Bright Field Microscopy to Detect Decoy Cells Due to BK Virus Infection in the Fresh and Unstained Urine Sediment in Kidney Allograft Recipients

José A. T. Poloni,^{1,2*} Gabriel G. Pinto,² Maria S. B. Giordani,¹ Elizete Keitel,^{2,3} Nadiana Inocente,² Carlos F. Voegeli,¹ Giovanni B. Fogazzi,⁴ Alessandro C. Pasqualotto,^{2,5} and Liane N. Rotta²

¹Central Laboratory of Clinical Analysis, Santa Casa de Misericórdia de Porto Alegre, Porto Alegre, Brazil

²Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil

³Department of Nephrology, Renal Transplant Unit, Santa Casa de Misericórdia de Porto Alegre, Porto Alegre, Brazil

⁴Research Laboratory on Urine Microscopy, Ospedale Maggiore, Milan, Italy

⁵Molecular Biology Laboratory, Santa Casa de Misericórdia de Porto Alegre, Porto Alegre, Brazil

Background: BK virus (BKV) may reactivate in kidney allograft recipients ultimately leading to BKV nephropathy and graft loss. Decoy cells (DCs) are one of the early marks of BKV reactivation, and these can be detected in the urine sediment. **Methods:** A cohort of 102 kidney transplant patients was followed during months 3 and 6 after the transplant procedure. Urine samples were obtained to detect the presence of DC in the fresh and unstained urine sediment under bright field microscopy (BFM), in concomitance to the determination of the amount of BK viruria by qPCR. **Results:** Decoy cells were found in 14.7% of patients (15/102). There was a strong agreement ($P < 0.001$) between qualitative DC detec-

tion by two experienced analysts and by qPCR. The positive predictive value, negative predictive value, specificity, and accuracy of BFM were 80%, 75%, 97%, and 75%, respectively. Test sensitivity was 16%. The comparative method was the qPCR. **Conclusions:** Despite its limited sensitivity, BFM of unstained urine sediment is an easily available, fast and cheap method to identify DCs in the population of kidney allograft recipients. The diagnostic performance of BFM on the hands of less experienced analysts deserves further investigation. *J. Clin. Lab. Anal.* **30**:1044–1050, 2016. © 2016 Wiley Periodicals, Inc.

Key words: bright field microscopy; decoy cells; polyomavirus BK; urine sediment analysis

INTRODUCTION

BK virus (BKV) is a DNA virus that infects up to 90% of the adult population (1). After initial exposure in childhood, the virus becomes latent in urothelial tissues (2). In transplant patients, BKV can reactivate in the context of potent immune suppression which may result in uncontrolled viral replication and eventually BKV-associated nephropathy (BKVAN) (3). Prompt detection of viral reactivation can improve outcomes and therefore active screening after transplantation is recommended (4).

Screening tests for BKV infection include identification of decoy cells (DCs), qualitative and quantitative

polymerase chain reaction (PCR) test to detect BKV DNA in urine and plasma, urine electron microscopy, and allograft biopsy. Once diagnosed with BKVAN, between 16% and 50% of transplant recipients will lose the graft (5).

Decoy cells appear in the urine presenting homogenous intranuclear inclusions that appear as glassy viral

*Correspondence to: José A. T. Poloni, Rua Prof. Annes Dias, 295, Zip code: 90.020-090, Porto Alegre, Brasil. E-mail: jatpoloni@yahoo.com.br

Received 10 January 2016; Accepted 9 March 2016

DOI 10.1002/jcla.21978

Published online in Wiley Online Library (wileyonlinelibrary.com).

material on bright field microscopy (BFM). DCs can be easily identified and counted on standard Papanicolaou-stained slides using cytospin. However, there are some variations to the method. Fogazzi et al. (6) identified DCs in the urine sediment of a kidney transplant recipient using phase contrast microscopy. A previous study of our group reported that DCs can also be detected in the urine sediment of kidney transplant patients by the means of BFM without any special stain (7). Since BFM is the main technology used to perform urinalysis in clinical analysis laboratories, the present study was conducted to evaluate the diagnostic utility of this basic technology to identify DCs due to BKV in the fresh and unstained urine sediment of kidney allograft recipients. Quantitative real-time PCR (qPCR) was used as the comparative reference method in this study.

SUBJECTS AND METHODS

Study Population

From April 2012 to March 2013, we prospectively enrolled kidney allograft recipients (from both live-donor and cadaveric transplants) attending Santa Casa de Misericórdia de Porto Alegre, a referral medical center for organ transplantation located in Porto Alegre, Southern Brazil. All patients were adults (age ≥ 18 years old).

Urine Sample Collection

Following a standard operational procedure, patients were asked to collect the first morning midstream urine for laboratorial testing. Urine samples were collected on sterile containers and divided into two 10-ml aliquots, in order to perform urine sediment analysis (for DCs detection) and quantification of BKV viruria. Clinical samples were obtained at the months 3 and 6 following the transplantation procedure.

DCs Examination by BFM in the Fresh and Unstained Urine Sediment

A conic tube containing urine (10 ml) was centrifuged at 1,500 rpm/5 min. The supernatant was removed using a Pasteur pipette till the mark of 0.5 ml. The urine sediment was homogenized using the same pipette and 20 μ l of the urine sediment was transferred to a slide and covered with a coverslip of 22 $\times$ 22 mm. Samples were analyzed by two experienced urine microscopists counting the number of DCs on 50 fields, using 400 $\times$ magnification lens (high power field) in a Zeiss Axiostar microscope (Jena,

Germany). Microscopists remained blinded for the results of each other until study analysis.

Viruria Determination

Urine samples were refrigerated at 4°C after collection for a maximum period of 24 hr. DNA was extracted from urine using QIAmp viral RNA mini kit (QIAGEN, Hilden, Germany). In a sterile falcon tube, 10 ml of homogenized urine was centrifuged at 3,500 rpm/10 min. The supernatant was discarded and a pellet of 140 μ l was used as recommended by the manufacturer.

Extracted DNA samples were stored at -80°C for later qPCR testing. An amplification internal control (CPE-DNA Internal Control-Nanogen, Buttiglieria Alta, Italy) was added to each sample before DNA extraction, as recommended by the manufacturer. The internal control was the human globin gene, which was amplified simultaneously with the target sequence. BKV DNA amplification was performed using a commercial kit (BKV Q-PCR Alert Ampliprobe, Nanogen, Buttiglieria Alta, Italy) in a 7500 thermal cycler qPCR System (Applied Biosystems, Foster City, USA). The following cycling steps were used: preholding stage 2 min at 50°C, initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 1 min and 60°C for 1 min. The standard curve was obtained using BKV Q-PCR Standard (Nanogen, Buttiglieria Alta, Italy) containing four known concentrations of BKV DNA (102–105 plasmid copies in 10-fold dilution steps). Results were read by comparing the cycle threshold (Ct) from the unknown sample with the standard controls. In addition to positive controls, in every run a negative BKV control as well as the internal control was added to the reaction (8).

Statistical Analysis

Descriptive statistics were used to summarize the data. Concordance between different diagnostic methods was assessed using the kappa (κ) value. The κ value also was used to evaluate the rate of agreement between observers. The correlation of DCs and viruria was tested using Spearman rank correlation coefficient. Variables were treated with Wilcoxon test with an internal confidence of 95%. *P* values of ≤ 0.05 were considered statistically significant. Data analysis was performed using the SPSS 19 software (IBM, New York, USA).

Ethical Aspects

The study was approved by the Institutional Review Board of the participating institutions, and followed the guidelines and regulatory standards for research

involving human participants of the Brazilian National Health Council (Resolution CNS/196). A written consent was obtained before entering the study.

RESULTS

A total of 189 urine samples were studied from 102 renal allograft recipients. Most patients were men (56.9%). Data from patients with DCs in the urinary sediment are summarized on Table 1. DCs were observed in 15 patients (14.7%), and 80.0% of these patients ($n = 12$) also had quantifiable BK viruria ($>700,000$ viral copies/ml). To the other three patients presenting DCs, viruria results were negative. To 87 patients, DCs were not found in the urine sediment and 15 of these patients presented viruria results $>700,000$ viral copies/ml.

Table 2 shows the overall agreement between observers for DC detection in unstained urine samples, as well as the correlation between diagnostic tests (unstained BFM and qPCR). Both analysts presented satisfactory agreement in the identification of DC qualitatively ($P < 0.001$) and quantitatively ($P < 0.001$), also, both analysts presented satisfactory agreement in the DC identification when compared to viruria ($P < 0.001$). The performance of the unstained urine sediment in terms of specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy is summarized on Table 3. Results of both analysts revealed elevated specificity (97–98%) and PPV (80–91%). However, NPV was in the range of (75–85%), and sensitivity was low (16–46%).

DISCUSSION

Human BKV has emerged in the last decade as a potential pathogen for renal transplant recipients. BKV infection occurs in early childhood without presenting any clinical syndrome. Subsequently it enters a latent stage. In immunocompromised hosts, such as renal transplant patients, BKV may reactivate. Even though reactivation is rarely followed by clinical manifestations, such as allograft dysfunction with an increase in serum creatinine or ureteric obstruction, nevertheless, renal graft damage may progress and may be irreversible (9). The prevalence of BKVAN after renal transplantation has been reported in 1% to 33% of the patients, depending on the study (10–21). At the Medical College of Wisconsin, from 1996 to 2004, the prevalence of BKVAN was 4% (10). In our study, the prevalence of BKVAN could not be established since BKVAN can only be proved when a kidney biopsy is performed. Kidney biopsy procedures in our study were conducted only under clinical indication and that occurred for 17 patients only (four of which had confirmed BKVAN). Circulating plasma BKV DNA has been seen in approximately 10–40% of renal transplant recipients (10, 11, 22). However, not all viremic patients develop clinical nephritis. BK viremia can be detected in nearly 100% of patients with BKVAN, by qPCR. However, the PPV for qPCR in the plasma for BKVN is only 60%. Viruria is seen in 30–40% of renal transplant recipients and the amount of BKV DNA in the urine is usually 100-fold higher than of plasma (10, 11, 23). Similar to plasma BKV

TABLE 1. Patients with Decoy Cells (DCs) Observed in the Unstained Urine Sediment Under Bright Field Microscopy (BFM), in Comparison to Viral Load (BKV) in the Urine

Sex	Age	Month 3			Month 6		
		DC analyst 1	DC analyst 2	Viruria (copies/ml)	DC analyst 1	DC analyst 2	Viruria (copies/ml)
M	44	556	774	2.6×10^{11}	40	31	1.9×10^{10}
F	44	21	22	6.1×10^{10}	0	0	1.1×10^8
F	43	0	0	8.9×10^8	5	3	1.2×10^{10}
M	43	2	5	6.0×10^9	0	0	3.7×10^7
M	37	1	0	7.3×10^6	0	0	1
F	25	1	2	0	0	0	1
M	46	1	3	9.5×10^9	0	0	4.6×10^9
M	34	12	11	2.4×10^{10}	NA	NA	NA
M	43	0	0	0	3	1	0
M	59	0	2	1	0	0	0
M	62	6	6	2.1×10^{10}	1	0	7.6×10^9
M	33	21	6	3.3×10^{10}	1	1	5.0×10^8
M	26	6	4	5.2×10^{10}	1	0	1.9×10^{10}
M	69	25	26	2.9×10^{10}	142	63	3.1×10^{11}
M	34	1	0	1.6×10^9	1	0	4.0×10^8

DC, decoy cell; F, female; M, male; NA, data not available (allograft rejection due to BKV and Cytomegalovirus co-infection).

TABLE 2. Interobservers' Comparison of Urine Microscopy Results and Comparison between Urine Microscopy and Viruria, Detected by qPCR

	Month 3	κ	Month 6	κ	<i>P</i> value
Agreement on DC identification between A1 and A2 (qualitative analysis)	A1 (<i>n</i> = 12) A2 (<i>n</i> = 11)	0.853	A1 (<i>n</i> = 8) A2 (<i>n</i> = 5)	0.752	<0.001
Intraclass correlation coefficient between A1 and A2 (quantitative analysis)	Value 0.947	–	Value 0.762	–	<0.001
Agreement between DC (A1) and viruria (qPCR)	DC (<i>n</i> = 12) Viruria (<i>n</i> = 24)	0.538	DC (<i>n</i> = 8) Viruria (<i>n</i> = 25)	0.333	<0.001
Agreement between DC (A2) and viruria (qPCR)	DC (<i>n</i> = 11) Viruria (<i>n</i> = 24)	0.429	DC (<i>n</i> = 5) Viruria (<i>n</i> = 25)	0.191	<0.001

A1, analyst 1; A2, analyst 2; DCs, decoy cells; qPCR, quantitative real-time polymerase chain reaction.

DNA, the presence of BKV DNA in the urine is associated with a poor PPV for BKVAN.

The hallmark of the BKV infection is the presence in the urine of DC which are infected cells shed into the urine from the renal tubules and uroepithelium (24).

It is quite logical to assume that qPCR is more sensitive tool to detect the presence of BKV in the urine, in comparison to DCs by BFM, a later finding. However, the performance of cheap, noninvasive quick methods such as unstained BFM has not been properly studied in the literature. Here, we demonstrated that on experienced hands DCs may be detected quite accurately under unstained BFM, and that correlates quite well with the amount of viruria, as detected by BKV qPCR.

TABLE 3. Performance of Decoy Cells Detection Under Bright Field Microscopy of Unstained Urine Sediment, in Comparison to Viruria (BKV) Detected by Real-Time Polymerase Chain Reaction (qPCR) Test

		Month 3 (<i>n</i> = 100)	Month 6 (<i>n</i> = 89)
Analyst 1	True positive (<i>n</i>)	11	7
	True negative (<i>n</i>)	75	63
	False positive (<i>n</i>)	1	1
	False negative (<i>n</i>)	13	18
Analyst 2	True positive (<i>n</i>)	9	4
	True negative (<i>n</i>)	74	63
	False positive (<i>n</i>)	2	1
	False negative (<i>n</i>)	15	21
Analyst 1	Positive predictive value (%)	91	88
	Negative predictive value (%)	85	78
	Sensitivity (%)	46	28
	Specificity (%)	98	98
	Overall accuracy (%)	86	78
Analyst 2	Positive predictive value (%)	81	80
	Negative predictive value (%)	83	75
	Sensitivity (%)	37	16
	Specificity (%)	97	98
	Overall accuracy (%)	83	75

Previous studies have shown that the PPV of urinary DCs may be as low as 20% to identify BKVAN. Thus, demonstration of urinary DCs suggests the presence of BKV in urothelium, but that does not confirm the presence of BKVAN (10). A previous study that compared urine cytology stained by Papanicolaou with kidney biopsies found 69% specificity, 85% sensitivity, 21% PPV, 98% NPV, and 70% overall accuracy (12). In the current study we used BKV viruria (>700,000 viral copies/ml) as the standard comparative diagnostic method. Both analysts presented satisfactory results in terms of PPV (80%), NPV (75%), specificity (97%), and overall accuracy (75%) even though the test sensitivity was only $\geq 16\%$ (Table 3). These results can be explained due to the ability of the molecular tests to detect small amounts of viral copies (that are present in high numbers within the DCs or free in the urine) which differs from the microscopic test that requires the clear evaluation of the cell morphology to provide the correct information. As seen on Table 2 the number of patients with viruria (as detected by BKV qPCR) was always higher than the number of patients with DCs in the urine sediment.

Sensitivity observed in the literature ranged from 25% to 100% (12–15, 19, 21), and the major part of the works found approximately a sensitivity value of 80%. PPV results from the present study compared with the PPV results available in the literature (5% to 21.2%) (12–15, 21) are higher. These can be explained because viruria was used as the comparative diagnostic method in the present work. All other studies compared their results to kidney biopsy, still the gold standard method to identify BKVAN.

Decoy cell detection is usually not part of the routine urinalysis performed in large clinical analysis laboratories. Since our laboratory performs urine analysis of approximately 60–70 kidney allograft recipients/day, we are able to identify DCs in 1–5 of these patients/day. The microscope used in our laboratory

Fig. 1. Large number of decoy cells stained by Papanicolaou stain in urine sediment. Bright field microscopy. Original magnification $400 \times$.

to perform urinalysis is equipped with phase contrast filters, however, the major part of the laboratories processing samples from kidney transplant recipients do

not work with such equipment. This study was conducted to standardize the procedure to search for DCs during simple routine urinalysis using BFM.

The classical description of the morphology of the DCs, using Papanicolaou staining or phase contrast microscopy, consists in nuclear enlargement, which confers a ground glass appearance and displacement of the nucleus toward the periphery of the cell as if the nucleus were escaping from the cell; chromatin margination, which is chromatin clumping along the nuclear membrane; abnormal chromatin patterns; single nuclear inclusion body surrounded by a peripheral halo, which confer a bird's eye appearance to the cells; cytoplasmic vesicles (25). Figure 1 shows a large number of DCs in the urine sediment stained by Papanicolaou stain. On the image it is possible to identify the characteristics explained previously and it helps on the comparison with the morphological aspects of the decoy cells unstained and seen under phase contrast microscopy and under BFM (Fig. 2).

The urine sediment profile of patients presenting DCs features commonly renal tubular epithelial cells (RTEC) and macrophages that cannot be misidentified as DCs.

Fig. 2. Three decoy cells presented under bright field microscopy (left panel) and under phase contrast microscopy (right panel). Original magnification $400 \times$.

Fig. 3. Structures 1 and 2 (Renal tubular epithelial cells); Structures 3 and 4 (Macrophages). Bright field microscopy. Original magnification $400\times$.

RTECs most commonly found in the urine sediment probably derive from the proximal segment of kidney tubules. They are round to oval in shape, have a large central or eccentric nucleus containing one or two nucleoli and a granular cytoplasm (Fig. 3, structures 1 and 2). Macrophages are roundish cells with very variable diameter and appearance. They contain one or more nucleus, which can be either in a central or in a peripheral location (Fig. 3 structures 3 and 4). The knowledge on the morphology of these structures is important to avoid errors in the identification of the DCs.

The comparison of the qualitative and quantitative results between the two analysts that performed the DCs search using BFM revealed a high rate of agreement (Tables 1 and 2). These results indicate that the information that can be provided on routine urinalysis is related to the already standardized qPCR test to quantify viruria related to BKV.

It is important to report that there was only one previous study that reported the finding of DCs in unstained urine sediment in a large group of patients and compared it to kidney biopsies, Papanicolaou stain of the urine sediment, and plasma qPCR. They concluded that the presence of decoy cells on urine sediment analysis was independently predictive of an

increased risk for BKV infection. DC shedding had a high NPV (98%) for BKV infection. Authors, however, did not inform if analysis was performed under BFM or under phase contrast microscopy (14). The other works on the identification of DC in the fresh and unstained urine sediment are basically case reports (6, 7).

In conclusion, despite its limited sensitivity, routine urine sediment analysis (fresh and unstained) is a method that can be used to identify DCs due to BKV reactivation on kidney allograft recipients. The analysis procedure is fast, cheap, noninvasive, and can lead to the early diagnosis of BKV reactivation, an important cause of graft loss on the large population of kidney allograft recipients. The ability of less trained observers to perform such diagnosis requires further validation.

ACKNOWLEDGMENTS

Central Laboratory of Clinical Analysis staff, Molecular Biology Laboratory staff, Nephrology Unit and Nephrology Ambulatory staff. CAPES and FAPERGS, which are governmental institutions, supported this study.

CONTRIBUTORSHIP STATEMENT

José Antonio Tesser Poloni, Elizete Keitel, Giovanni Battista Fogazzi, Alessandro Comarú Pasqualotto, and Liane Nanci Rotta – Planned, conducted, and reported the work described in the article. Gabriel Godinho Pinto – Conducted and reported the work described in the article. Maria Solange Bressan Giordani and Nadiana Inocente – Conducted the work described in the article. Carlos Franco Voegeli – Reported the work described in the article.

REFERENCES

- Comoli P, Hirsch HH, Ginevri F. Cellular immune responses to BK virus. *Curr Opin Organ Transplant* 2008;13:569.
- Chesters PM, Heritage J, McCance DJ. Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infect Dis* 1983;147:676.
- Hirsch HH, Brennan DC, Drachenberg CB, et al. Polyomavirus-associated nephropathy in renal transplantation: Interdisciplinary analyses and recommendations. *Transplantation* 2005;79:1277.
- Drachenberg CB, Papadimitriou JC, Wali R, et al. Improved outcome of polyoma virus allograft nephropathy with early biopsy. *Transplant Proc* 2004;36:758.
- Hirsch HH, Steiger J. Polyomavirus BK. *Lancet Infect Dis* 2003;3:611.
- Fogazzi GB, Cantu' M, Saglimbeni L. "Decoy cells" in the urine sediment due to polyomavirus BK infection: Easily seen by phase-contrast microscopy. *Nephrol Dial Transplant* 2001;16:1496–1498.
- Poloni JAT, Bruno RM, Voegeli CF. Common digital camera and urinary sediment analysis: A tool to be explored. *Nephrol Dial Transplant Plus* 2011;4:455–456.
- Pinto GG, Poloni JA, Carneiro LC, Baethgen LF, Barth AL, Pasqualotto AC. Evaluation of different urine protocols and DNA extraction methods for quantitative detection of BK viruria in kidney transplant patients. *J Virol Methods* 2013;188:94–96.
- Koukoulaki M, O'Donovan M, Pursglove S, Alexopoulou D, Hadjiconstantinou V, Drakopoulos S. Prospective study of urine cytology screening for BK polyoma virus replication in renal transplant recipients. *Cytopathology* 2008;19:385–388.
- Hariharan S. BK virus nephritis after renal transplantation. *Kidney Int* 2006;69:655–662.
- Hirsch HH, Knowles W, Dickenmann M, et al. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med* 2002;347:488–496.
- Ranzi AD, Prolla JC, Keitel E, et al. The role of urine cytology for "decoy cells" as a screening tool in renal transplant recipients. *Acta Cytol* 2012;56:543–547.
- Viscount HB, Eid AJ, Espy MJ, et al. Polyomavirus polymerase chain reaction as a surrogate marker of polyomavirus-associated nephropathy. *Transplantation* 2007;84:340–345.
- Yeo FE, Yuan CM, Swanson SJ, et al. The prevalence of BK polyomavirus infection in outpatient kidney transplant recipients followed in a single center. *Clin Transplant* 2008;22:532–541.
- Vidas Z, Misić M, Pacić A, Jurenec F, Knotek M, Kardum-Skelin I. The value of urinar decoy cells finding in patients with kidney transplantation. *Coll Antropol* 2010;34:153–157.
- Maia TM, Silva SF, Silva SL, Holanda MC, Nascimento JM, Ferreira MV. Polyomavirus-infected decoy cells in cytocentrifuged urine cytology specimens from renal transplant recipients. *Acta Cytol* 2011;55:445–448.
- Chakera A, Dyar OJ, Hughes E, Bennett S, Hughes D, Roberts IS. Detection of polyomavirus BK reactivation after renal transplantation using an intensive decoy cell surveillance program is cost-effective. *Transplantation* 2011;92:1018–1023.
- Chung BH, Hong YA, Kim HG, et al. Clinical usefulness of BK virus plasma quantitative PCR to prevent BK virus associated nephropathy. *Transpl Int* 2012;25:687–695.
- Huang G, Chen WF, Wang CX, et al. Noninvasive tool for the diagnosis of polyomavirus BK-associated nephropathy in renal transplant recipients. *Diagn Microbiol Infect Dis* 2013;75:292–297.
- Abdelsalam NF, Hashad DI, Salem MA, El-Wakil HS, Adam AG. Occurrence of the polyomavirus among kidney transplant recipients: A single-center study. *Saudi J Kidney Dis Transpl* 2014;25:285–293.
- Nankivell BJ, Renthawa J, Jeffreys N, et al. Clinical utility of urinary cytology to detect BK viral nephropathy. *Transplantation* 2015;99:1715–1722.
- Brennan DC, Agha I, Bohl DL, et al. Incidence of BK with tacrolimus vs. cyclosporine and impact of preemptive immunosuppression reduction. *Am J Transplant* 2005;5:582–594.
- Hussain S, Orentas R, Walczak J, et al. Prevention of BKV nephritis by monitoring BK viremia in renal transplant recipients: A prospective study. *Graft* 2004;7:28–30.
- Nickeleit V, Hirsch HH, Zeiler M, et al. BK-virus nephropathy in renal transplants-tubular necrosis, MHC-class II expression and rejection in a puzzling game. *Nephrol Dial Transplant* 2000;15:324–332.
- Singh HK, Bubendorf L, Mihatsch MJ, Drachemberg CB, Nickleleit V. Urine cytology findings of polyomavirus infections. *Adv Exp Med Biol* 2006;577:201–212.