The sensitivity and specificity of WT1-positive cells in the present study (sensitivity = 43.0% and specificity = 100%) were similar to those in a previous study on adult patients with glomerular disease (sensitivity = 50.0% and specificity = 100%) [13]. The results of the present study indicated that im-
munoenzyme staining using the PDX antibody can detect glomerular disease with a higher accuracy than that using WT1 antibody. Some researchers may prefer PDX immunofluorescence staining, but because immunoenzyme staining is routinely performed as a pathologic examination, we consider it will be better suited for routine tests [14].

This study targeted 79 cases of glomerular diseases, but the number of cases was tiny in most of the glomerular disease types. Therefore, we compared the numbers of PDX- and WT1-positive cells among the 3 groups (IgA nephropathy, HSPN, and MGAs groups), which had a relatively large number of cases. As a result, the numbers of PDX- and WT1-positive cells were significantly higher in the IgA nephropathy and HSPN groups than that in the MGAs group. On the other hand, there were no significant differences in these positive cell numbers between the IgA nephropathy and HSPN groups. Our results were similar to those of previous studies on the detection of PDX-positive cells using immunofluorescence staining [7, 29–31].

The lack of correlations of PDX- and WT1-positive cell counts with renal function markers likely has several explanations. The number of urinary PDX-positive cells was significantly higher in patients with focal segmental glomerulosclerosis than in those with membranous nephropathy or minimal change nephrotic syndrome in prior research [29, 30]. The number of urinary PDX-positive cells was significantly higher in patients with active IgA nephropathy or HSPN than in those with inactive disease cases [7, 31]. Furthermore, in minimal change nephrotic syndrome, it is known that a large amount of protein appears in the urine even though PDX- and WT1-positive cells are rarely observed [13, 29, 30]. In IgA nephropathy, hematuria is usually present, though proteinuria is rare in the early stages. As mentioned previously, urinary PDX-positive cells and some renal function markers reflect both glomerular disease type and activity, but the current study population included patients with various disease types and activities. These findings potentially explain the lack of correlations of PDX- and WT1-positive cell counts with serum creatinine, urine protein-to-creatinine ratio, and occult blood in the urine in the present study. From this result, we would like to note that our method is a noninvasive biomarker independent of other tests, and glomerular disease can be detected with high accuracy using our cutoffs for PDX-positive cells.

In conclusion, because our urinary podocytes detection method can be standardized and it detected glomerular disease with high accuracy, we believe this method could emerge as a noninvasive routine laboratory test for various glomerular diseases. Future work should focus on the relationship between the activity of individual glomerular diseases and PDX-positive cell counts.

Acknowledgement

The authors would like to thank Yuki Morisato, Rinako Miyagi, Maiko Takahashi, Junko Kitagawa, Shinya Otahara, Nobuo Murai, Yuuko Tasaki, Masayuki Satou, Mika Nakajima, Ryutaro Serizawa, and Yukari Kiji for their excellent technical assistance.

Statement of Ethics

This study was approved by the Ethics Committee of Shizuoka Children’s Hospital (no. 2020-28) and Kobe University Graduate School of Health Sciences (no. 977) and conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from each patient’s parent or legal guardian.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was financially supported by the Shizuoka General Hospital Encouragement Research Grant and the Japanese Society of Clinical Cytology Grant.

Author Contributions

Methodology: Ohsaki H. Formal analysis: Sakane J., Inoue T., Nakamura A., and Ohsaki H; resources: Kitayama H., Yamada M., Miyama Y., Kawamura H., Iwafuchi H., and Ohsaki H; writing – original draft: Ohsaki H; writing – review and editing: Kitayama H., Nakamura A., Kamoshida S., and Ohsaki H; and supervision: Ohsaki H.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.
References

1. Drumond MC, Deen WM. Structural determinants of glomerular hydraulic permeability. *Am J Physiol*. 1994;266(1 Pt 2):F1–12.

2. Ikuma D, Hiromura K, Kajiyama H, Suwa J, Ikeuchi H, Sakairi T, et al. The correlation of urinary podocytes and podocalyxin with histological features of lupus nephritis. *Lupus*. 2018;27(3):484–93.

3. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol*. 2007;17(9):428–37.

4. Yamaguchi Y, Iwano M, Suzuki D, Nakatani K, Kimura K, Harada K, et al. Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am J Kidney Dis*. 2009;54(4):563–64.

5. Miyauchi M, Toyoda M, Kobayashi K, Abe M, Kobayashi T, Kato M, et al. Hypertrophy and loss of podocytes in diabetic nephropathy. *Intern Med*. 2009;48(18):1615–20.

6. Reidy K, Susztak K. Epithelial-mesenchymal transition and podocyte loss in diabetic kidney disease. *Am J Kidney Dis*. 2009;54(4):590–3.

7. Sun D, Zhao X, Meng L. Relationship between urinary podocytes and kidney diseases. *Ren Fail*. 2012;34(3):403–7.

8. Al Hussain T, Al Mana H, Hussein MH, Akhtar M. Podocyte and parietal epithelial cell interactions in health and disease. *Adv Anat Pathol*. 2017;24(1):24–34.

9. Shankland SJ, Anders HJ, Romagnani P. Glomerular parietal epithelial cells in kidney physiology, pathology, and repair. *Curr Opin Nephrol Hypertens*. 2013;22(3):302–9.

10. Hara M, Yanagihara T, Takada T, Itoh M, Matsuno M, Yamamoto T, et al. Urinary excretion of podocytes reflects disease activity in children with glomerulonephritis. *Am J Nephrol*. 1998;18(1):35–41.

11. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV. Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol*. 2003;285(1):F40–8.

12. Beyer-Boon ME, Voorn-den Hollander MJ. Cell yield obtained with various cytopreparatory techniques for urinary cytology. *Acta Cytol*. 1978;22(6):589–93.

13. Ohsaki H, Matsunaga T, Fujita T, Tokuhara Y, Kamoshida S, Sofue T. Quantifying podocytes and parietal epithelial cells in human urine using liquid-based cytology and WT1 Immunoenzyme staining. *Bio Protoc*. 2018;8(9):e2827.

14. Fujita T, Sofue T, Moritoki M, Nishijima Y, Tokuhara Y, Wakisaka H, et al. Urinary WT1-positive cells as a non-invasive biomarker of crescent formation. *Cytopathology*. 2017;28(6):524–30.

15. Gong Y, Symmans WF, Krishnamurthy S, Patel S, Sniege N. Optimal fixation conditions for immunocytochemical analysis of estrogen receptor in cytologic specimens of breast carcinoma. *Cancer*. 2004;102(1):34–40.

16. Davies RC, Calvio C, Bratt E, Larsson SH, La mond AI, Hastie ND. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. *Genes Dev*. 1998;12(20):3217–25.

17. Caricasole A, Duarte A, Larsson SH, Hastie ND, Little M, Holmes G, et al. RNA binding by the Wilms tumor suppressor zinc finger proteins. *Proc Natl Acad Sci U S A*. 1996;93(15):7562–6.

18. Niksic M, Slight J, Sanford JR, Caceres JF, Sanford JR, et al. RNA binding by the Wilms tumor suppressor zinc finger proteins. *Proc Natl Acad Sci U S A*. 1996;93(15):7562–6.

19. Shu Y, Yoshida F, Inagaki T, Sonoda J, Watanabe T, et al. Podocyte injury promotes progressive nephropathy in Zucker diabetic fatty rats. *Lab Invest*. 2002;82(1):25–35.

20. Achenbach J, Mengel M, Tossidou I, Peters I, Park JK, Haubitz M, et al. Parietal epithelia cells in the urine as a marker of disease activity in glomerular diseases. *Nephrol Dial Transplant*. 2008;23(10):3138–45.

21. Ohse T, Vaughan MR, Kopp JB, Krofft RD, Marshall CB, Chang AM, et al. De novo expression of podocyte proteins in parietal epithelial cells during experimental glomerular disease. *Am J Physiol Ren Physiol*. 2010;298(3):F702–11.

22. Wang P, Li M, Liu Q, Chen B, Ji Z. Detection of urinary podocytes and nephrin as markers for children with glomerular diseases. *Exp Biol Med*. 2015;240(2):169–74.

23. Wright RG, Halford JA. Evaluation of thin-layer methods in urine cytology. *Cytopathology*. 2001;12(5):306–13.

24. Kimura M, Toyoda M, Saito N, Kaneyama N, Miyatake H, Tanaka E, et al. A liquid-based cytology system, without the use of cytocentrifugation, for detection of podocytes in urine samples of patients with diabetic nephropathy. *J Diabetes Res*. 2019;2019:9475637.

25. Kimura M, Toyoda M, Saito N, Kaneyama N, Miyatake H, Tanaka E, et al. Corrigendum to “A liquid-based cytology system, without the use of cytocentrifugation, for detection of podocytes in urine samples of patients with diabetic nephropathy”. *J Diabetes Res*. 2020;2020:3623147.

26. Nakamura T, Ushiyama C, Suzuki S, Hara M, Shimada N, Ebihara I, et al. The urinary podocyte as a marker for the differential diagnosis of idiopathic focal glomerulosclerosis and minimal-change nephrotic syndrome. *Am J Nephrol*. 2000;20(3):175–9.

27. Hara M, Yanagihara T, Kihara I. Urinary podocytes in primary focal segmental glomerulosclerosis. *Nephron*. 2001;89(3):342–7.

28. Hara M, Yanagihara T, Kihara I. Cumulative excretion of urinary podocytes reflects disease progression in IgA nephropathy and Schönlein-Henoch purpura nephritis. *Clin J Am Soc Nephrol*. 2007;2(2):231–8.