

Surveillance Renal Allograft Biopsy on Diagnosis of BK Virus Nephropathy in Chinese Renal Transplant Recipients

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ABSTRACT

Objective: We prospectively investigated 121 renal allograft biopsy specimens performed in our center. **Methods:** BKVAN was diagnosed by light microscopic examination and a positive immunohistochemistry staining of anti-SV40 large T antibody in a biopsy specimen. **Results:** Of the 121 patients, nine were diagnosed with BKVAN (7.4%). Nine patients with BKVN classically presents as allograft dysfunction with an asymptomatic rise in serum creatinine, about 3 to 39 months posttransplant. Urinary decoy cells are positive in two patients (22.2%). The histologic changes of BKVN are not pathognomonic, and can be mistaken for allograft rejection, *i.e.*, tubulointerstitial nephritis with varying degrees of inflammatory infiltrates, tubulitis and tubular atrophy, and fibrosis. Typical findings on histology are focal interstitial mononuclear inflammatory cell infiltrates, presence of plasma cells, necrotic tubular epithelium, and presence of homogenous intranuclear inclusion bodies. Immunohistochemistry with SV40 staining were positive in allograft. Graft loss occurred in one patient and the other 8 showed progressive allograft dysfunction. **Conclusion:** The definitive diagnosis of BKV disease requires renal biopsy. Immunohistochemistry with SV40 staining has been used as an indirect method to document the presence of BKVAN.

Keywords: Renal Transplantation; Biopsy; BK Virus; BK Virus Associated Nephropathy; Diagnosis

1. Introduction

BK virus associated nephropathy (BKVAN) is one of the most serious complications after renal transplantation [1,2]. Most renal transplant recipients with BKVN manifest with renal dysfunction [3,4]. In recent years, routine post-transplant protocol biopsy has also detected BKVN in the absence of serum creatinine elevation. Progressive renal failure has been reported in approximately 30% - 60% of cases. BKVAN has not attracted the attention of clinical transplant physician over the years. With the extensive use of new immunosuppressive agents, the infection ratio of BK virus after renal transplantation continues to rise, currently there are few effective therapeutic drugs and the rates of failure and loss caused by BKVAN are high, and the leading risk factor causing BKVAN and renal allograft loss is not clear, which is a difficult problem in dire need of resolve [5,6]. The BKVAN diagnosis mainly depends on the transplant renal biopsy. BK virus can cause acute necrosis of renal tubular epithelia, and early diagnosis of BKVAN and use of effective treatment can prevent irreversible kidney damage caused by virus and improve prognosis of BKVAN, therefore early diagnosis of BKVAN has become current research focus

[7,8]. Celik *et al.* took the initiative in regular biopsy after renal transplantation, which contributed to timely diagnosis of BKVAN [9]. Currently, there are transplant centers which have begun to carry out some research on BK virus infection after renal transplantation, but clinical and pathological characteristics studies on BKVAN in China have rarely reported and the renal transplantation insufficiency and even renal transplantation loss caused by BKVAN are lack of enough attention. In this paper, 9 cases of patients diagnosed as BKVAN through pathology of transplanted kidney by biopsy in our hospital were studied because of chronic renal allograft dysfunction so as to provide a reference for clinical, pathological and treatment outcomes of patients with BKVAN.

2. Methods

2.1. Objects

Among 121 cases of subjects confirmed with histopathologic biopsies of kidney transplants with decreased graft functions in our unit, from August 2005 to April 2011 period, 9 patients accorded with the inclusion criteria. All subjects have signed informed consent.

2.2. Inclusion Criteria

1) More than 3 months after renal transplantation, increased serum creatinine ($SCr \geq 1.5$ mg/dl or greater than 30% in increase of serum creatinine values above the baseline), and slow clinical progression (increase in serum creatinine < 1 mg/week), accompanied with or without hematuria or proteinuria; 2) Vascular and post-renal factors were excluded by color doppler ultrasound of patients with transplanted kidney. All patients were carried out with blood routine, urine routine, liver and kidney function, blood coagulation function and concentration measurements of cyclosporine A (CsA) or FK506 (tacrolimus, Tac) and mycophenolate mofetil (MMF) in blood before biopsy. Decoy cells were detected in urine. All patients gave their written informed consent.

2.3. Pathological Diagnosis of Renal Graft Biopsy

Renal graft biopsy was carried out under the guidance of B ultrasound. The specimens were qualified if the renal tissues contained 2 vessels and more than 10 renal glomerules, and were confirmed with conventional light microscopy and immunofluorescence examination. Light-microscopic staining included HE, PAS, PASM and Masson trichrome staining. The pathological diagnosis of transplanted kidney can accord with Banff 97 criteria [10].

2.4. Main Outcome Measueres

Immunofluorescence. Direct fluorescent assay was used to observe the deposition intensities and sites of IgG, IgA, IgM, C3, C4, C1q, and C4d. All frozen tissue sections were confirmed with C4d immunofluorescence staining, and the staining intensities were in accordance with Banff 2001 criteria, in which C4d negative was defined as $< 25\%$ of PTC C4d deposition, reversible focal positive was defined as 25% to 50% of PTC C4d deposition and C4d asystematic positive was defined as $> 50\%$ of PTC C4d deposition [11].

Immunohistochemistry. Enrolled patients were carried out with CD4, CD8, CD68, HLA-DR and BK virus immunohistochemical staining. Paraffin immunohistochemistry and Envision two-step method were applied: the paraffin sections were deparaffinized by normal method, treated by 3% hydrogen peroxide to inhibit the endogenous peroxidase, placed into a pressure cooker with 1ml EDTA solution for antigen retrieval, blocked in normal 10% bull serum solution, washed and added with monoclonal mouse anti-human CD3 (1:100), CD68 (1:100), CD20 antibody (1:100) and HLA-DR antibody (1:50), respectively, incubated at room temperature for 2 h, added with Envision and incubated at room temperature for 40 min, colored with DAB for 5 min, counter-

stained with hematoxylin for 2 min, dried and sealed with neutral gum. Read under a Nikon 8100 microscope. SV240 large T antigen was used for positioning (anti-polyomavirus antibody staining).

The infiltration degrees of CD4, CD8, and CD68-positive cells were classified according to the following criteria: 16 high power fields with a total area of 1 mm^2 were randomly selected for counting, the sum of counting > 300 was defined as 3 points, 200 - 300 was defined as 2 points, 100 - 200 was defined as 1 point, and < 100 was defined as 0 point.

HLA-DR expression was judged on the basis of 50 tubular cross-sections, HLA-DR positive tubular accounted for a percentage of the whole tubular. $> 50\%$ was defined as 3 points, 25% - 50% was defined as 2 points, 10% - 25% was defined as 1 point, and $< 10\%$ was defined as 0 point.

2.5. Criteria of BK Virus-Associated Nephropathy [12,13]

SV240 large T antigen was located in the nuclear of renal tubular epithelial cell. Positive expression was brown and negative cells were not detected. BKVAN was diagnosed through their positive expression in combination with HE staining.

2.6. Statistical Analysis

The measurement data is represented by mean \pm standard deviation ($\bar{x} \pm s$), $P < 0.05$ has significant difference, $P < 0.01$ has obviously significant difference.

3. Results

3.1. Clinical Data

Renal allograft biopsy was implemented in 121 patients followed by BKV large T antigen staining, 9 positive cases were found and diagnosed as BKVAN with incidence rate of 7.4%. According to staining results of BKV large T antigen staining of renal allograft biopsy, the patients were divided into two groups: BKVAN group ($n = 9$) and non-BKVAN group ($n = 112$). There was no significant difference in gender, age, tissue matching, postoperative acute rejection, and serum creatinine levels when renal biopsy (**Table 1**). But the incidences of pulmonary infection, FK506 renal toxicity, and chronic renal allograft insufficiency before biopsy in BKVAN group were obviously higher than that in non-BKVAN group. 9 patients all had slowly progressive increase in serum creatinine at the onset of disease, without hematuria and proteinuria, and other specific clinical symptoms.

Decoy cells were found in urine of 2 patients (positive rate of 22.2%), and Decoy cells in urine were detected for 3 times, and the results showed that there were a large

Table 1. Comparison between BKVAN and non-BKVAN groups.

	BKVAN group	Non-BKVAN group	P value
Gender (F/M)	6/3	75/37	NS
Age (years)	36.8 ± 10.1	37.4 ± 11.2	NS
Postoperative onset time (months)	9.25 ± 3.66	11.4 ± 5.63	NS
Historical events			
Acute rejection	1 (12.5%)	14 (12.5%)	NS
Pneumonia	3 (37.5%)	13 (11.6%)	<0.05
FK506 nephrotoxicity	2 (22.2%)	10 (8.9%)	<0.05
Immunosuppressive schema			
CsA + MMF + Pred (CMP)	1 (11.1%)	13 (11.6%)	NS
Tac + MMF + Pred (TMP)	7 (77.8%)	99 (88.4%)	NS
Tac + MMF + Pred + TII (TMPT)	1 (11.1%)	0	
Serum creatinine level			
atrenal biopsy (mg/dl)	2.7 ± 1.5	2.5 ± 1.2	NS

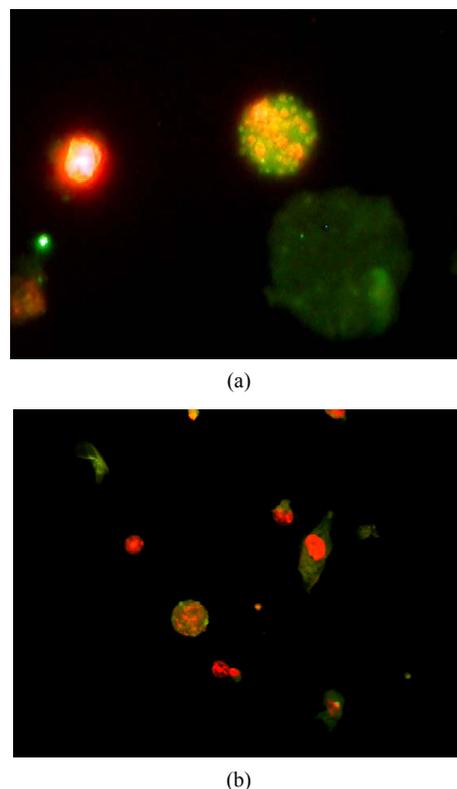
number of visible exfoliated cells and scattered BKV-positive cells. Green fluorescence can be visible in the cell nuclear (**Figures 1(a)** and **(b)**). The results are as follows: urinary protein < 0.10 g/24 hour, urinary sediment RBC < 10000.0/ml, white blood cells < 0 - 1/HP; NAG enzyme > 42.1 U/g-Cr (<17.0 U/g-Cr), RBP > 11.94 mg/L, KIM-1 > 11.46 µg/L (<0.4 µg/L), IL-18 22.93 ng/L (<20 ng/L), and NGAL > 25.18 µg/L (<20 µg/L).

3.2. Pathological Features of BKVAN

The typical pathological features of BKVAN are as follows: different phenotypes of basophilic BK virus inclusion bodies appeared renal tubular epithelial cells, infected renal tubular epithelial cells exfoliated towards renal tubular lumen, resulting in bare spot on the base membrane (**Figures 2(a)** and **(b)**). Interstitial prominent presented as multifocal gathered infiltrating cells, with mononuclear cells and plasma cells as main, and their surrounding tubulars often had tubulitis, multifocal thickening and atrophy of tubular basement membrane (**Figures 2(c)** and **(d)**). Later pathological changes mainly include fibrosis, with a few of BKV-infected cells (**Figure 2(d)**).

Immunofluorescence: Immunofluorescence of IgG, IgM, IgA, C3, C4, C1q, and C4d were negative.

Immunohistochemistry: A large aggregation of CD3, CD4, CD8, CD68 positive cells, CD3 809 ± 201/mm², CD4 348 ± 110/mm², CD8 295 ± 89/mm², and CD68 679.5 ± 209/mm², can be seen in renal interstitial from all

**Figure 1. Decoy cells in urine (indirect IF, ×400).**

renal pathology. In one patient combined with rejection, HLA-DR and high IL-2R expression were 19.6% and 148/mm², respectively; in patients combined without rejection, HLA-DR and IL-2R expression were mostly less than 5%. 9 patients were implemented with SV-40 large T antigen staining of renal tissues, positive scattered BKV tubules can be seen in renal cortex and medulla, that is the nuclear of tubular epithelial cells was brown, the middle was transparent, and the peripheral was burr-like, and exfoliated cell BKV expression can be seen in lumen of renal tubules (**Figures 3(a)** and **(b)**).

Ultrastructural characteristics of polyoma virus inclusion body: Under electron microscopy, virus-like particles arranged in dense crystalline with a diameter of 40 - 45 nm can be seen in renal tubular epithelial cells or endothelial cells (**Figures 4(a)** and **(b)**).

3.3. Concentration of Immunosuppressant and BKVAN

Nine patients were carried out with three-point MPA AUC 0 - 12 examination when biopsy, and the results showed significantly higher than patients who were not infected with BKV, 75.85 ± 20.49 (51.04 - 96.33) mg·h/L vs. 36.3 ± 11.9 (34.5 - 55.1) mg·h/L, respectively. FK506 trough concentrations were also significantly higher than that in control group, 9.28 ± 2.38 (9.8 - 11.05) ug/L vs. 5.95 ± 1.14 (5.6 - 7.2) ug/L.

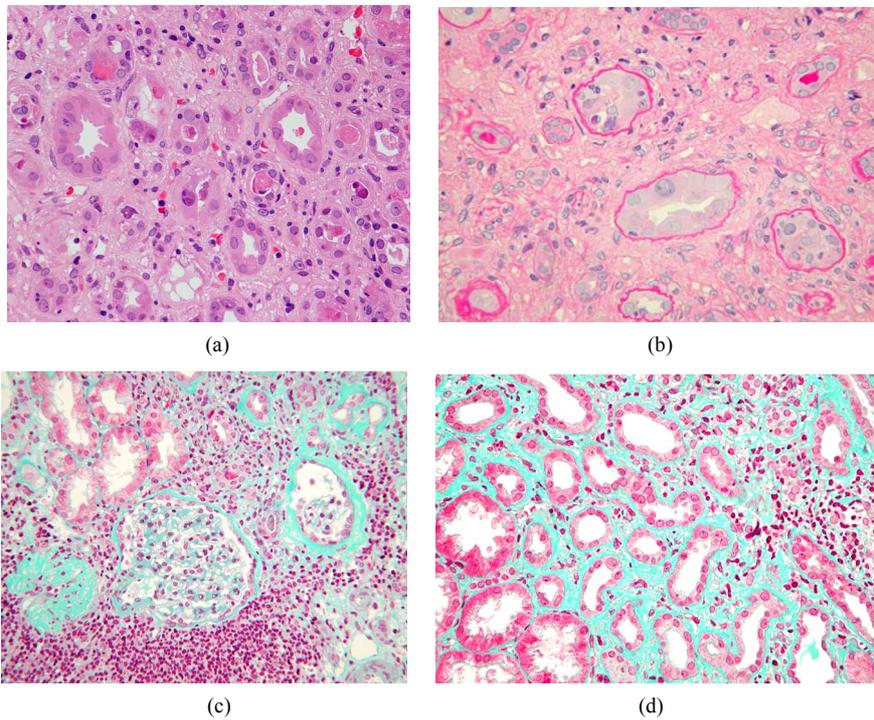


Figure 2. Pathological features of BKVAN.

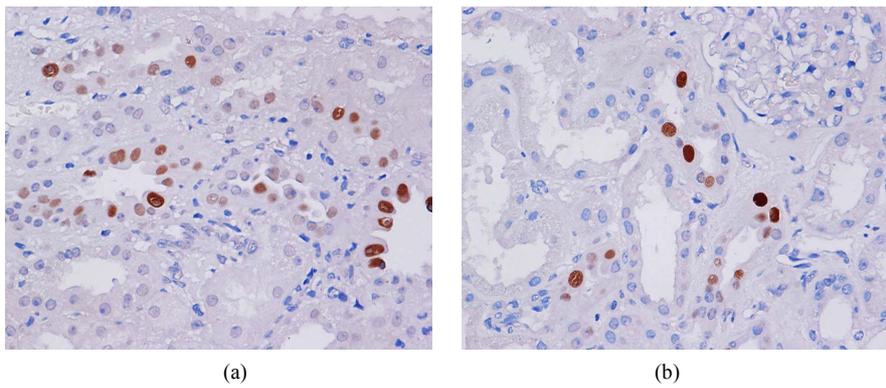


Figure 3. BKV test in frozen section of renal tissues (IH, $\times 400$).

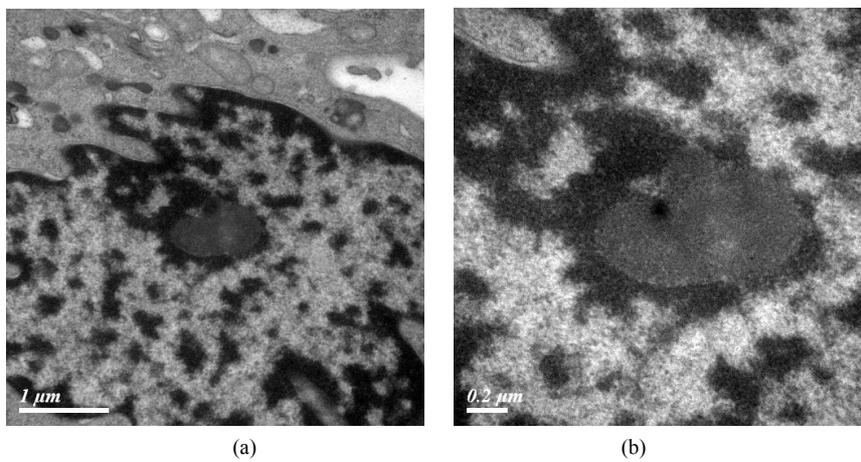


Figure 4. BKVAN ultrastructural changes.

3.4. BKVAN Follow-Up Treatment

After 9 patients were diagnosed as BKVAN, FK506 was reduced, triptolide and MMF were stopped and converted to leflunomide (10 - 40 mg/d) to maintain the therapy. The longest follow-up was 5 years and the shortest was 6 months, average follow-up of 26.60 ± 13.76 months. The function of transplanted kidney was stable and serum creatinine was remained at 1.46 - 4.57 mg/dl, but the serum creatinine levels in most patients can not be reduced to the level before onset. There were 1 case of renal transplantation loss, 3 cases of improved, 2 cases of stable, and 3 cases of deteriorated, and the average serum creatinine doubling time was longer than 22.4 ± 15.60 months. There was no death during the whole follow-up.

4. Discussion

BKVAN is a group of diseases paid attention gradually and is continuously reported on a global scale. The incidences of PVAN reported by various transplant centers were 2% to 9.3% [14]. It was reported that progressive renal failure occurred in 30% to 60% of patients, of which 45% of the patients developed to irreversible renal failure. In the data of this group, 121 patients underwent BKV large T antigen staining and it was found that 9 patients were positively diagnosed as BKVAN, with positive rate of 7.4%, which was similar with the related reported international incidence.

The study found that BKVAN mostly occurred in the period of 3 to 13 months after renal transplantation, average of 10 months. There was no characteristic symptoms in clinical, and asymptomatic elevated serum creatinine were often seen as the main features, accompanied by urine detection indicators caused by renal tubular damage, such as increase in urinary NAG enzyme and urine RPB protein levels. Decoy cells can be found in urine in this group, with positive rate of 22.2%, and we found that other reasons, such as adenovirus infection, also can cause positive decoy cell in urine, because the urinary exfoliated cells had variations in the number and morphology. Therefore, the specificity of this test was lower and can be used as screening index for BKVAN patients [15-17]. Why BKVAN patients mainly acted as renal tubular injury without proteinuria and erythrocyturia still needs further study. The above situations were mainly caused because BK virus can lie in the renal tubular epithelial cells and urinary transitional epithelial cells after BK virus latent infection and reproduce in a large number and damage renal tubular when the host immunity were declined [12,18,19].

The diagnosis of BKVAN needed to be established on the basis of renal allograft biopsy [20,21], and the typical pathology mainly presented as different phenotypes of basophilic BK virus inclusion bodies occurred in the nu-

clear of renal tubular epithelial cells, infected renal tubular epithelial cells exfoliated towards renal tubular lumen, resulting in bare spot on the base membrane (**Figures 2(a)** and **(b)**). Interstitial prominent presented as multifocal gathered infiltrating cells, with mononuclear cells and plasma cells as main, and their surrounding tubulars often had tubulitis, multifocal thickening and atrophy of tubular basement membrane (**Figures 2(c)** and **(d)**). Parts of pathological changes took fibrosis as main, only a small number of BKV-infected cells. 9 cases of patients with BKVAN were confirmed by electron microscopy, and the electron microscopy showed that virus-like particles arranged in dense crystalline with a diameter of 40 - 45 nm can be seen in renal tubular epithelial cells or endothelial cells. According to morphological changes, inflammatory cell infiltration, tubular atrophy and interstitial fibrosis, Hirsch *et al.* [9] divided the pathological process of BKVAN into three phases through semi-quantitative evaluation: Phase I: mild pathological changes, such as positive stained large T antigen of focal tubular epithelial cells and virus inclusion bodies, no extensive necrosis and inflammatory infiltration can be seen. Phase II: extensive multifocal diffuse pathological changes of cells, necrosis associated with inflammatory response and initial signs of interstitial fibrosis can be seen. Infiltrated inflammatory cells included polymer-phonuclear cells, monocytes and plasma cells. Phase III: renal interstitial fibrosis, scarring and even calcification can be seen. BKVAN observed in this study was mainly Phase II. Therefore, it has extremely important clinical significance for patients with renal allograft dysfunction to carry out renal allograft biopsy for detecting BKVAN in early phase and staging.

Nine patients had obviously abnormal renal function in case of biopsy. One patient was initially diagnosed as acute rejection. As the early pathological features of BKVAN often similar to the performance of interstitial nephritis, it was difficult to distinguish from acute rejection only through routine staining and light microscopy, especially for mild rejection. Therefore, it becomes necessary for using immune staining on the basis of light microscopy. In this study, kidney tissue SV-40 large T antigen staining was used, which has advantage of specificity. Immunohistochemical staining of transplanted kidney SV-40 large T antigen was widely recognized "gold standard" for the diagnosis of BKVAN [22]. 9 patients were implemented with SV-40 large T antigen staining of renal tissues, positive scattered BKV tubules can be seen in renal cortex and medulla, that is the nuclear of tubular epithelial cells was brown, the middle was transparent, and the peripheral was burr-like, and exfoliated cell BKV expression can be seen in lumen of renal tubules (**Figures 3(a)** and **(b)**). In addition, the acute rejection combined with BKVAN also should be discrimi-

nated from the secondary interstitial non-specific changes by virus, because under the high load conditions, serious viral infection often can lead to renal tubular epithelial cell necrosis, polymorphonuclear leukocyte infiltration and plasma cells, which could also lead to higher rate of misdiagnosis of this disease. It is interesting to find that all kidney immunofluorescence IgG, IgM, IgA, C3, C4, C1q, and C4d staining were negative, a large aggregation of CD3, CD4, CD8, CD68 positive cells, CD3 $809 \pm 201/\text{mm}^2$, CD4 $348 \pm 110/\text{mm}^2$, CD8 $295 \pm 89/\text{mm}^2$, and CD68 $679.5 \pm 209/\text{mm}^2$, can be seen in renal interstitial from all renal pathology. In one patient combined with rejection, HLA-DR and high IL-2R expression were 19.6% and $148/\text{mm}^2$, respectively; in patients combined without rejection, HLA-DR and IL-2R expression were mostly less than 5%. Therefore, the transplanted kidney tissue HLA-DR, IL-2R expression and C4d staining to identify BKVAN and is an important indicator of acute rejection [23]. Therefore, HLA-DR, IL-2R expression in the transplanted kidney tissues and C4d staining were important indicators for identify BKVAN and acute rejection [23].

It is reported that long-term high-dose application of FK506, MMF and other immunosuppressive agents can promote BK viral replication [24,25]. Multiple transplant centers revealed that after previous CsA + azathioprine (Aza) immunosuppressive regimen was transferred to FK506 and MMF, the BKN incidence increased significantly. Large doses of FK506 (whole blood concentration of 8 $\mu\text{g}/\text{L}$) or MMF can increase the incidence of BKVAN by 13 times. The results of a prospective study showed that the incidence rate of BKVAN in patients with transplanted kidney who accepted FK506 or MMF-based immunosuppressive therapy was about 5%. Therefore, FK506 or MMF-based treatment programs have the risk of increasing BKN. In this study, except for 1 patient with CsA + MMF + Pred, the remaining patients were administrated with immunosuppressive regimen of FK506 combined with MMF, and the blood concentration of FK506 and MPA were significantly higher than that in the control group. Whether FK506 and MPA concentrations are risk factors still needs further large sample of clinical observation. However, BK virus infection was not associated with the application of particular immunosuppressant, more likely it was related to the overall state of over-immunosuppression.

Although for the treatment of BKVAN, it is reported [26-28] that cidofovir and laifumite can be effectively used for antiviral treatment, it is still a lack of accepted, standard treatment. Reduction of immunosuppressive agents is the core principle of treatment [9]: for all patients diagnosed as BKVAN, the dosage of FK506 was lowered, mycophenolate mofetil was stopped and converted to low dose of laifumite. The longest follow-up

was 5 years and the shortest was 6 months, average follow-up of 26.60 ± 13.76 months. The function of transplanted kidney was stable and serum creatinine was remained at 1.46 - 4.57 mg/dl, but the serum creatinine levels in most patients can not be reduced to the level before onset. There were 1 case of renal transplantation loss, 3 cases of improved, 2 cases of stable, and 3 cases of deteriorated, and the average serum creatinine doubling time was longer than 22.4 ± 15.60 months. There was no death during the whole follow-up. The long-term effect of laifumite in the treatment of patients with BKVAN still needs further observation.

5. Conclusion

High-dose immunosuppression or immune dysfunction is the main reason for the occurrence BKVAN. BKVAN patients are characterized by slowly progressive increase in serum creatinine accompanied by increased tubular injury index in clinical. BKVAN diagnosis mainly depends on the transplant renal biopsy pathology, and using SV-40 large T antigen staining and electron microscopy can improve the diagnostic rate of BKVAN and the detections of C4d, IL-2R and HLA-DR antigen of transplanted kidney tissues are extremely important for identification and diagnosis of BKVAN. Laifumite can effectively control most of the lesions progress, but its long-term effect needs further observation.

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