Non-invasive urinary sediment double-immunostaining predicts BK polyomavirus associated-nephropathy in kidney transplant recipients

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Background: The positive predictive value (PPV) of urinary decoy cells for diagnosing BK polyomavirus associated-nephropathy (BKPyVAN) is low. This study was designed to increase the PPV of urinary decoy cells for diagnosing BKPyVAN in kidney transplant recipients.

Methods: A total of 105 urine sediment samples from 105 patients with positive BK viruria and decoy cells were evaluated by automatic double-immunostaining with anti-HGD (a renal tubular marker) antibody + anti-SV40-T antibody or anti-S100P (an urothelial marker) antibody + anti-SV40-T antibody.

Results: Of the 105 patients, 76 (72.4%) had both HGD(+)/SV40-T(+) cells and S100P(+)/SV40-T(+) cells (group A), 24 (22.9%) had only S100P(+)/SV40-T(+) cells (group B), and 5 (4.6%) had only S100P(–)/ HGD(–)/SV40-T(+) cells (group C). Seventy patients in group A (92.1%), 3 patients in group B (12.5%), and no patients in group C were diagnosed with BKPyVAN. The area under the ROC curve of predicting BKPyVAN by decoy cells was 0.531 (0.431–0.630), with an optimal cut-off value of 29 (per 10 high power field), a sensitivity of 45.8% (95% CI: 34.0–58.0%), and a specificity of 68.8% (95% CI: 50.0–83.9%). Besides, the area under the ROC curve of predicting BKPyVAN by plasma BKPyV load was 0.735 (95% CI: 0.632–0.822), with an optimal cut-off value of 1,000 copies/mL, a sensitivity of 61.1% (95% CI: 48.9–72.4%) and a specificity of 84.2% (95% CI: 60.4–96.6%). In contrast, the PPV, negative predictive value, sensitivity, and specificity of HGD(+)/SV40-T(+) cells for diagnosing BKPyVAN were 92.1% [95% confidence interval (CI): 83.0–96.7%], 89.7% (95% CI: 71.5–97.3%), 95.9% (95% CI: 87.7–98.9%), and 81.3% (95% CI: 63.0–92.1%) respectively.

Conclusions: Double-immunostaining with anti-HGD or anti-S100P and anti-SV40-T antibodies helps to identify the origin of decoy cells and diagnose BKPyVAN.

Keywords: BK polyomavirus; BKPyVAN; decoy cells; double-immunostaining; kidney transplantation

Introduction

BK polyomavirus associated-nephropathy (BKPyVAN) is one of the main complications affecting renal transplant function and prognosis (1). In kidney transplant recipients, the incidence of BKPyVAN fluctuates between 1% and 10% (2). Based on the Banff classification criteria, BKPyVAN was divided into grade A, grade B and grade C (3). The 2-year renal graft survival is 90%, 70% and 50% for grade A, grade B and grade C respectively (4). Currently, the main screening tests for BK polyomavirus (BKPyV) infection are urine cytology examination, and qualitative and quantitative polymerase chain reaction (PCR) for detecting BKPyV DNA (5). Some centers examine VP1 messenger RNA by real-time PCR (6). Clinical studies showed that the incidence of BKPyV viruria was 20% to 57% (7,8), the incidence of urinary decoy cells was 13% to 42% (9,10), and the incidence of BKPyV viremia was 7% to 29% in kidney transplant recipients (7,11,12). The negative predictive value (NPV) of urine BKPyV DNA for BKPyVAN is nearly 100%, but the positive predictive value (PPV) is only 40% (13). BKPyV viruria usually precedes BKPyV viremia by about 4 weeks, and precedes BKPyVAN by about 12 weeks (13,14). Sustained BKPyV viremia (plasma BKPyV DNA loads >10,000 copies/mL for >4 weeks) had high specificity and sensitivity (both >93%) for BKPyVAN (13,15,16). Urinary polyomavirus-Haufen has been reported to be an accurate indicator for BKPyVAN as it has a 97% PPV and a 100% NPV (17). However, the urinary polyomavirus-Haufen test has not been verified by multiple centers, and requires electron microscopy, which is not routinely available in many centers. As such, kidney biopsy still serves as the golden standard for diagnosing BKPyVAN (5). However, kidney biopsy is an invasive procedure and has limitations including a high risk of bleeding, it can't be repeated frequently, and has a false negative rate of 36.5% (18).

BKPyV inclusion-bearing epithelial cells, termed "decoy cells", can be identified on urine cytological examination, using Papanicolaou staining (19). Although, the NPV of urine decoy cells for the diagnosis of BKPyVAN is close to 100%, it's PPV is only 29% (13). Our previous study observed that a significant number of kidney transplant recipients with positive urinary decoy cells did not have or develop BKPyVAN (20). Therefore, methods of increasing the PPV of urine decoy cells for BKPyVAN is valuable for therapeutic decision.

We speculate that if biomarkers that are specific to renal tubular epithelial cells or urothelial cells could be detected on urinary decoy cells, the source of the decoy cells could be identified. Homogentisate 1, 2-dioxygenase (HGD) is a specific biomarker of tubular epithelial cells (https://www.proteinatlas.org/ENSG00000113924-HGD/tissue) (21), and S100 calcium binding protein P (S100P) is a specific biomarker of urothelial cells (https://www.proteinatlas.org/ENSG00000163993-S100P/tissue) (22-24). In voided urinary sediment samples from patients with biopsy-proven BKPyVAN, we observed HGD(+)/SV40-T(+) cells and S100P(+)/SV40-T(+) cells by double-immunostaining. It can be reasonably considered that HGD(+)/SV40-T(+) cells represent BKPyV-infected tubular epithelial cells, and S100P(+)/SV40-T(+) cells represent BKPyV-infected urinary transitional epithelial cells.

We hypothesize that the presence of urinary HGD(+)/SV40-T(+) cells indicate an active intra-renal BKPyV infection. This study was performed to identify the source of urinary decoy cells by double-immunostaining using anti-S100P or anti-HGD, and anti-SV40-T antibodies. Furthermore, we sought to determine if the presence of urinary HGD(+)/SV40-T(+) cells were predictive of BKPyVAN.

Methods

Study population

This retrospective cross-sectional study was performed in a single center. The inclusion criteria included: a history of kidney transplantation, urinary decoy cells ≥1/10 HPF (high power field), and urinary BKPyV DNA load >10^3 copies/mL. The exclusion criteria included: recurrent BKPyVAN, repeated kidney transplant, and lack of renal biopsy. Isolation of urinary sediment cells was performed within 3 days before/after renal biopsy. This study was approved by the Ethics Committee and the Research Board of our institution.

Urine cytology

Papanicolaou staining method was used for urinary cytological smears. Decoy cells were counted as the number per 10 HPFs (25). The criteria for the diagnosis of decoy cells was an obviously enlarged and malformed nucleus of BKPyV-infected cells, with the presence of basophilic viral inclusions.

Virological studies

Urine and plasma BKPyV load were quantitatively measured by quantitative PCR (MJ Research, Waltham, MA, USA). Specimen collection and processing, PCR primers, TaqMan

probe (targeting the BKPyV VP1 gene), plasmid standard containing the targeted BKPyV VP1 gene, amplification protocols, PCR precautions, and quality assurance were performed as previously described (26). Urine or plasma BKPyV load were presented as BKPyV genome copies per milliliter. The lower limit of quantitation was determined at 1,000 copies per milliliter.

Pathological diagnosis of BKPyVAN

The pathological diagnosis of BKPyVAN was confirmed by immunohistochemical (IHC) staining with anti-SV40-T large T antigen monoclonal antibody, as previously described (27). The histological features of BKPyVAN were classified using the American Society of Transplantation (AST) schema; BKPyVAN was classified as A, B, and C based on the guidelines published by Hirsch *et al.* (28).

Antibodies

Using the database of the Human Protein Atlas (http://www.proteinatlas.org/) (29), we screened more than 40 biomarkers that are mainly expressed in either transitional epithelium or in renal tubular epithelium. Based on our review, we selected S100P as a specific marker of transitional epithelial cells (https://www.proteinatlas.org/ENSG00000163993-S100P/tissue) (22-24), and HGD as a specific marker of renal tubular epithelial cells (https://www.proteinatlas.org/ENSG00000113924-HGD/tissue) (21). BKPyV was detected by anti-SV40-T large antigen antibody. The results of double-immunostaining with anti-S100P antibody + anti-SV40-T antibody or anti-HGD antibody + anti-SV40-T antibody on ureterocystic and renal tissue with BKPyVAN and without BKPyVAN were presented in *Figure S1* and *Figure S2*.

Preparation of cell block

Patients were instructed to collect a total of 600 mL of fresh urine in a sterile bottle from 10:00 pm to 8:00 am. To prevent cell lysis, 60 mL of 4% formaldehyde solution was added to the urine specimen. The urine specimen was centrifuged for 10 min at 500 ×g within 2 h after collection. The urine sediment was transferred to EP tube with 2 mL of 4% formaldehyde solution. After centrifuging (at 500 ×g for 2 min), the supernatant was discarded.

The Anbiping kit (LBP Medicine Science & Technology Co., Ltd., Guangzhou, China, Batch number 1708001)

was used for cell block preparation. The procedure was performed strictly in accordance with the instructions. A total of 0.15 mL of Reagent A was added to the urine sediment. The mixture was shaken and centrifuged for 2 min at 500 ×g. After discarding the supernatant, 0.15 mL of Reagent B was added to the urine sediment. After standing for 1 min, the cell mass was transferred to a cassette for dehydration (Leica ASP300S). The detailed procedure of dehydration was as follows.

- ❖ Neutral formalin, 10 min,
- ❖ 70% ethyl alcohol, 20 min,
- ❖ 80% ethyl alcohol, 20 min,
- ❖ 90% ethyl alcohol I, 20 min,
- ❖ 90% ethyl alcohol II, 20 min,
- ❖ 100% ethyl alcohol I, 20 min,
- ❖ 100% ethyl alcohol II, 20 min,
- ❖ Xylene I, 20 min,
- ❖ Xylene II, 20 min,
- ❖ Paraffin I, 30 min,
- ❖ Paraffin II, 30 min,
- ❖ Paraffin III, 30 min.

Paraffin-embedded section was sliced at 3 μm (Leica, RM2235) and then was dried at 65 ℃ for 30 min (Leica, HI1220).

Double immunohistochemical staining

Double-immunostaining was performed using a fully automatic IHC staining machine (Leica Band-Max, Germany). The automatic double staining protocol (Sequential double staining) was set as follows.

Part 1 Equivalent to the following operating procedure step V) to step XIX) (Bond Polymer Refine IHC protocol, Leica, Microsystems, batch number 62798)
- ❖ Marker 1 (anti-SV40-T mouse monoclonal antibody, 1:1,000, Catalogue Number DP02, Merck Millipore, USA) 15 min,
- ❖ Post Primary, 8 min,
- ❖ Polymer, 8 min,
- ❖ Peroxide Block, 5 min,
- ❖ Mixed DAB Refine, 5 s,
- ❖ Mixed DAB Refine, 5 min.

Part 2 Equivalent to the following operating procedure step XXII) to step XXVI) (Bond Polymer Refine Red IHC protocol, Leica, Microsystems, batch number 65131)
- ❖ Marker 2 (anti-S100P rabbit monoclonal antibody,

1:5,000, Catalogue Number ab133554, Abcam, Cambridge, UK; or anti-HGD rabbit monoclonal antibody, 1:3,200, Catalogue Number ab131035, Abcam, Cambridge, UK) 15 min,
❖ Post Primary alkaline phosphatase (AP) 20 min,
❖ Polymer AP 30 min,
❖ Mixed Red Refine 10 min,
❖ Mixed Red Refine 5 min,
❖ Hematoxylin 6 min.

The detailed procedure of double-immunostaining (anti-SV40-T + anti-S-100P or anti-SV40-T + anti-HGD) was as follows

(I) Deparaffinizing with Bond solution,
(II) Rinsing with 100% alcohol for 1 min, 3 times,
(III) Rinsing with Bond wash solution for 1 min, 3 times,
(IV) Antigen retrieval with Bond epitope retrieval solution for 20 min (pH 6.0, 100 ℃), followed by washing with wash buffer for 1 min, 3 times,
(V) Incubating with anti-SV40-T antibody, 15 min (37 ℃),
(VI) Rinsing with Bond wash solution for 1 min, 3 times,
(VII) Post-primary, 8 min,
(VIII) Rinsing with Bond wash solution for 1 min, 3 times,
(IX) Polymer, 8 min,
(X) Rinsing with Bond wash solution for 1 min, 3 times,
(XI) Peroxide block, 5 min,
(XII) Rinsing with Bond wash solution for 1 min, 3 times,
(XIII) Mixed DAB Refine, 5 s,
(XIV) Mixed DAB Refine, 5 min,
(XV) Rinsing with Bond deionized water solution for 1 min, 3 times,
(XVI) Rinsing with Bond wash solution for 1 min, 3 times,
(XVII) Incubating with the anti-S100P antibody or anti-HGD, 15 min (37 ℃),
(XVIII) Rinsing with Bond wash solution for 1 min, 3 times,
(XIX) Post Primary AP, 20 min,
(XX) Rinsing with Bond wash solution for 1 min, 3 times,
(XXI) Post Primary AP, 20 min,
(XXII) Rinsing with Bond wash solution for 1 min, 3 times,

(XXIII) Polymer AP, 30 min,
(XXIV) Rinsing with Bond wash solution for 1 min, 3 times,
(XXV) Mixed Red Refine, 10 min,
(XXVI) Mixed Red Refine, 5 min,
(XXVII) Rinsing with Bond deionized water solution for 1 min, 3 times,
(XXVIII) Rinsing with Bond wash solution for 1 min, 3 times,
(XXIX) Hematoxylin, 6 min
(XXX) Rinsing with Bond deionized water solution for 1 min, 3 times,
(XXXI) Air drying,
(XXXII) Transparentizing with xylene,
(XXXIII) Mounted with resin.

IHC slides were reviewed by 2 independent cytopathologists who were blinded to the histological diagnosis and clinical data.

Statistical analysis

Clinical data was expressed as count (percentage), or as mean ± standard deviation. Sensitivity, specificity, PPV, NPV, and 95% confidence intervals (CIs) of urinary HGD(+)/SV40-T(+) cells for diagnosing BKPyVAN were calculated using renal allograft biopsy findings as the gold standard. Correlation analysis between HGD(+)/SV40-T(+) cells and various pathological damage indicators was evaluated by a Chi-square test. IBM SPSS version 20 software (IBM Corporation, Somers, New York, USA) was used for statistical analyses. Values of $P<0.05$ were considered statistically significant.

Results

Patient characteristics

Demographic and transplant characteristics are shown in *Table 1*. A total of 105 urine samples from 105 patients [44 females, 61 males; median age 40 years, interquartile range (IQR): 31–46 years] were studied morphologically by double-immunostaining. The median time of detecting positive decoy cells, namely patients' enrollment, was 10.9 months (IQR: 5.2–24.6 months) after kidney transplantation. According to the pathological results, all patients were divided into 73 cases of BKPyVAN and 32 cases of non-BKPyVAN. The BKPyVAN group consists of 5 cases of stage A, 60 cases of stage B, and 8 cases of stage
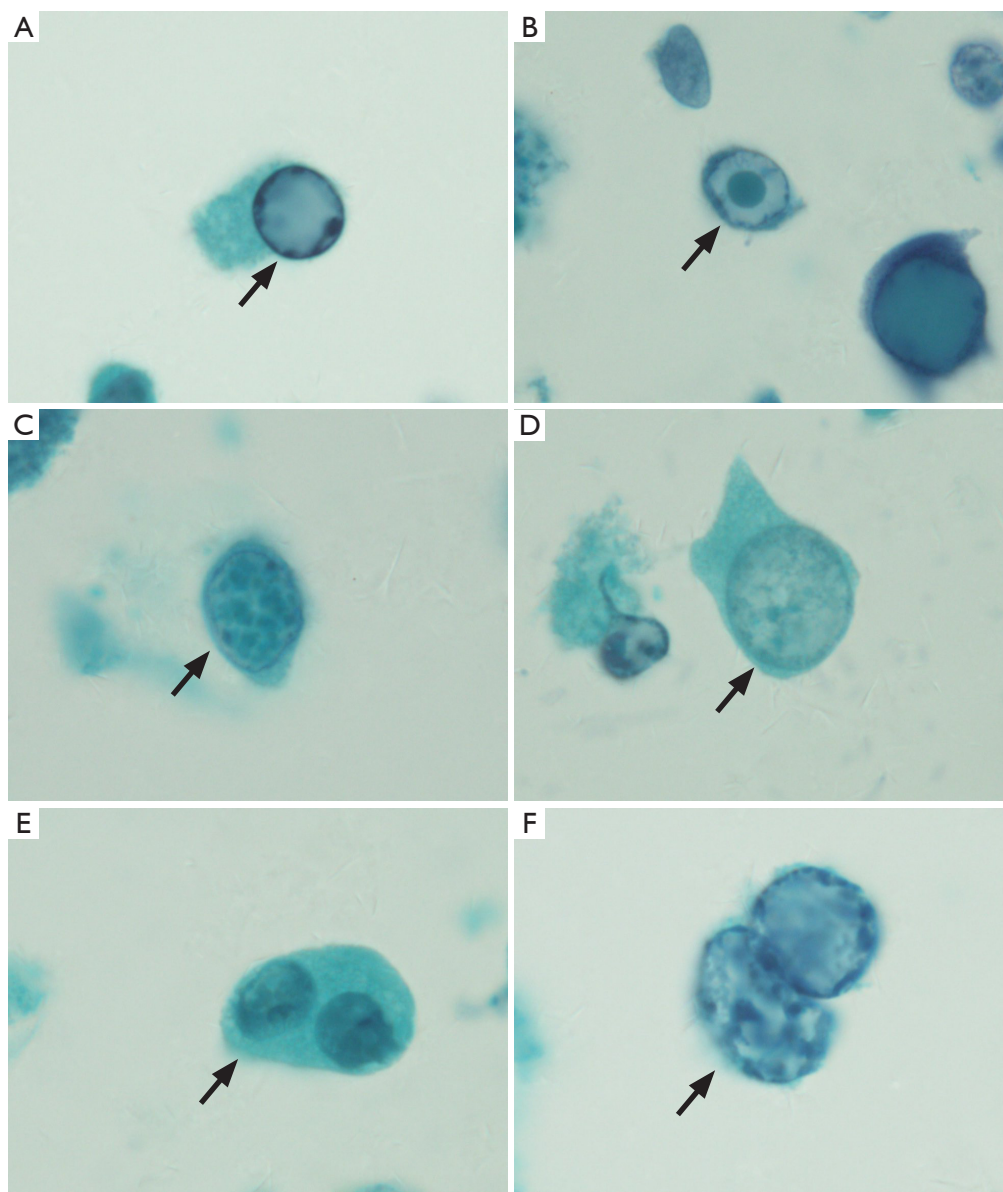
Figure 1 On Papanicolaou staining (×1,000), the decoy cells exhibited 4 classical morphological types. (A) Type 1 classic decoy cell contained enlarged, basophilic, homogeneous amorphous ground glass-like intra-nuclear inclusion body (arrow); (B) type 2 decoy cell contained a single nuclear inclusion body surrounded by a peripheral halo, appearing as a bird's eye showing (arrow); (C) type 3 decoy cell contained scattered clumped chromatin (arrow); (D) type 4 decoy cell contained cytoplasmic vesicles with fine-grained chromatin and nucleoli (arrow); (E) decoy cell contained two nucleus (arrow); (F) inclusion-bearing cell with degenerative changes in the cytoplasm, which appeared an apoptotic scenario (arrow).

C. The median initial serum creatinine level was 162.0 (125.3–235.0) µmol/L, the number of decoy cells was 23/10 HPF (IQR: 11–39), and the BKPyV viral load in the urine was $1.0×10^9$ copies/mL (IQR: $5.3×10^7$–$4.2×10^9$) and in the plasma was $1.9×10^3$ copies/mL (IQR: $0–2.6×10^4$).

Urinary decoy cells

On Papanicolaou staining, the decoy cells exhibited 4 classical morphological types (30). Type 1 classic decoy cell contained enlarged, basophilic, homogeneous amorphous ground glass-like intra-nuclear inclusion body (*Figure 1A*).
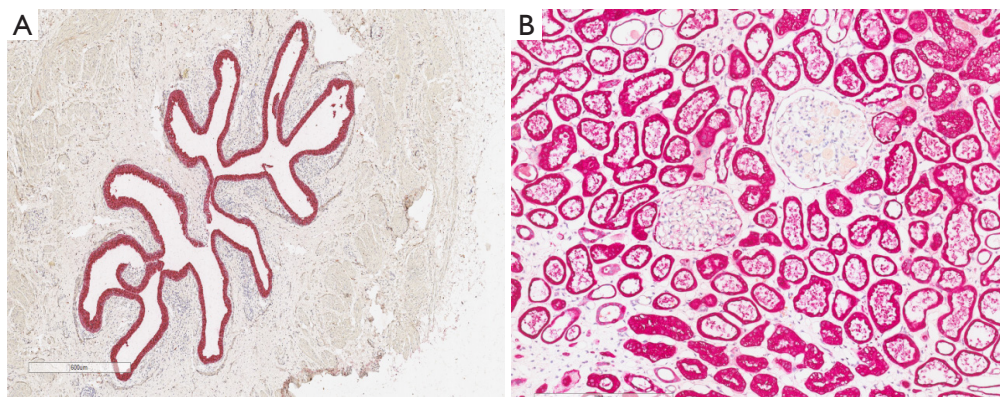
Figure 2 Confirmation of S100P(+) cells and HGD(+) cells in normal tissue. (A) Positive anti-S100P immunostaining was detected in the cytoplasm of urothelial cells (red, ×100); (B) positive anti-HGD immunostaining was detected in the cytoplasm of tubular epithelial cells (red, ×400).
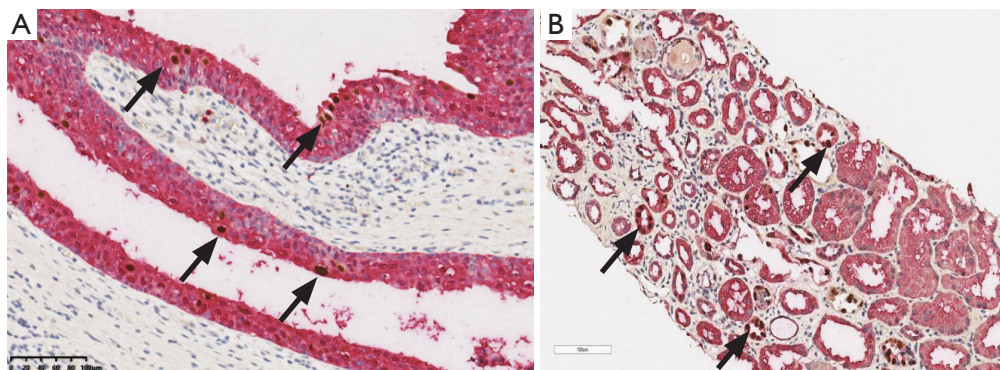
Figure 3 Confirmation of S100P(+)/SV40-T(+) cells and HGD(+)/SV40-T(+) cells in renal tissue with BKPyVAN. Tissue stained for S100P(+), HGD and SV40-T using immunostaining. (A) The cytoplasm was positive for S100P (red) and the nuclei were positive for SV40-T (brown) in BKPyV-infected urothelial cells (arrow) (×400); (B) the cytoplasm was positive for HGD (red) and the nuclei were positive for SV40-T (brown) in BKPyV-infected renal tissue (arrow) (×200).

Type 2 decoy cell contained a single nuclear inclusion body surrounded by a peripheral halo, appearing as a bird's eye showing (*Figure 1B*). Type 3 decoy cell contained scattered clumped chromatin (*Figure 1C*). Type 4 are decoy cell contained cytoplasmic vesicles with fine-grained chromatin and nucleoli (*Figure 1D*).

S100P(+) cells and HGD(+) cells in renal tissue without BKPyVAN

To confirm the specificity of S100P for urothelial epithelium and HGD for tubular epithelium, tissue sections from normal bladder, ureter, and kidney were examined. Positive anti-S100P immunostaining was detected in the cytoplasm of urothelial cells (red) (*Figure 2A*). Inversely, positive anti-HGD immunostaining was detected in the cytoplasm of tubular epithelial cells (red) (*Figure 2B*).

S100P(+)/SV40-T(+) cells and HGD(+)/SV40-T(+) cells in renal tissue with BKPyVAN

The tissue sections from BKPyV-infected kidneys, ureter, and bladder were examined to confirm the co-expression of HGD/SV40-T in tubular epithelial cells and S100P/SV40-T in urothelial cells. In BKPyV-infected urothelial cells, the cytoplasm was positive for S100P (red) and the nuclei were positive for SV40-T (brown) (*Figure 3A*). On the other hand, in BKPyV-infected kidney samples the cytoplasm was positive for HGD (red) and the nuclei were positive for SV40-T (brown) (*Figure 3B*).
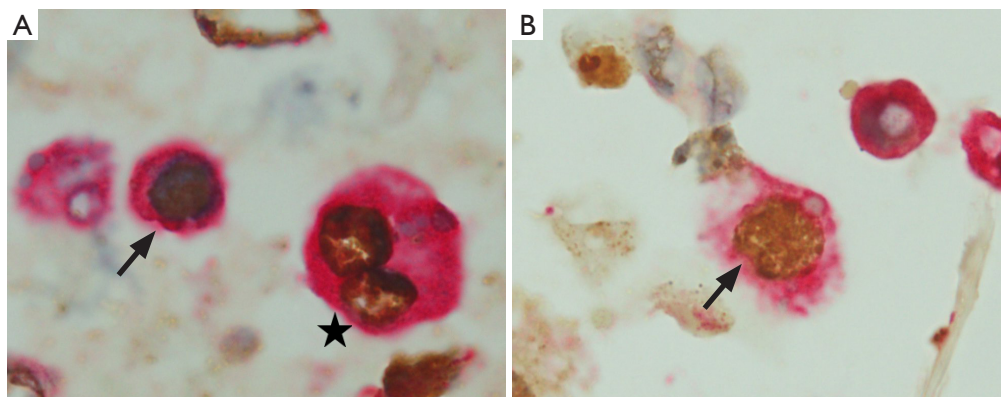
Figure 4 Confirmation of S100P(+)/SV40-T(+) cells and HGD(+)/SV40-T(+) cells in urine cell block by double-immunostaining (×1,000). Double-immunostaining with anti-SV40-T antibody and anti-S100P antibody showed a cell with red cytoplasm (S100P) and brown nuclei (SV40-T) (A, arrow) and a cell contained two nucleus (A, asterisk). Double-immunostaining (×1,000) with anti-SV40-T antibody and anti-HGD antibody showed a cell with red cytoplasm (HGD) and brown nuclei (SV40-T) (B, arrow).

Table 1 Demographic and transplant characteristics of 73 patients of BKPyVAN and 32 patients of non-BKPyVAN

Parameters	BKPyVAN (n=73)	non-BKPyVAN (n=32)	Total (n=105)	P
Gender				0.301
Male (%)	40 (54.79)	21 (65.63)	61 (58.10)	
Female (%)	33 (45.21)	11 (34.38)	44 (41.90)	
Age (years)	41.0 (IQR: 34.0–46.0)	33.0 (IQR: 28.0–47.0)	39.5 (IQR: 31.0–46.3)	0.201
ESRD etiology				0.353
Chronic glomerulonephritis (%)	30 (41.10)	16 (50.00)	46 (43.81)	
IgA nephropathy (%)	21 (28.77)	5 (15.63)	26 (24.76)	
Others (%)	22 (30.14)	11 (34.38)	33 (31.43)	
Immunosuppressive regimen				0.220
Tac-MPA (%)	72 (98.63)	30 (93.75)	102 (97.14)	
CsA-MPA (%)	1 (1.37)	2 (6.25)	3 (2.86)	
Serum creatinine at biopsy (μmol/L)	174.0 (IQR: 134.0–268.0)	133.0 (IQR: 110.0–150.0)	162.0 (IQR: 124.5–236.0)	<0.001
Decoy cells count (/10HPF)	27.0 (IQR: 10.0–45.0)	25.0 (IQR: 12.0–43.0)	25.5 (IQR: 11.0–45.0)	0.503
Urine BKPyV-DNA loads (copies/mL)	$1.7×10^9$ (IQR: $5.9×10^7–4.8×10^9$)	$2.1×10^8$ (IQR: $2.0×10^7–1.9×10^9$)	$1.0×10^9$ (IQR: $5.3×10^7–4.2×10^9$)	0.052
Plasma BKPyV-DNA loads (copies/mL)	$5.6×10^3$ (IQR: $1.3×10^3–6.0×10^4$)	0 (IQR: $0–8.8×10^2$)	$1.9×10^3$ (IQR: $0–2.6×10^4$)	<0.001

BKPyVAN, BK polyomavirus associated-nephropathy; EDSR, end stage renal disease; Tac, tacrolimus; MPA, mycophenolic acid; CsA, cyclosporine A; HPF, high power field; IQR, interquartile range.

S100P(+)/SV40-T(+) cells and HGD(+)/SV40-T(+) cells in cell block

Typical decoy cells detected by double-immunostaining are shown in *Figure 4*. Double-immunostaining results were qualitatively reported as either present (one or more cells) or absent. In urine samples, the BKPyV-infected (SV40-T-positive) cells were categorized as S100P(+)/SV40-T(+) (*Figure 4A*) and HGD(+)/SV40-T(+) (*Figure 4B*), and HGD(–)/

S100P(-)/SV40-T(+). Of the 105 patients, 76 (72.4%) patients had HGD(+)/SV40-T(+) cells and S100P(+)/SV40-T(+) cells simultaneously (group A), 24 (22.9%) patients had S100P(+)/SV40-T(+) cells only (group B), and 5/109 (4.6%) patients had S100P(–)/HGD(–)/SV40-T(+) cells only (group C). All patients with HGD(+)/SV40-T(+) cells also had S100P(+)/SV40-T(+) cells. Conversely, among patients with S100P(+)/SV40-T(+) cells, only 76 (76%) patients also had HGD(+)/SV40-T(+) cells. In most cases, the proportion of S100P(+)/SV40-T(+) cells was significantly higher than that of HGD(+)/SV40-T(+) cells.

Predictive value of urinary HGD(+)/SV40-T(+) cells for the diagnosis of BKPyVAN

In group A, 70 (92.1%) patients were diagnosed with BKPyVAN. In group B, 3 (12.5%) patients were diagnosed with BKPyVAN. In group C, no patient was diagnosed with BKPyVAN. The PPV, NPV, sensitivity, and specificity of urinary HGD(+)/SV40-T(+) cells for predicting BKPyVAN were 92.1% (95% CI: 83.0–96.7%), 89.7% (95% CI: 71.5–97.3%), 95.9% (95% CI: 87.7–98.9%), and 81.3% (95% CI: 63.0–92.1%).

Correlation analysis between HGD(+)/SV40-T(+) cells and various pathological damage indicators showed that HGD(+)/SV40-T(+) cells were correlated with the extent of SV40-T (P<0.001) and tubulitis (P=0.001), but not with interstitial inflammation (P=0.734), total interstitial inflammation (P=0.739), tubular atrophy (P=0.366), or interstitial fibrosis (P=0.505).

Diagnostic performance of urinary decoy cells and BKPyV viremia for the diagnosis of BKPyVAN

The area under the ROC curve of predicting BKPyVAN by decoy cells was 0.531 (0.431–0.630), with an optimal cut-off value of 29 (per 10 high power field), a sensitivity of 45.8% (95% CI: 34.0–58.0%), and a specificity of 68.8% (95% CI: 50.0–83.9%). Besides, the area under the ROC curve of predicting BKPyVAN by plasma BKPyV loads was 0.735 (95% CI: 0.632–0.822), with an optimal cut-off value of 1000 copies/mL, a sensitivity of 61.1% (95% CI: 48.9–72.4%) and a specificity of 84.2% (95% CI: 60.4–96.6%).

Discussion

The detection of decoy cells in urine is generally used as a simple and inexpensive technique for screening BKPyV viruria after kidney transplantation. However, Hirsch *et al.* reported that the PPV of a "positive" decoy cells for predicting BKPyVAN was 25% to 30% (13). Decoy cells may be derived from urothelium and/or the transplanted kidney (31), and it is difficult to identify the source of decoy cells by morphological appearance alone. Double-immunostaining with anti-P16 and anti-Ki67 antibodies is widely used for screening and diagnosing cervical precancerous lesions and cervical cancer (32). Inspired by this idea, we put forward a hypothesis that detecting decoy cells with double-immunostaining targeting tubule-specific or urothelium-specific markers can identify the source of the decoy cells.

S100P is a member of the S100 calcium-binding protein P family, and functions by regulating cell growth and differentiation (33). In the urinary system, S100P mainly expresses in renal pelvic urothelium, ureteral epithelium, and bladder urothelium, but not in tubular epithelium or glomerular parietal epithelial cells (https://www.proteinatlas.org/ENSG00000163993-S100P/tissue). HGD is a member of the 1 ensembl protein family, and functions by regulating the catabolism of the amino acids tyrosine and phenylalanine (34). In the urinary system, HGD is mainly expressed in the cytoplasm of proximal and distal tubular, and collecting duct epithelium, but is not expressed in renal pelvic urothelium, ureteral epithelium, and bladder urothelium cells (https://www.proteinatlas.org/ENSG00000113924-HGD/tissue). In our study, IHC staining results confirmed that HGD was only expressed in renal tubular epithelial cells, and S100P was only expressed in ureter and bladder epithelial cells. Therefore, it is reasonable to believe that HGD and S100p are useful markers for differentiating renal tubular epithelial cells from urothelial cells, and that the presence of HGD antigen in the cytoplasm of decoy cells strongly indicates that these cells are detached tubular epithelial cells.

Our results showed that the qualitative detection of HGD(+)/SV40-T(+) cells in a fixed urine cell block predicted BKPyVAN with high PPV (92.1%) and high NPV (89.7%). Actually, the PPV may even be higher because of the local distribution of BKPyV replication in renal allograft and potential sample error. In this study, 3 patients had positive urinary HGD(+)/SV40-T(+) cells, but negative anti-SV40-T IHC staining in renal allograft biopsy specimens, which we believe to be misdiagnosed BKPyVAN because of sample error.

Some researchers believe that BKPyV remains latent in tubular epithelial cells and urothelial cells. Once host
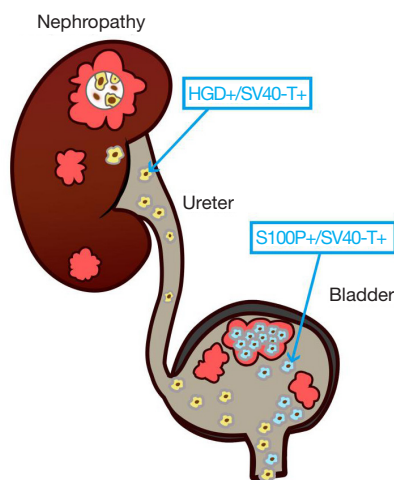
Figure 5 Conceptual model showing anatomical sources of decoy cells. BKPyV infection in renal tubular epithelium and bladder epithelium both lead to cell detachment, forming decoy cells. The renal-derived decoy cells are positive for HGD and SV40-T, while the bladder-derived decoy cells are positive for S100P and SV40-T. When BKPyV involves the bladder epithelium but not tubular epithelium, only S100P(+)/SV40-T(+) cells can be detected in the urine cell block. When BKPyVAN occurs, HGD(+)/SV40-T(+) cells, representing virus-infected tubular epithelial cells, can be detected in urine cell block as specific biomarker for "intrarenal" BKPyVAN.

immunity is inhibited, the latent BKPyV reactivates and replicates, resulting in the host cells dropping into the urine to form decoy cells (35). Others believe that decoy cells mainly originate from bladder epithelial cells and travels in a retrograde manner along the ureter to infect tubular epithelial cells (36). In our study, HGD(+)/SV40-T(+) and S100P(+)/SV40-T(+) double-immunostaining was performed on urine sediment respectively, and the results showed that the proportion of S100P (+)/SV40-T (+) cells was greater than that of HGD(+)/SV40-T(+) cells. In addition, S100P(+)/SV40-T(+) cells were found in all patients with HGD(+)/SV40-T(+) cells. However, in 100 patients with S100P(+)/SV40-T(+) cells, HGD(+)/SV40-T(+) cells were not found in 24 patients. We believe that this group of patients have an early BKPyV-activated infection, only involving the bladder and/or ureteral transitional epithelial cells. As such, our results demonstrated that in most patients with urinary BKPyV infection, decoy cells are predominantly derived from the urothelium. This phenomenon appears to be inconsistent

with the results of a previous study in which only anti-S100P and anti-SV40-T antibodies, but no antibody aimed at renal tubular epithelial cells, were used for detecting decoy cells (37). In that study, the authors examined urine samples from 7 kidney transplant recipients for S100P(+)/SV40-T(+) cells and S100P(−)/SV40-T(+) cells, and the S100P(+)/SV40-T(+) cells accounted for a relatively low proportion of all SV40-T(+) cells, so they indirectly inferred that S100P(−)/SV40-T(+) cells are derived from the renal tubular epithelium and most decoy cells are tubular-derived cells.

Notably, in a few cases, BKPyV can infect the glomeruli. Both Celik *et al.* and Ravindran *et al.* found that BKPyV could involve glomeruli in biopsy of transplanted kidney (38,39). In addition, Alcendor *et al.* and Popik *et al.* also demonstrated that BKPyV could infect and replicate in glomerular cells *in vitro* (40,41). Similarly, in our previous study, we also found that BKPyV could infect glomerular parietal epithelial cells, and this phenomenon often indicated more serious pathological damage and worse prognosis of transplanted kidney function (42). Moreover, our results showed that BKPyV infection in renal tubular epithelial cell is concurrent in all patients with glomerular BKPyV involvement. This may be because BKPyV usually spreads in a retrograde way. Theoretically, the combination of HGD and SV40-T is enough to demonstrate whether BKPyV replicate in transplanted kidney or not. Of course, we also expect to find a specific marker of glomerulus, which can provide us with clues of glomerular BKPyV infection.

The novel, non-invasive urine-based BKPyV-Haufen test has been reported to diagnose BKPyVAN with a PPV and NPV both greater than 95% (17). BKPyV-Haufen form in virus-infected renal tubules are flushed out of the kidney, and appear in the urine as a direct marker of intra-renal viral disease (17,43). Similar to the detection of BKPyV-Haufen aggregates, urinary HGD(+)/SV40-T(+) cells detach from virally injured tubular epithelium and subsequently are flushed out of the kidney. Thus, the presence of urinary HGD(+)/SV40-T(+) cells directly reflect BKPyV replication in renal allografts. Detection of urinary polyomavirus-Haufen requires an experienced electron microscopist, and electron microscopy can't be routinely performed in many transplant centers. In contrast, double-immunostaining is simple to perform, can be done at almost every transplant center, and even an inexperienced pathologist can make a diagnosis. The technique can be used as a screening tool for BKPyVAN during outpatient follow-up, and as a supplementary test when a patient is suspected to have BKPyVAN but biopsy samples are negative.

In conclusion, urinary decoy cell identification via double-immunostaining represents a reliable alternative method for determining the origin of decoy cells in urine samples and for predicting BKPyVAN (*Figure 5*). We believe that this non-invasive method combined with PCR can provide useful information for diagnosing BKPyVAN when renal biopsy specimens are IHC staining negative for anti-SV40-T. Moreover, regular monitoring of urine HGD(+)/SV40-T(+) cells after transplantation cells can further demonstrate its diagnostic performance for BKPyVAN.

Acknowledgments

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are responsible for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee and the Research Board, the First Affiliated Hospital of Sun Yat-sen University {No. [2019]220}.

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Cite this article as: Chen XT, Chen WF, Hou XT, Yang SC, Yang HF, Li J, Deng RH, Huang Y, Nuertai Y, Wang CX, Qiu J, Huang G. Non-invasive urinary sediment double-immunostaining predicts BK polyomavirus associated-nephropathy in kidney transplant recipients. Ann Transl Med 2020;8(5):235. doi: 10.21037/atm.2020.01.15
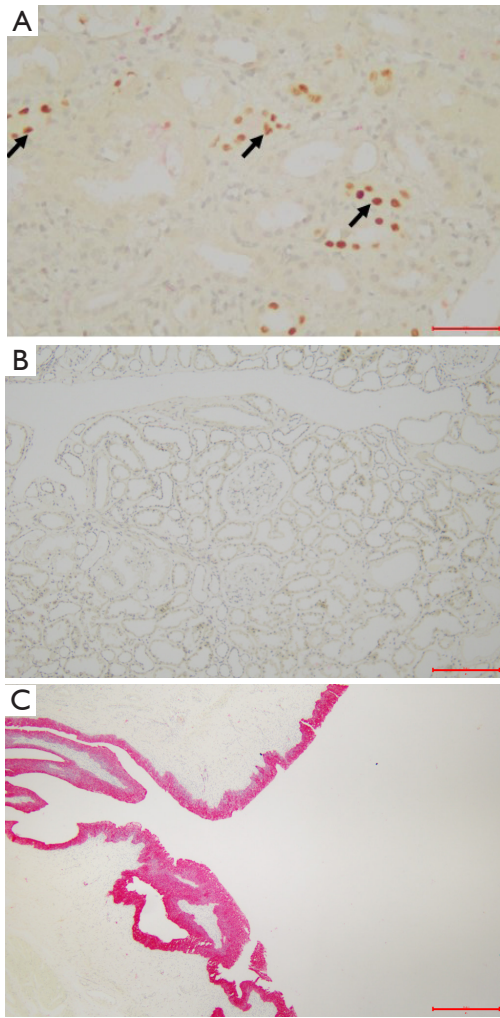
Figure S1 Confirmation of S100P expression in transitional epithelium. Double-immunostaining with anti-SV40-T antibody and anti-S100P antibody showed that SV40-T (brown) was expressed in renal tubular epithelium of renal tissue with BKPyVAN (A, ×200, arrow), but S100P was not expressed in renal tubular epithelium of renal tissue with BKPyVAN (A, ×400) or without BKPyVAN (B, ×100). (C) S100P (red) was expressed in transitional epithelial cells (C, ×40).
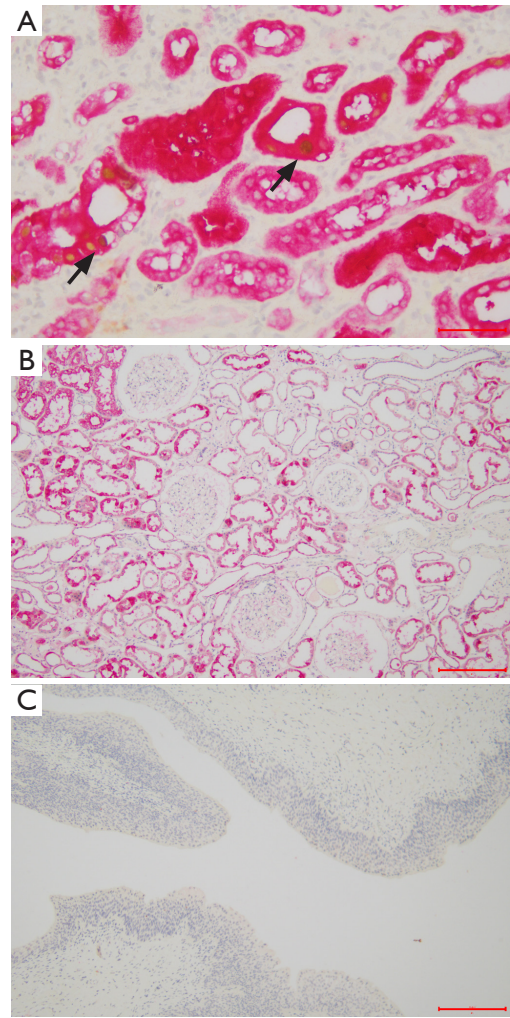
Figure S2 Confirmation of HGD expression in renal tubular epithelium. Double-immunostaining with anti-SV40-T antibody and anti-HGD antibody showed SV40-T was expressed in the nucleus and HGD was expressed in renal tubular epithelium of renal tissue with BKPyVAN (A, arrow, ×400) and renal tissue without BKPyVAN (B, ×100). (C) HGD was not expressed in transitional epithelial cells (C, ×100).