

Simultaneous immunostaining with anti-S100P and anti-SV40 antibodies revealed the origin of BK virus-infected decoy cells in voided urine samples

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Abstract

Background: Methods for determining the origin of BK virus (BKV)-infected cells in clinical urine samples (decoy cells) have not been established, although they could enhance diagnosis of BKV infection in immunocompromised patients.

Methods: We performed simultaneous immunostaining with anti-S100P (a urothelial marker) and anti-SV40 antibodies in 66 clinical urine samples exhibiting SV40 positivity and a decoy-cell appearance in Papanicolaou staining. The clinical urine samples included seven cases of renal transplantation, 47 cases of cancer therapy, and 12 cases of non-neoplastic disease.

Results: BKV-infected cells were categorized as SV40(+)/S100P(+) and SV40 (+)/S100p(-). SV40(+) and S100p(-) cells were found in 55 cases (83.4%); nine cases (13.6%) carried both SV40(+)/S100p(-) and SV40(+)/S100P(+) cells. The former were identified as BKV infection in renal tubules and the latter in both the renal tubules and urothelial epithelia. The remaining two cases (3.0%) had only SV40(+)/S100P(+) cells, of urothelial origin.

Conclusion: Simultaneous immunostaining with anti-S100P and anti-SV40 is a useful method for determining the origin of BKV-infected cells in clinical urine samples from immunocompromised patients such as renal transplantation recipients.

Key Words: BK virus-infected cells, S100P, SV40, Simultaneous immunostaining, Urine cytology

Introduction

In urine cytology, BK virus (BKV)-infected cells are detected as decoy cells, most of which are thought to originate from the renal tubules [1-3,5]. The detection of decoy cells is generally used as a supporting technique for BKV nephropathy in kidney-transplantation patients [4-6]. BKV infection is common in adults with a reported prevalence of more than 80%; the infection usually occurs in early childhood. Reactivations may occur in conditions associated with impaired immunity [7-9]. BKV infection usually occurs in the urothelial cells of the bladder and ureter and can spread to renal-collecting ductal epithelial cells and renal tubular epithelial cells in immunocompromised individuals [2,10]. We suggest there are two types of BKV-infected cells in clinical urine samples: renal tubular epithelial cells and urothelial cells. Bone marrow transplant patients with BKV infection often suffer from hemorrhagic cystitis, and kidney transplantation is associated with BKV nephropathy; the treatments for each of these conditions differ [11,12]. Identifying the site of BKV infection is important for determining the appropriate therapeutic strategy.

Detection of decoy cells by urine cytology is generally performed for diagnosis of BKV infection, in addition to direct detection of BKV by PCR [13,14]. Although PCR is highly sensitive and specific, it cannot identify the site of BKV infection [15]. It is difficult to discriminate between renal tubular epithelial cells and urothelial cells by cytological morphology and a specific antibody to distinguish these cells from each other was not available until recently.

In this study, we attempted to identify the origin of BKV-infected cells in clinical urine samples by simultaneous immunostaining. We used anti-S100P (an urothelial marker) and anti-SV40 antibodies [16-18]. Anti-SV40 antibody is commonly used to identify BKV infection because it provides the advantage of cross-reactivity between SV40 and BKV [19-21].

Material and Methods

Materials

75 The institutional research board of Okayama Medical Center approved this study. Among the 1039 clinical urine specimens submitted to the Okayama Medical Center for cytological examination from September 2012 to June 2013, 66 cases were SV40 positive and exhibited decoy cell morphology by Papanicolaou staining. These samples represented seven cases of renal transplantation, 47 cases of cancer therapy, and 12 cases of non-neoplastic disease. The
80 decoy cells displayed four primary morphological types: Type 1 are classic decoy cells, which contain nucleus with large, homogenous, amorphous ground-glass like intranuclear inclusion bodies and a condensed rim of chromatin. Type 2 are decoy cells containing nucleus with granular intranuclear inclusions surrounded by a clear halo, i.e., cytomegalovirus (CMV)-like. Type 3 are multinucleated decoy cells with granular chromatin. Type 4 are decoy cells
85 containing vesicular nuclei with clumped chromatin and nucleoli [10,22]. We excluded SV40-negative cells exhibiting a decoy-cell appearance and SV40-positive cells without a decoy-cell appearance. To confirm the specificity of S100P for urothelial epithelium, we examined autopsy specimens of normal tissue sections from the bladder and kidney cases with no kidney and urinary tract disease. We examined the tissue sections of BKV-infected kidney
90 to confirm that the renal tubular epithelia were positive for SV40 and negative for S100P.

Confirmation of S100P immunostaining

Non-fixed autopsy specimens of bladder mucous membrane and renal parenchyma were swabbed with cotton. Each sample was suspended in physiological saline, and renal tubular
95 epithelial cell and urothelial cell suspensions were established. The samples were fixed with CytoRich Red (Becton Dickinson, Franklin Lakes, NJ) and liquid-based cytology slides were

prepared according to SurePath™ liquid-based cytology (SP-LBC) protocols (Becton Dickinson, Franklin Lakes, NJ) [23]. The characteristics of the anti-S100P antibody are shown in Table 1. With the exception of the primary antibody, immunostaining was performed under the same conditions as those used for simultaneous immunostaining (Table 2). The Vulcan Fast Red Chromogen Kit 2 (Biocare Medical, Concord, CA) was used for counterstaining. Formalin-fixed paraffin-embedded sections of urinary bladders and kidneys sampled from autopsy cases were immunostained with anti-S100P antibody and compared to the cytology samples.

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Simultaneous immunostaining with the primary antibody cocktail

Urine samples were obtained by SP-LBC. Simultaneous immunostaining was conducted in each of the 66 cases as described in Table 2. Heat-induced antigen retrieval using a pressure cooker was performed with Target Retrieval Solution (pH 9.0, Dako, Carpinteria, CA) for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The anti-S100P (Atlas antibodies, Sweden) and anti-SV 40 (Calbiochem, CA) primary antibodies were mixed prior to the reaction (Table 1), which was completed in one step for 40 min at room temperature. Secondary antibodies were reacted with MACH2 double stain 2 (Biocare Medical, Concord, CA) for 30 min. Enzyme reactions were visualized by incubation with 3-3'-diaminobezidine-4HCl and the Vulcan Fast Red Chromogen Kit 2 [24].

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Results

Confirmation of S100P immunostaining

Positive S100P immunostaining was detected in the cytoplasm of bladder urothelial cells (Fig. 1-a), but not in the renal tubule epithelial cells (Fig. 1-b). Similarly, suspended urothelial cells were S100P-positive and suspended renal tubular epithelial cells were S100P-negative

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(Fig. 1-c, d). Almost all suspended urothelial cells (96%) were positive for S100P, whereas suspended renal tubular epithelial cells were completely negative. Small numbers of S100P-positive cells in the suspended renal tubular epithelial cells were also positive for LCA (leukocyte common antigen), indicating that they were leukocytes.

Simultaneous immunostaining with the primary antibody cocktail

The cytoplasm of the renal pelvic urothelial cells was positive for S100P (red) in BKV-infected kidney samples (Fig. 2-a, b). The nuclei of BKV-infected renal tubular epithelial cells were positive for SV40 (brown), but the cytoplasm was negative for S100P (Fig. 2-a, c). The BKV-infected (SV40-positive) cells in urine cytology samples were categorized as SV40(+)/S100P(+) (Fig. 2e) and SV40(+)/S100p(-) (Fig. 2f). SV40(+)/S100p(-) cells were found in 55/66 cases (83.4%), and 9/66 (13.6%) cases had both SV40(+)/S100P(+) and SV40(+)/S100p(-) cells. The remaining two cases (3.0%) had only SV40(+)/S100P(+) cells.

Discussion

In this study, urothelial cells and renal tubular epithelial cells in clinical urine samples were differentiated by S100P-immunostaining. S100P is a member of the S100 protein family, and plays important roles in cell cycle control and differentiation [16-18]. S100P was initially identified in the placenta, and subsequently identified in the mucosal epithelia of the gastrointestinal tract, epithelial cells of the prostate gland, white blood cells, and urothelial cells of the bladder and renal pelvis [16-18]. In addition, S100P has recently been recognized as a urothelial marker for the renal pelvic urothelium and bladder urothelium [16-18]. In this study, 96% of suspended urothelial cells were positive for S100P, and the suspended renal tubular epithelial cells were completely negative. In the urinary tract, squamous cells and renal tubular epithelial cells are negative for S100P, but can be distinguished from each other by

their morphology. We conclude that S100P is a useful marker for differentiation of urothelial cells from renal tubular epithelial cells in clinical urine samples.

In this study, BKV-infected cells in clinical urine samples were classified as SV40(+)/S100P(+) or SV40(+)/S100P(-). In BKV-infected kidney tissue, the infected renal tubular epithelial cells were SV40(+)/S100P(-) (Fig. 2c). Therefore, we conclude that SV40(+)/S100P(+) cells originate from the urothelia and SV40(+)/S100P(-) cells originate from the renal tubular epithelia. In this study, BKV-infected cells of renal tubular origin [SV40(+)/S100P(-) cells] were detected in 97% of cases, 83.4% of which contained only BKV-infected cells of renal tubular origin, and the remaining 13.6% of cases contained BKV-infected cells of both renal tubular and urothelial origin. Thus, the presence of decoy cells in clinical urine samples indicates BKV infection of renal tubules and our results confirm that BKV-infected cells in clinical urine samples correspond to renal tubule epithelial cells. Ruomei et al. used BKV-infected urothelial cells in vitro to demonstrate that urothelial cells are vulnerable to BKV-infection, but the virus replication rate is lower than in renal tubular epithelial cells [12]. In addition, urothelial cells have one of the lowest turnover rates in the body [25]. Thus, the appearance of BKV-infected urothelial cells in the urine is by far more infrequent than that of renal tubular epithelial cells. This explains the large number of renal tubule-type BKV-infected cells found in this study. We also identified BKV-infected urothelial cells in some of the clinical urine samples. It seems likely that decoy cells in hemorrhagic cystitis correspond to BKV-infected urothelial cells. We examined these cases and found many BKV-infected urothelial cells (unpublished data).

This study shows that simultaneous immunostaining for S100P and SV40 is a quick, useful method for determining the origin of BKV-infected cells in clinical urine samples. As a screening tool for urinary tract BKV infections, we believe this method can add value to cytological examinations and may be useful for analyzing renal biopsy specimens in patients

with suspected BKV nephropathy, in whom BKV is detected by PCR. BKV-infected bone marrow transplant patients tend to suffer from hemorrhagic cystitis, while kidney-transplant tends to be associated with BKV nephropathy [11,12]. The treatments for these conditions differ, so it is important to identify the infected site before choosing a therapeutic strategy. A few cases of the BKV-related urothelial carcinoma have been reported in recent years [26]; we believe our method may provide useful clues to the origin of BKV-infected cells in these cases.

The merits of this method include low cost and convenience; the method requires only a few staining steps and can be completed in as little as 4 h from sample preparation to staining and identification. In addition, the method requires no special equipment or techniques, and is practical for use in facilities with limited experience.

Conclusion

Simultaneous immunostaining with anti-S100P and anti-SV40 antibodies can be used to determine the origin of BKV-infected cells in urine specimens. In this study, BKV-infected cells of renal tubule origin were detected in almost all cases of BKV infection (97%), and the presence of decoy cells in clinical urine samples indicate renal tubular infection. This method can be combined with PCR for better efficacy in comparison to current methods of serial observations of transplant patients.

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Figure Legends

Figure 1. S100P immunostaining

Note the intensely positive cytoplasmic immunostaining for S100P of the bladder epithelium.

265 (a). Renal tubular cells were negative (b). Almost all cells in the suspended urinary bladder mucosa cells were positive for S100P (c). Suspended renal medullary cells were negative (d). [a, b: DAB staining, 20×; c, d: Fast Red staining, 40×]

Figure 2. Simultaneous immunostaining for S100P and SV40

270 Upper row: BKV-infected kidney samples. The cytoplasm of the renal pelvic urothelial cells is positive for S100P (a, b). The nuclei of BKV-infected renal tubule epithelial cells are positive for SV40 and the cytoplasm is negative for S100P (a, c). [a: 2×, b, c: 20×]

Lower row: Decoy cells in urine cytology samples. The BKV-infected cells, i.e.,

SV40-positive cells were classified as S100P-positive (+) (e) and S100P-negative (-) (f) [d:

275 Papanicolaou stain 100×, e, f: 100×]; S100P(red)/SV40(brown)

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Tables

Table1. Monoclonal antibodies

Antibody	Manufacturer	Clone	Subtype	Dilution
SV40 LT-ag	Calbiochem	Ab-2	Mouse	1:200
S100P	Atlas antibodies	polyclonal	Rabbit	1:3000

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Table2. Summary of Simultaneous immunostaining Protocol

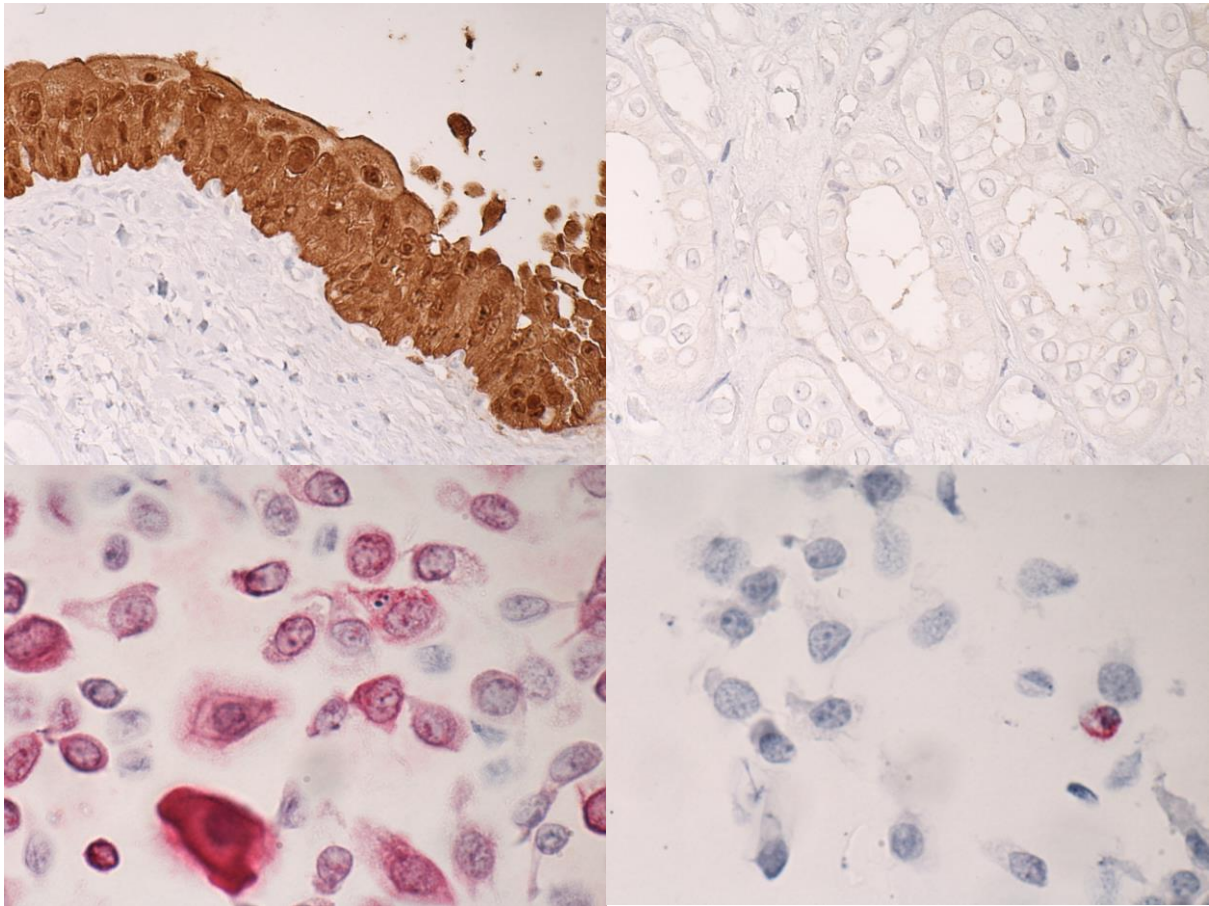
- 310 1. Heat-induced antigen retrieval with pressure cooker for 10 min
(Target Retrieval Solution , pH 9.0, Dako)
2. Blocking with 3% hydrogen peroxide for 10 min
3. Primary antibody reaction for 40 min
4. Washing with TBS
- 315 5. Secondary antibody reaction for 30 min (MACH 2 detection, Biocare Medical)
6. Washing with TBS
7. DAB
8. Washing
9. Vulcan Fast Red Chromogen Kit 2 (Biocare Medical)
- 320 10. Washing
11. Counterstain in hematoxylin
12. Washing
13. Dehydrate rapidly in 3 changes of 100% alcohol and clear in 2 changes of xylene
14. Mounting with Malinol (Muto Purechemicals, Tokyo, Japan)

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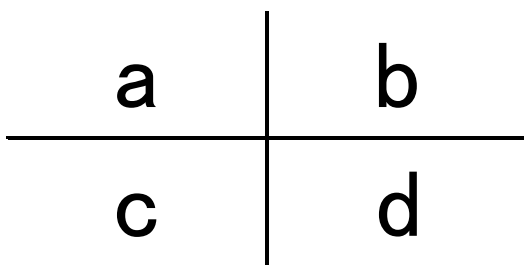
TBS; tris-buffered saline, DAB; indicates 3,3-diaminobenzidine

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Figure1. S100P single immunostaining

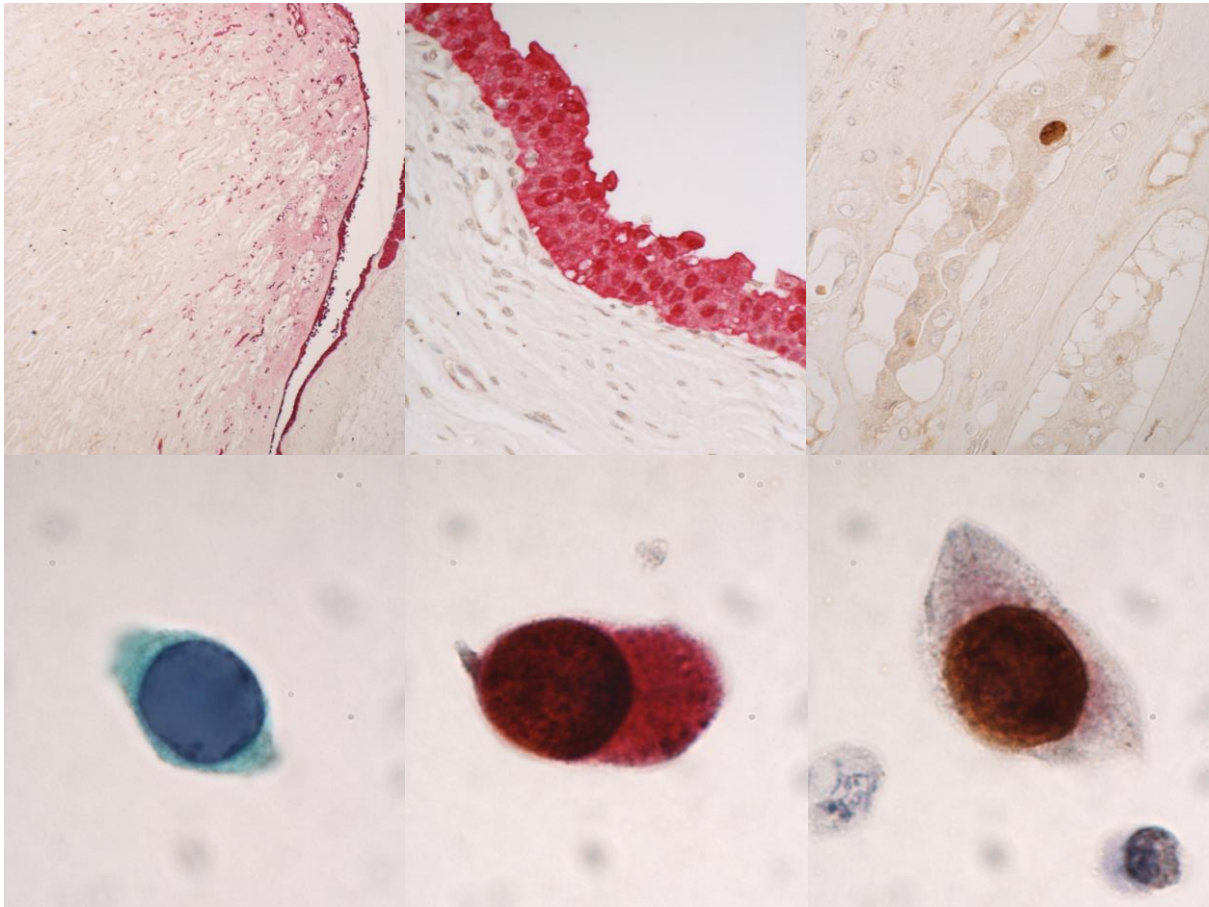


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Figure2. Simultaneous immunostaining for S100P and SV40.



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a	b	c
d	e	f