Original Article

Study of Urinary WT1 Positive Cells as a Biomarker for Detection of Glomerular Diseases

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ABSTRACT

Introduction: Glomerular diseases form a large part of the morbidity and mortality associated with kidney diseases. Podocyte injury and loss is implicated in a number of these diseases. It has been suggested that the detection of proteinuria cannot distinguish between a persistent defect of the glomerular barrier and ongoing injury, whereas podocyturia may be more specific for 'active' injury. This study was done to assess if detection of WT1 positive cells in urine by immune-histochemistry is a viable biomarker of renal disease.

Methodology: A total of 96 consecutive renal biopsies with clinically suspected glomerular disease were evaluated for presence of WT1 positive cells in their urine using immune-histochemistry on cell blocks.

Results: Out of 96 patients 23 had WT1 positive cells in their urine. Positivity for WT1 varied with different disease types.

Conclusion: Staining for WT1 on urinary cell blocks is a cost effective and comparatively easy method for detecting glomerular diseases.

Keywords: Podocyturia, WT1 positive cells, glomerular disease.

INTRODUCTION

Renal diseases in India are on the rise. We are not far behind US, which has seen a 30% increase in chronic kidney disease (CKD) prevalence in the last decade. Although there is limited data from India, various studies and meta-analyses have estimated the prevalence to be around 3-4%. As the Indian population ages, this is bound to increase.[1,2] Glomerular diseases form a large part of the morbidity and mortality associated with kidney diseases. There are inherent problems with the classical biomarkers of kidney injury. Proteinuria is not specific to glomerular injury and also it has limited ability to monitor disease progression or response to therapy. Over the last two decades, it has been noted that podocytes are shed in urine in glomerular diseases. The earliest studies, done by Hara et al,[3] detected podocyte injury in IgA nephropathy and Henoch Schonlein purpura (HSP) nephritis using immuno staining against podocalyxin and documented presence of podocytes in the urine. Podocyturia, or the presence of podocytes in urine, can be detected with an increasing set of novel biomarkers such as detection of various proteins (podocalyxin, nephrin, podocin, WT1), podocyte specific mRNA, or exosomal transcription factors.[4] Most of these novel biomarkers are not easy to analyze in a small lab, and require either flourescence microscopy, or deal with mRNA or transcriptome analysis. The method described in this study is compatible with the various cytology methods that are already present in a small laboratory and require only immuno-histochemistry against WT1 protein for podocyturia detection. In the present study, we attempted to find out whether detection of podocyturia by immunohistochemistry of WT1 protein is a viable option in a small lab.

METHODS

Renal biopsies of 96 consecutive patients with clinically suspected glomerular disease were evaluated for presence of WT1 positive cells in their urine. First urine sample of the patients was collected in the morning on the next day of the biopsy procedure. Next day freshly voided morning urine samples were immediately sent to laboratory for processing. 10-20 ml of urine was centrifuged at 2500 rpm

for 15 minutes. Supernatant was discarded and acetic acid, alcohol, and formalin (AAF) solution was added to the sediment and kept for four hours. This was again centrifuged at 2500 rpm for 15 minutes. Supernatant was discarded and formalin was added to the sample and kept overnight. The sample was processed as a cell block the next day. The slide so obtained after sectioning was stained by hematoxylin and eosin (H&E) and then examined for its cellular contents.

4μ section was taken on albumin coated slides for immuno-enzyme staining. 1% hydrogen peroxide in methanol was used for blocking endogenous enzyme activity. Heat induced epitope retrieval was done using a decloaking chamber. Slides were then incubated with ready-to-use antiWT1 antibody (Biocare Medical) for 60 minutes in humidity chamber at room temperature. Universal HRP polymer and DAB chromogen of Biocare Medical were used to visualise the stain. Single 3μ tissue section from a renal biopsy with minimal change disease was taken as positive control and stained with WT1 in parallel.

RESULTS

A total of 96 patients, who underwent renal biopsy over a period of two years at our institute were evaluated for presence of WT1 positive cells in their urine. The patients' age ranged from 9 months to 70 years of age. WT1 positive cells showed diffuse or granular cytoplasmic staining and were negative for nuclear stain (Figure 1). The cells were three to five times that of a neutrophil in size.

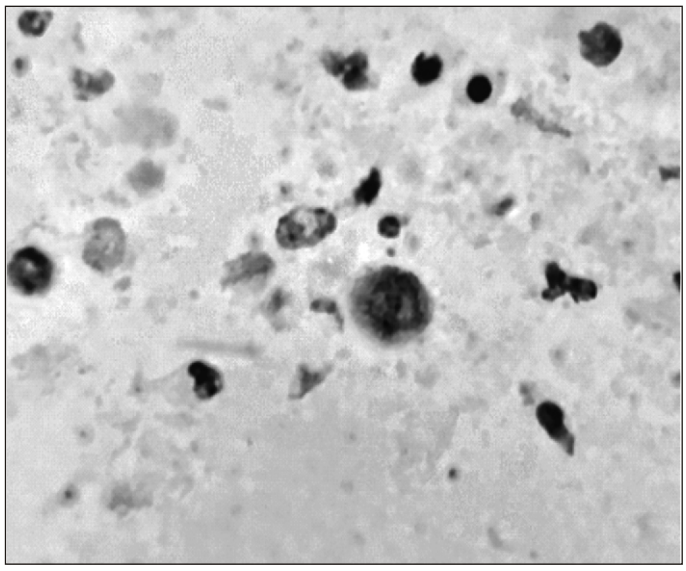

Figure 1: Single WT1 positive cell on urinary cell block with IHC staining using anti-WT1 antibody (x400).

Table 1 shows the number of cases with WT1 positive cells in different glomerular diseases that were examined. Maximum percentage of cases with WT1 cells positive was seen in IgA nephropathy and Pauci-immune Cresentic Glomerulonephritis (PICGN), followed by Henoch Schonlein Purpura (HSP) nephritis, C3D glomerulopathy, and membranous glomerulopathy. Low positivity was seen in diseases like Focal Segmental Glomerular Sclerosis (FSGS) and renal amyloidosis. None of the cases of minimal change disease (22 cases), acute tubular injury (4 cases), chronic glomerulosclerosis (4 cases), glomerular organized deposit disease (GODD) (3 cases), and IgG4 related TIN (1 case) had any WT1 positive cells in cell blocks prepared from urine. All the three cases of GODD were negative for anti-SAA antibody. They could not be further delineated because electron microscopy was not available at our centre.

No correlation was found between positivity of WT1 cells in urine with age, degree of hematuria, serum creatinine, proteinuria, or blood urea level by nonparametric independent tests (Table 2).

DISCUSSION

Podocytes are involved in many glomerular diseases and are shed in active renal disease. Podocytes contain many specific proteins that help them in their function. These proteins can be detected in podocytes in urine and serve as biomarkers to assess podocyturia. Podocalyxin[5], nephrin[6], podocin[6], CD80[7], synaptopodin[8], and WT1[9] are some of the different biomarkers that have been described in the literature. Although many of the studies done earlier used immunofluorescence technique for detection of these proteins, we preferred immunohistochemistry over immunofluorescence because of several reasons. Although simple to perform, immunofluorescence studies require the use of fluorescence microscope. Since immuno-fluorescence fades with time, photography for documentation is necessary. Standardization is also difficult to achieve, because of high inter-observer variability and background noise due to immunostain positive cell debris. WT1 was chosen over other proteins for immuno-histochemistry because WT1 antibody is usually already present in an immuno histochemistry lab owing to its frequent use in other diseases.

Earlier believed to be positive only in podocytes, WT1 is now known to be positive in podocytes, parietal epithelial

cells, and crescent cells, according to data published in several studies.[3,10-14] The pattern of positivity is predominantly nuclear, however cytoplasmic fractions can also show positivity for WT1.[15] In the present study, cells showed cytoplasmic positivity and nucleus did not take up any stain. This can be explained by the fact that antibodies to nuclear staining give less reproducible results when alcohol based fixatives are used.[16] Early morning urine sample was taken to increase the chances of detecting podocytes that might have shed off overnight. A mixture of alcohol, acetic acid, and formalin (AAF solution) for processing of cell blocks was used. Studies done earlier used immunocytochemistry for WT1 and detected WT1 positive cells in liquid based cytology (LBC) prepared slides of urine of patients.[9,14] Thus, WT1 is an established biomarker for detecting podocyte injury. Since the source of WT1 positive cells in the urine is glomerulus itself, it is of utmost importance to process urine as soon as possible, taking two hours at the latest. This is to avoid further degeneration of WT1 positive cells that have already been in urine for a long time.

There is considerable disagreement for positivity of urinary podocytes in MCD. Hara et al[17] compared podocalyxin positive cells in urine of several glomerulopathy patients and detected positivity in seven out of eight cases of MCD. However, Nakamura et al[18] failed to detect urinary podocalyxin positivity in 12 cases of active MCD and 8 cases of MCD in remission. In the same study, all of the 15 cases of FSGS were urinary podocalyxin positive. On this basis, they suggested presence of urinary podocalyxin positive cells as a marker for differentiating between MCD and FSGS. This discrepancy may be due to difficulty in differentiating between cell debris and intact immuno-positive cells in immuno-fluorescence staining. Ohsaki et al[9] did not detect WT1 positive cells in urine in all of the four patients of MCD in their study. All of the 22 patients of MCD in the present study did not show presence of WT1 positive cells in urine. This could probably be used as a marker in differentiating FSGS from MCD patients.

Data on presence of podocytes in urine in patients of renal amyloidosis and GODD is sparse, if any. We had a large number of renal amyloidosis cases as we took consecutive cases and renal amyloidosis is quite common in the local population. Acute tubular injury (five cases) and IgG4 TIN (one case), although fall out of the umbrella of 'glomerular disease', were included in the present study because of initial clinical suspicion of a possibility of glomerular injury in the patients. None of these cases had presence of WT1 expressing cells in their urine.

The present study had four cases of chronic glomeru-

Table 1: WT1 positive cells in various diseases

Disease	Number of Cases	WT1 positive cells in urine	Percentage
Minimal Change Disease	22	0	0.00
Renal Amyloidosis	13	1	7.69
Lupus Nephritis Class IV	10	4	40.00
Focal Segmental Glomerulosclerosis	9	3	33.33
Immune Mediated Glomerulonephritis	8	3	37.50
HSP Nephritis	5	3	60.00
C3 Dominant Glomerulopathy	4	2	50.00
Membranous Glomerulopathy	4	2	50.00
Acute Tubular Injury	4	0	0.00
Chronic Glomerulosclerosis	4	0	0.00
Glomerular Organized Deposit Disease (GODD)	3	0	0.00
IgA Nephropathy	2	2	100.00
Lupus Nephritis Class V+IV	2	1	50.00
Hypertensive Nephropathy	2	1	50.00
IgG4 related TIN	1	0	0.00
Lupus Nephritis class II	1	0	0.00
Lupus Nephritis class III	1	0	0.00
Total	96	23	23.96

Table 2: Correlation between positivity of WT1 cells in urine and various clinical parameters

Clinical Parameters	Null Hypothesis	p value	Decision
Age (n=96)	The distribution of 'Age' is the same across categories of 'Cases with WT1 expressing cells in urine.'	0.356	Retain the null hypothesis
Proteinuria (n=79)	The distribution of 'Proteinuria' is the same across categories of 'Cases with WT1 expressing cells in urine.'	0.192	Retain the null hypothesis
Serum creatinine (n=67)	The distribution of 'Serum creatinine' is the same across categories of 'Cases with WT1 expressing cells in urine.'	0.804	Retain the null hypothesis
Blood urea (n=52)	The distribution of 'Blood urea' is the same across categories of 'Cases with WT1 expressing cells in urine.'	0.237	Retain the null hypothesis
Degree of hematuria (n=42)	The distribution of 'Hematuria' is the same across categories of 'Cases with WT1 expressing cells in urine.'	0.077	Retain the null hypothesis

p value < 0.05 : Significant

losclerosis, none of which showed presence of WT1 positive cells in urine. This is in contrast to the study done by Kubo K et al[19] who detected WT1 mRNA in 7 out of total 20 patients of chronic glomerulosclerosis. This can be attributed to the fact that WT1 mRNA detection has higher levels of sensitivity.

Presence of WT1 positive cells in urine of FSGS, C3 dominant glomerulopathy, hypertensive nephropathy, immune mediated glomerulonephritis, and PICGN has not been much documented. However, presence of podocytes in urine of FSGS patients has been very well established using different podocyte specific markers such as podocalyxin, WT1 mRNA etc. in various other studies.[20-22]

There was no correlation between presence of WT1 positive cells in urine and age, degree of hematuria, serum creatinine, proteinuria, or blood urea in the present study as shown in table 2. Results from previous studies have been somewhat conflicting. Although Hara et al[17] showed a significant correlation between podocalyxn positive cells in urine and proteinuria and hematuria, several studies done later, found no relationship between podocyturia and albu-minuria, and considered podocyturia to be a more sensitive marker than proteinuria to assess glomerular damage.[10,12] This study was limited by its small sample size. Also, an analysis of number of podocytes in urine on follow up of the patients was not undertaken as this was a cross sectional study. Hence, outcome could not be predicted.

CONCLUSION

Although there are a number of proteins specific to podocytes that can be used as a biomarker to assess glomerulopathy non-invasively, immune-enzyme staining for WT1 on urinary cell blocks is a cost effective and easily available method, not requiring any additional setup in small to medium labs.

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