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Original Article

The impact of everolimus on the BKV-associated oncogenesis

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Purpose: Human polyomaviruse BK (BKV) has been considered as an oncogenic virus that may cause various human tumors. Considering that BKV is known to persist in urothelial cells and can reactivate in immunocompromised hosts, urothelial cell carcinoma is likely to be associated with BKV infection, especially in renal transplant patients (RTRs). However, the strategies regarding how to adjust immunosuppressant in RTRs and the role of everolimus (Evr) in BKV-associated oncogenesis are uncertain.
Method: Using BKV-infected renal urothelial SV-HUC-1 cell models, we investigated the effect of Evr (a mammalian target of rapamycin [mTOR] inhibitor) on BKV inducing the normal urinary urothelial cell migration in vitro. After the infection of the SV-HUC-1 cells with BKV, the cells were then exposed to different concentrations of Evr.
Results: We found that the infected SV-HUC-1 cells had transformed cell characteristics, such as increased colony formation, wound healing ability, migratory and invasion capacity, and MMP9 activity. Evr improved the transformation of the infected-SV HUC-1 cells by inhibiting the mTOR related pathway but not that of Ras signaling.
Conclusion: In routine practice, mTOR inhibitors may be an ideal immunosuppressant for RTRs with BKV infection or urothelial cell carcinoma.

Keywords: BK virus, everolimus, migration, invasion, urothelial cell carcinoma

1. Introduction:

Human polyomaviruses BK (BKV) has been demonstrated to be an oncogenic virus in many cell and animal studies (1-3). The early region of BKV encodes two known oncoproteins: large tumor antigen (TAg) and small tumor antigen (tAg), which may exert their transformation activity by interacting with cellular tumor suppression proteins, such as p53, and inhibiting protein phosphatase 2A, respectively (4-6). People with compromised immune system, such as organ transplant patients, are prone to suffer from a reactivation of BKV. This leads to an increase in urine viral loads and some known diseases, such as hemorrhagic cystitis and BK nephropathy(7.8). Furthermore, there have been a few studies that have discussed an the association of BKV with the development of human bladder urothelial cell carcinoma (UCC); however, the evidence until now has been limited (9).

The mammalian target of rapamycin (mTOR) is frequently activated in human cancers (10.11). In cell and animal studies, mTOR inhibitors, such as sirolimus or everolimus (Evr), have been proved to inhibit cell proliferation, migration, and invasion, attenuating tumorigenesis and the speed of tumor growth (12). Furthermore, they have

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been widely used as immunosuppressants in organ transplantation and have been reported to reduce the incidence of post-transplantation malignancies, including urothelial carcinoma, in kidney transplant recipients (13). In addition, mTOR inhibitors have been proved to lead to less replication of BKV in renal transplant recipients as compared to other immunosuppressants (14). Therefore, whether using an mTOR inhibitor as the main immunosuppressant in renal transplant patients decreases BKV-associated oncogenesis is of great interest for the transplant immunology. Our study is tested the possible biologic effect of an mTOR inhibitor on BKV induced cell transformation and its potential mechanism.

2. Materials and methods:

2.1. Cell line

SV-HUC-1 cells derived from transformed normal human urinary tract epithelial cells were grown in vitro in Ham's F12 medium supplemented with 7% v/v fetal bovine serum (FBS). The cell lines were supplemented with 100 U/ml penicillin G and 100 mg/ml streptomycin and kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed twice a week, and the cells were subcultured when confluence was achieved.

2.2. BKV infection

The BKV was a gift from Dr. Meilin Wang, of the School of Medicine, CSMU. For the infection, $5x10^5$/mL SV-HUC-1 cells were seeded on a 60 mm culture dish and co-cultured with 5 μL BKV for 3 days. Then, the medium was discarded and the samples were washed three times with REBM containing 0.5 % FBS. Fresh medium was added and the samples were incubated for another 5 days.

2.3. Cell viability

An MTT colorimetric assay was performed to determine cell viability. The cells were then seeded in 24-well plates at a density of 5×10^4 cells/well and treated with various concentrations of Evr from 0 -100 μM at 37 °C for 1 to 5 days. After the exposure period, the media were removed, followed by the washing of the cells with PBS. Thereafter, the medium was changed and the samples incubated with 20 μL MTT (5 mg/mL) for 4 h. The viable cell number/dish was directly proportional to the production of formazan, which following solubilization with isopropanol, can be measured spectrophotometrically at 563 nm.

2.4. Colony formation assay

Colony formation test was performed to evaluate the long-term proliferative potential of 2 BKV-infected cell lines following Evr treatment. Cells were seeded in 6-well plates at a density of 1000 cells/well and cultured for 7–10 days at 37°C with 5% CO_2. The medium was changed every 3 days until visible colonies were formed. Then the colonies were fixed with 4% paraformaldehyde at 4°C for 15 min and stained using Giemsa for 30 min. The samples were washed with PBS and dried out at room temperature. The number of focus was counted.

2.5. Wound-healing assay

Wound-healing assay was performed as described previously (15). The cells were plated in six-well plates at a density of 5×10^5 cells/well and serum was starved for 12 hours. The wounds were scratched with pipette tips and washed with phosphate-buffered saline to remove the suspended cells. The media containing 1% serum and different concentrations of Evr were used for cell culture. The assay was performed for 48 hours at 37°C and 5% CO_2. Wounds were photographed with an Axioskop microscope (Zeiss, Olympus CK-2, Hamburg, Germany) at 48 hours.

2.6. Invasion assay

The polycarbonate membrane filters (8 mm pores) were coated with 25 mg/50 mL Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Medium containing 10% fetal bovine serum was applied to the lower chamber as a chemoattractant, and then Evr-treated cells were seeded on the upper chamber with 8-μm pore polycarbonate filters at a density of 1×10^5 cells/well in 50 μL of serum-free medium. After incubation for 12 hours, the cells that had migrated to the lower

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surface of the membrane were fixed with methanol and stained with 5% Giemsa solution, and the cells on the upper surface of the membrane were removed with a cotton swab carefully. The migrated cells were counted in five high-power fields (200×) by using a light microscope. All samples were tested in triplicate, and the data are expressed as the mean ± SD.

2.7. Zymography assay

The levels of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) released in the cultured medium were detected by gelatin zymography assays as described previously (16). First, serum-free conditioned medium was prepared with a 5x loading buffer containing 0.01% SDS without 2-mercaptoethanol. The prepared samples were subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 140 V for 3 h in an ATTO apparatus. Gels were washed twice with 50 mL distilled water containing 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove the SDS after electrophoresis. The gel was then incubated in 50 mL reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM CaCl2, 0.02% NaN3) overnight at 37°C, stained with Coomassie brilliant blue R-250 and destained with methanol-acetic acid-water (5, 7.5 and 87.5%, vol/vol/vol).

2.8. RT PCR

Total RNA was isolated from cells by using a guanidinium chloride procedure and complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification were performed as previously described (17). For reverse transcription, 4 μg of total cellular RNA was used as templates in the reaction buffer containing 4 μL deoxynucleoside triphosphate (2.5 mM), 5 μL oligo dT20 (10 pmol/mL) and 1 μL RTase (200U/mL), and the reaction was performed at 42°C for 1 h. Then, the cDNA was stored at 4°C after reaction for 5 min at 990C. Afterward, 10 μL cDNA product was amplified by PCR with the following primers:

BKV LTag, 5'-CTGTCCCTAAAACCCTGCAA-3# (sense) and 5'-GCCTTTCCTTCCATTCAACA-3# (antisense), urokinase-type plasminogen activator (uPA), 5'-CACGCAAGGGGAGATGAA-3# (sense) and 5'-ACAGCATTTTGGTGGTGACTT-3# (antisense); tissue inhibitor of metalloproteinase-1 (TIMP-1), 5'-CTGTTGTTGCTGTGGCTGATA-3# (sense) and 5'-CCGTCCACAAGCAATGAG T-3# (antisense) and glyceraldehyde 3- phosphate dehydrogenase (GAPDH), 5'-GAAGGTGAAGGTCGGAGTC- 3# (sense) and 5'-GAAGATGGTGATGGGATTTC-3# (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromid.

2.9. Western blotting assay

Western blotting was performed as described previously (18). Proteins (60 μg) were electrophoresed on 8 -12.5% polyacrylamide gels, and the gels were transferred onto nitrocellulose membranes (Millipore, MA, USA) that were incubated with the relevant antibodies. The following antibodies were used: phospho-mTOR, mTOR, phospho-S6K, S6K, Ras, phospho-ERK, ERK, and β-actin (Cell Signaling Technology, Danvers, MA, USA). β-actin expression was used as reference band.

2.10. Statistical Analysis

All the data are presented as means ± SD. Statistical analysis was performed using an unpaired Student's *t* test. A P value of less than 0.05 was considered to represent a significant difference.

3. Results:

3.1. BKV replication in uuman urinary tract epithelial cells

To determine the replication effect of BKV in human urinary tract epithelial cells, SV-HUC-1 cells were infected with BKV at a concentration of 1.0×10^6 copy/mL and intracellular or extracellular BKV DNA was measured by a real-time PCR. After the BKV infection, the intracellular BKV load had rapidly increased by day 4 post-infection (Fig. 1). Then, the BKV rapidly replicated after day 4 post-infection and was released into the extracellular culture medium a day later, suggesting that the BKV had infected the SV-HUC-1 cells successfully.

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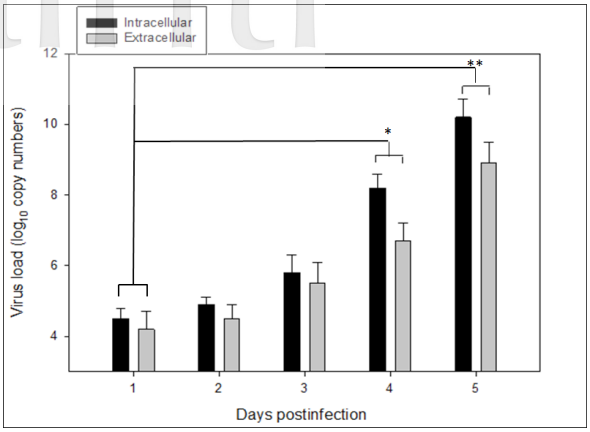


Fig 1. Expression of extracellular and intracellular BKV load from SV-HUC-1 cells. SV-HUC-1 cells were seeded for 1 day and infected with the BKV. Cell and cell free supernatants harvested at 1, 2, 3, 4, and 5 days were measured for intracellular and extracellular BKV DNA respectively. *P < 0.01 compared with the extracellular virus load by the t-test. A representative result of three independent experiments is shown.

3.2. Growth inhibitory effects of Evr on SV-HUC-1 cells infected with BKV

The time and dose dependence of the cytotoxic effects of Evr in the SV-HUC-1 cells were determined using the MTT assay. As seen in Fig. 2, Evr showed a significant dose- and time-dependent inhibition of the growth of the SV-HUC-1 cells. The concentration of Evr inhibiting 50% of SV-HUC-1 cell viability (IC_{50}) after Evr exposure for 4 days was over 100μM, indicating the low cytotoxicity of Evr on the SV-HUC-1 cells. A colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. It can also be used to measure the sensitivity of human

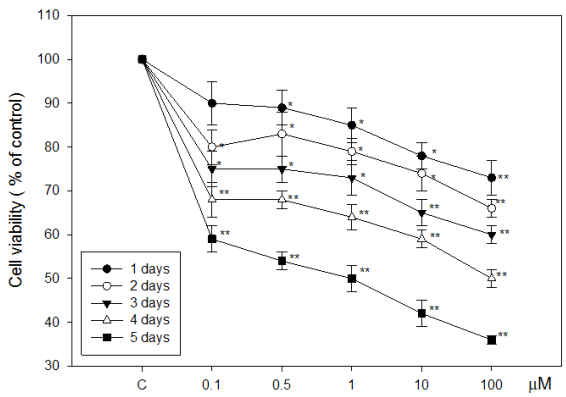


Fig 2. Effects of different durations and concentrations of Evr exposure on the viability of SV-HUC-1 cells. The cells (1×10^5 cells/mL) were treated with various concentrations of Evr for 1–5 days. Cell viability was determined by MTT assay, which was measured spectrophotometrically at 563 nm. Values were expressed as the mean ± SD of three independent experiments. *P < 0.01 compared with the untreated controls, **P < 0.001 compared with the untreated controls.

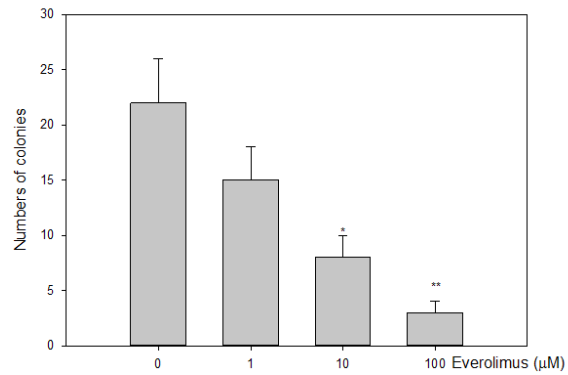


Fig 3. Evr decreased the colony numbers of BKV-infected SV-HUC-1 cells. Colony forming assay was performed on BKV-infected SV-HUC-1 cells exposed for 10 days to increasing concentrations (0-100 μM) of Evr. Three independent experiments were representative. *P<0.01, **P<0.001, compared with the untreated group.

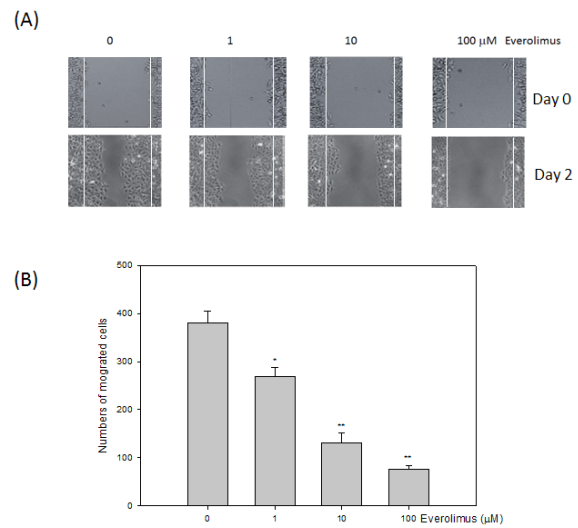


Fig 4. Evr inhibited the wound healing ability of BKV-infected SV-HUC-1 cells. After treatment BKV-infected SV-HUC-1 cells with different concentrations of Evr for 1 day, cells were cultured in a six-well plate after monolayer wounding with a yellow P200 pipette tip. The healing ability was observed using phase contrast microscopy on an inverted microscope after 2 days.

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tumors to anticancer drugs. Here, the role of Evr on regulating the colony formation ability of SV-HUC-1 cells infected by BKV was analyzed. The colony formation ability of enhanced SV-HUC-1 infected by BKV was dose dependently inhibited by Evr (Fig. 3).

3.3. Evr suppressed the migration and invasion of BKV-infected SV-HUC-1 cells

A wound-healing assay was performed to investigate the effects of Evr on BKV-infected SV-HUC-1 cell migration. The cells were treated with Evr (0, 1, 10, or 100 μM) for 48 h. As shown in Fig. 4, the wound-healing ability in BKV-infected SV-HUC-1 cells decreased gradually with increasing concentrations of Evr. Meanwhile, the effects of Evr on the invasion of BKV-infected SV-HUC-1 cells were investigated using transwell assays. After treatment with Evr at 10 μM and 100 μM for 12 h, the invasion capacity of the cells was 33.3% ($P < 0.01$) and 20.1% ($P < 0.001$) of the cell invasion at 0 μM, respectively (Fig. 5). These results demonstrate the inhibitory effect of Evr on the migration and invasion of BKV-infected SV-HUC-1 cells.

3.4. Evr inhibited the activity of MMP-2 and MMP-9 in BKV-infected SV-HUC-1 cells

To explore whether Evr-regulated BKV-infected SV-HUC-1 cell migration and invasion were associated with the activity of MMP2 and MMP9, gelatin zymographic analysis was performed. BKV-infected SV-HUC-1 cell treated with Evr (0, 1, 10, or 100 μM) for 24 h and the medium was collected to elvaluate the MMP2 and MMP9 activities. The level of MMP9 activity decreased in the presence of 1 μM Evr (Fig. 6). With the concentration of Evr increasing to 10 μM and 100 μM, the inhibitory effects on the activity of MMP9 appeared to be more obvious. In the presence of Evr, the level of MMP9 activity significantly decreased whereas the MMP2 expression slightly reduced in high dose of Evr treatment. Besides, MMPs are activated by proteolysis of pro-MMPs by uPA and inhibited by TIMP1, the uPA and TIMP1 mRNA level were also assayed. As expected, the reduced uPA and increased TIMP-1 were observed in a concentration-dependent manner (Fig. 7).

3.5. Evr regulated the expression of migration-related proteins of BKV-infected SV-HUC-1 cells

Ras and mTOR are well-established pathways that modulate MMPs expression to induce bladder-related cancer. Thus, the effects of Evr on Ras

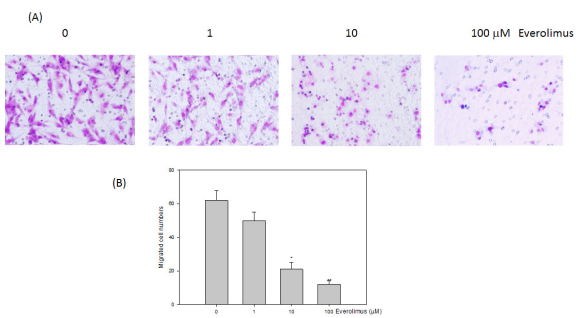


Fig 5. Evr suppressed the invasion of BKV-infected SV-HUC-1 cells. Invasion potential was assayed by transwell assay after cells were exposed to various concentrations of Evr. Cells were seeded in transwell insert matrigel-coated upper part and incubated for 12 h. Data represent the results of three independent experiments (mean ± SD). Th statistical difference was compared with the untreated group. (* $p < 0.05$; ** $p < 0.01$).

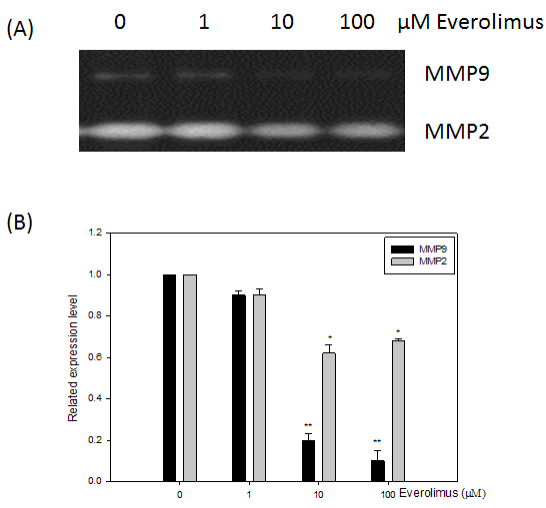


Fig 6. Evr inhibited the activity of MMP2 and MMP9 in BKV-infected SV-HUC-1 cells. Cells were treated with the indicated concentrations of Evr. The conditioned medium was prepared and collected. The activity ofMMP2 and MMP9 was measured using gelatin zymography. Each value expressed as the mean ± SD of three independent experiments and is expressed relative to the control. *$P < 0.01$ and **$P < 0.001$ compared with the untreated controls.

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and mTOR signaling pathways were examined using western blot analysis. Fig. 8 showed that Evr inhibited phosphorylated mTOR in low dose and significantly reduced the expression of total mTOR and phosphorylated S6K, mTOR downstream protein. However, Ras and phosphorylated ERK were trifling reduced, indicating that Evr suppressed migration of BKV-infected SV-HUC-1 cells via mTOR but not Ras pathway.

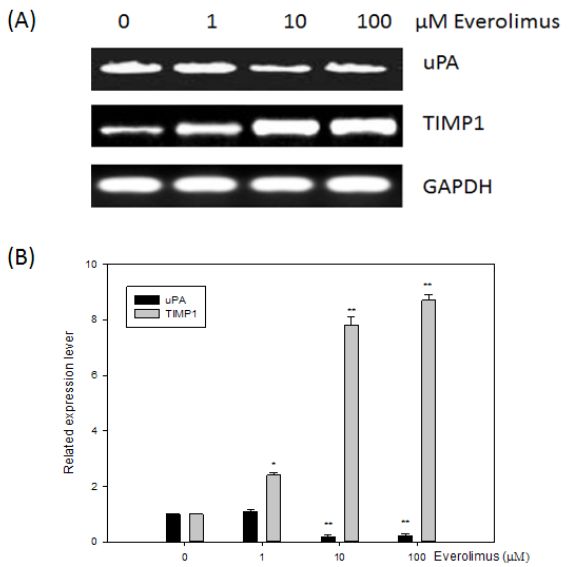


Fig 7. Effects of Evr on the mRNA levels of protease and tissue inhibitor using RT–PCR. Cells were treated with the indicated concentrations of Evr. The uPA, and TIMP1 mRNA levels were measured by RT–PCR and GAPDH was used as an internal control. *P < 0.01 and **P < 0.001 compared with the untreated controls.

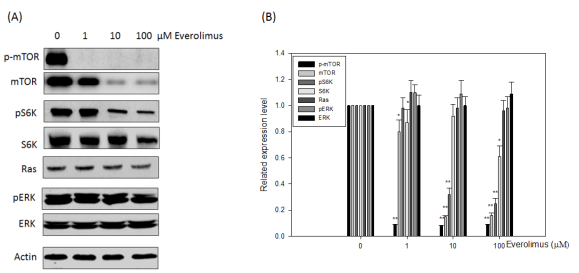


Fig 8. Evr regulated the expression of migration-related proteins of BKV-infected SV-HUC-1 cells. Total cell lysate was extracted and the expression of p-mTOR, mTOR, pS6K, S6K, Ras, pERK and ERK were assayed by western blotting. Actin was used as an internal control. *P < 0.01 and **P < 0.001 compared with the untreated controls.

4. Discussion:

The reactivation of latent BKV is an important complication of immunosuppression seen in renal transplant recipients. All viruses are extremely dependent on the cell protein synthesis of their host for constituent proteins and genomic replication. Some viruses kill the cell and then go on to infect other cells, but BKV does not, and rather it persists within cells in one form or another. Furthermore, nephropathy from BKV infection (BK nephropathy) has been increasingly recognized as a cause of kidney allograft loss, and this is thought to be related to the newer, more potent immunosuppressive agents. The antiviral effect and ability to preserve protective immunity of mTOR inhibitors have been demonstrated in several studies (19.20). Evr, one mTOR inhibitors, regulates cell protein synthesis, inhibits neointimal and smooth muscle cell proliferation, and reduces fibrogenesis after the onset of graft damage (21) Interestingly, Evr is relatively devoid of nephrotoxicity (22). Liancini et al (23) found that sirolimus, also known as rapamycin, can reduce BKV large T-antigen expression and its genome replication (dose dependently) within a few days. In this study, we used the anti-migratory effect of an mTOR inhibitor on BKV-associated oncogenesis via the inhibition of MMP9 secretion regulated by the mTOR related pathway. This will help clinicians to choose the immunosuppressant for patients with BKV infection or UCC.

BK nephropathy has been diagnosed in renal transplant patients receiving maintenance immunosuppressants, including calcineurin inhibitors, antimetabolites, mTOR inhibitors, and corticosteroids (24-27). Before 1995, BK nephropathy was rarely identified as a clinical problem in renal transplantation. New immunosuppressive agents have thus been regarded as a causative factor for the occurrence of BKV reactivation. Brennan et al. prospectively evaluated the differences between viremia, viruria, and BK nephropathy with different immunosuppressive therapies.[28] Patients were randomly assigned to receive tacrolimus, cyclosporine, azathioprine, or mycophenolate mofetil (MMF). By year 1, 35% of patients developed BK viruria and 11.5% BK viremia. Neither the development of BK viruria nor viremia

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was affected by the choice of immunosuppressants. In summary, these results demonstrated that noy immunosuppressant has been proven to be efficacious for BK virus reactivation. In addition, studying the genetic background of urothelial carcinoma of the bladder has revealed that chromosomal alterations are not seen at the same level in other types of cancer. This is especially the case for mutations of the genes involved in the PI3K/AKT/mTOR signaling pathway, which occupies a major place in the etiology of these tumors.[29-32] Therefore, the inhibition of mTOR could be a solution to BK nephropathy and provide a superior strategy for overcoming the development of the bladder UCC induced by BKV infection.

The early diagnosis of BKV infection with monitoring of viremia and viruria to guide the cautious adjustment of immunosuppressants continues to be the most efficacious tool to control BK nephropathy. Furthermore, considering that BKV may be associated with the development of urothelial carcinoma of the bladder, it is reasonable to extrapolate that Evr could decrease the developed of urothelial carcinoma. Here, we have revealed the possibility that Evr may be an ideal immunosuppressant for renal transplant patients with ongoing BKV infection or UCC.

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