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Original Article

DNA binding activity of nuclear factor of activated T cells in mononuclear cells from renal transplant patients with and without BK virus viruria

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ABSTRACT

Objectives: Renal transplant patients receive calcineurin inhibitors to suppress the calcineurin-nuclear factor of activated T cells (NFAT) pathway. The DNA binding activity of NFAT and its relationship to the reactivation of BK virus (BKV) has not been evaluated in renal transplant patients.
Patients and Methods: The DNA binding activity of NFAT cytoplasmic 1 (NFATc1) was measured by enzyme-linked immunosorbent assay in peripheral blood mononuclear cells from 26 renal transplant patients and 26 healthy controls. At the same time, their urinary BKV viral load was measured by real-time polymerase chain reaction.
Results: The activity of NFATc1 was lower in renal transplant patients without BKV viruria [BKV (−)] than in healthy controls, while it trended to be higher in renal transplant patients with BKV viruria [BKV (+)] than in BKV (−) patients. The tacrolimus blood levels did not differ between BKV (+) and BKV (−) renal transplant patients or correlate with NFATc1 activity.
Conclusion: NFATc1 DNA binding activity was lower in renal transplant patients without BKV viruria than in those who were BKV (+). However, there was no relationship between tacrolimus blood levels and NFATc1 activity in renal transplant patients.
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1. Introduction

Calcineurin inhibitors, such as cyclosporine and tacrolimus, are important immunosuppressive drugs in the prevention of renal allograft rejection. Tacrolimus binds to the intracellular immunophilin FK506 binding protein (FKBP12), and this FKBP12-tacrolimus complex inhibits the phosphatase activity of calcineurin [1]. In immune cells, the major target molecule of calcineurin is the transcription factor nuclear factor of activated T cells (NFAT). After dephosphorylation by calcineurin, activated NFAT induces the transcriptional activation of many important genes involved in the immune response, including interleukin-2, interferon-γ, and granulocyte macrophage colony-stimulating factor. Tacrolimus inhibits calcineurin activity, thus suppressing the production of these cytokines [2]. Although tacrolimus is highly effective in preventing allograft rejection, it has some common adverse effects, including nephrotoxicity, neurotoxicity, and diabetogenicity [3]. In addition, the therapeutic range of tacrolimus is very narrow, and the drug is known to have a large degree of inter- and intra-individual pharmacokinetic variability [4]. Therapeutic drug monitoring of trough blood concentrations is used clinically to guide the adjustment of tacrolimus. However, allograft rejection and tacrolimus-related adverse effects have occasionally developed in patients whose blood levels were within the therapeutic range [5]. This may be because the blood levels of tacrolimus do not always accurately reflect the effective level of tacrolimus-induced immunosuppression. Recent studies have attempted to evaluate the effective level of tacrolimus-induced immunosuppression by measuring calcineurin phosphatase activity and monitoring NFAT-regulated gene expression [6–8]. However, we could find no study that investigated the DNA binding activity of NFAT in renal transplant patients.

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Human polyomavirus BK virus (BKV) infects up to 90% of the general population, but significant clinical manifestations are rare except in patients with impaired immunity [9]. BKV primary infection usually occurs in childhood without specific symptoms, and the initial infection is followed by a nonreplicative state, primarily in the urogenital tract. Asymptomatic reactivation and low-level replication with viruria are observed in 5% of healthy individuals. BKV reactivation in renal transplant patients may be associated with nephropathy causing renal allograft failure [10,11]. The reactivation of BKV is related to the use of immunosuppressants in renal transplant patients [12]. Therefore, in clinical practice, it is crucial to adjust the dose of an immunosuppressant to a level that prevents acute rejection without leaving the patient susceptible to new infections and reactivation of BKV in the urinary tract.

In the present study, we first compared the DNA binding activity of NFAT cytoplasmic 1 (NFATc1) in peripheral blood mononuclear cells (PBMCs) of renal transplant patients with and without BKV viruria [BKV (+) and BKV (−), respectively] and healthy controls to determine the level of tacrolimus-induced immunosuppression. We then correlated BKV reactivation in renal transplant patients and NFAT activity with the blood levels of tacrolimus.

2. Patients and methods

2.1. Patients

Twenty-six renal allograft recipients and 26 sex- and age-matched healthy volunteers were recruited for the present study. All participants gave informed consent and the study was approved by the local Internal Review Board and Ethics Committee of Buddhist Dalin Tzu Chi General Hospital, Chiayi, Taiwan (No. B09602035). The personal and clinical data of the renal transplant patients were recorded. Most of the transplant patients were prescribed a protocol of a tacrolimus-based triple regimen that combined tacrolimus with mycophenolic acid/mycophenolate mofetil and prednisolone and did not include induction therapy. The blood level of tacrolimus was adjusted to 10–15 μg/L during the first month post-transplantation, 8–12 μg/L the second month, and 6–8 μg/L thereafter. Acute rejection was noted in five (19.2%) of the patients, and most of these cases were controlled by pulse therapy. Tacrolimus was given every 12 hours and blood samples were collected in the morning (6:00–7:00 AM) just prior to the morning dose. Midstream urine specimens were collected in sterile containers without transport medium, immediately frozen, and stored at −20°C until analyzed. Tacrolimus blood levels were routinely measured by microparticle enhancement immunoassay (Abbott Diagnostics, North Chicago, IL, USA; detection limit <1.5 μg/L).

2.2. Measurement of the nuclear NFATc1 DNA binding activity of PBMCs

Heparinized venous blood obtained from patients and healthy volunteers was mixed with a 2% dextran solution (molecular weight 464 kDa; Sigma-Aldrich, St. Louis, MO, USA) at a ratio of four parts blood to one part dextran, and the resulting solution was incubated at room temperature for 30 minutes. A leukocyte-enriched supernatant was collected and layered over a Ficoll-Hypaque density gradient solution (specific gravity 1.077; Pharmacia Biotech, Uppsala, Sweden). After centrifugation of the leukocyte-enriched supernatant at 250× *g* for 25 minutes, PBMCs were aspirated from the interface. The PBMC suspension should have contained 90% lymphocytes, 5–8% monocytes and 2–5% other cells, which we confirmed this with Giemsa staining. The PBMC cell concentration was adjusted to 2×10^9/L in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL; 10% fetal bovine serum-RPMI). Trypan blue dye exclusion was performed to demonstrate that the viability of the PBMCs was >95%. NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) were used to prepare nuclear extracts from PBMCs according to the manufacturer's protocol. The DNA binding activity of NFATc1 in the nuclear extract was detected with a sensitive multi-well colorimetric assay kit (Active Motif, Carlsbad, CA, USA), and all samples were measured in the same plate at the same time.

2.3. Quantification of BKV viral load in urine by real-time polymerase chain reaction

The tacrolimus blood levels in BKV (+) and BKV (−) renal transplant patients were compared. First, seven patients were excluded who were taking cyclosporine ($n = 2$) or rapamycin ($n = 5$) to prevent a potential confounding effect of these immunosuppressants. Therefore tacrolimus blood levels were correlated with BKV viruria or NFATc1 DNA binding activity in the PBMCs of 19 renal transplant patients in the following analysis.

DNA was extracted from midstream urine with a QIAamp DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Then, BKV viral copy numbers were quantified by real-time polymerase chain reaction (PCR) using an ABI 5700 sequence detection (Applied Biosystems, Foster City, California, USA) system as previously described [13]. Primers for the BKV capsid protein-1 gene were based on those used in a study by Randhawa et al [14] and were: forward primer, 5′-GCA GCT CCC AAA AAG CCA AA-3′; reverse primer, 5′-CTG GGT TTA GGA AGC ATT CTA-3′. All patient samples were tested in duplicate, and the number of BKV copies was calculated from a standard curve. In all real-time PCR assays, the correlation coefficient of the standard curve was >0.980. Standard precautions were used to prevent contamination during PCR amplification. Both template-free control lanes and negative control samples (DNA extracted from healthy human mononuclear cells) were included in each run.

2.4. Statistical analysis

All data are represented as mean ± standard deviation. Statistical significance was assessed by the unpaired Mann–Whitney *U* test and Fisher's exact test. Multiple linear or logistic regression was applied to calculate the correlation coefficient and significance between different parameters. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. BKV reactivation rate and viral load in urine from renal transplant patients and healthy controls

Data from the renal transplant patients and healthy volunteers are shown in Table 1. The prevalence of BKV viruria tended to be higher in renal transplant patients (42.3%, 11/26) than in healthy controls (15.4%, 4/26; $p = 0.064$). The mean urine BKV viral load [log(urine BKV DNA copy number/mL)] was significantly higher in transplant patients than controls (2.80 ± 3.54 vs. 0.84 ± 2.05, $p = 0.0264$). Besides the current use of immunosuppressants, all other parameters measured were similar between the two groups. The patients were then further divided into subgroups based on the presence or absence of BKV viruria [BKV (+) or BKV (−), respectively] to make comparisons between these subgroups that related to tacrolimus.

Table 1
Personal and clinical data of renal transplant patients and healthy volunteers.

	Transplant patients ($n = 26$)	Healthy controls ($n = 26$)
Age (y, mean ± SD)	43.6 ± 11.6	35.9 ± 7.5
Sex (F:M)	12:14	16:10
Prevalence of BK viruria (%)	42.3% (11/26) *	15.4% (4/26)
Urine BKV viral load [log(urine DNA copy numbers)]	2.80 ± 3.54 **	0.84 ± 2.05
Acute rejection	19.2% (5/26)	—
Immunosuppressants		
Prednisolone	92.3% (24/26)	—
Tacrolimus (FK-506)	73.1% (19/26)	—
Cyclosporine	7.7% (2/26)	—
Mycophenolate mofetil/ Mycophenolic acid	46.2% (12/26)	—
Rapamycin	19.2% (5/26)	—
Duration of treatment (months, mean ± SD)	45.0 ± 22.5	—

*$p = 0.064$ compared with controls; **$p = 0.0264$ compared with controls.
SD = standard deviation.

3.2. NFATc1 DNA binding activity in PBMCs from BKV (+) and BKV (−) renal transplant patients and controls

Calcineurin inhibitors such as tacrolimus and cyclosporine are the most common immunosuppressive drugs used in renal transplant patients. NFATc1 is a transcription factor downstream of calcineurin that is the key target of these immunosuppressive drugs. The nuclear NFATc1 DNA binding activity was lower in PBMCs from the BKV (−) group than PBMCs from healthy controls (0.20 ± 0.03 vs. 0.23 ± 0.03, $p = 0.0292$; Fig. 1). Conversely, a trend of increased NFATc1 activity was found in PBMCs from BKV (+) patients compared with those from BKV (−) patients (0.24 ± 0.08 vs. 0.20 ± 0.03, $p = 0.0775$).

3.3. Correlation between clinical parameters and NFATc1 DNA binding activity in PBMCs from renal transplant patients

Correlation of different clinical parameters with the NFATc1 DNA binding activity in PBMCs from renal transplant patients was attempted by univariate and multivariate linear regression analysis. The NFATc1 DNA binding activity tended to be positively associated with BKV viruria ($p = 0.078$) and female gender ($p = 0.097$). After adjusting for age, the NFATc1 DNA binding activity still tended to be positively associated with BKV viruria ($p = 0.086$) and female gender ($p = 0.086$; Table 2).

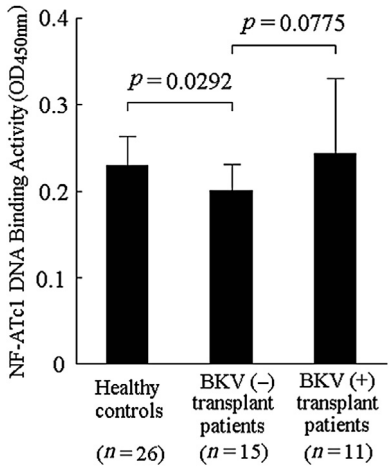

Fig. 1. Comparison of the nuclear DNA binding activity of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) in peripheral blood mononuclear cells of renal transplant patients with/without BK virus viruria [BKV (+) and BKV (−), respectively] and healthy controls.

3.4. Comparison of tacrolimus blood levels in BKV (+) and BKV (−) renal transplant patients

From the results above, it was tentatively concluded that the reactivation of BKV in the urinary tract was positively associated with NFATc1 DNA binding activity and that this activity was suppressed by calcineurin inhibitors. Thus, the next step was to measure tacrolimus blood levels in 19 renal transplant patients after excluding those who were taking rapamycin or cyclosporine. Surprisingly, tacrolimus blood levels did not differ between BKV (+) and BKV (−) renal transplant patients (Fig. 2A). Also, the percentage of renal transplant patients with BKV viruria was quite similar in those with lower (<5.5 μg/L) and higher tacrolimus drug levels [≥ 5.5 μg/L; 44% (4/9) vs. 50% (5/10); $p = 1.000$; Fig. 2B].

3.5. Correlation between tacrolimus blood levels and NFATc1 DNA binding activity in PBMCs from renal transplant patients

There was no correlation between the level of tacrolimus in the blood and NFATc1 DNA binding activity in PBMCs from19 renal transplant patients after excluding those taking rapamycin or cyclosporine ($R^2 = 0.155$, $p = 0.527$; Fig. 3).

4. Discussion

Calcineurin inhibitors are the primary immunosuppressants used as prophylaxis against allograft rejection in renal transplantation. We focused on the calcineurin inhibitor tacrolimus in the present study. Although monitoring tacrolimus blood levels may be useful in preventing allograft rejection and infections, some patients still experience acute rejection or become infected when tacrolimus levels are within the therapeutic range [5]. In fact, the blood concentration of tacrolimus does not accurately reflect the level of tacrolimus-induced immunosuppression in clinical practice. This discrepancy may be due to genetic polymorphism in the

Table 2
Univariate and multivariate liner regression models for assessing the correlations among different clinical parameters and the NFATc1 DNA binding activity in peripheral blood mononuclear cells from renal transplant patients.

	Univariate analysis Fold change (95% confidence interval)	Multivariate analysis Fold change (95% confidence interval)
Age (per 10 y)	0.008 (−0.014–0.031)	0.0004 (−0.017–0.025)
Sex (male/female)	−0.040[a] (−0.089–0.008)	−0.040[b] (−0.087–0.006)
Prevalence of BK viruria (yes/no)	0.043[c] (−0.005–0.091)	0.042[d] (−0.006–0.090)
Tacrolimus (yes/no)	0.016 (−0.041–0.073)	—
Cyclosporine (yes/no)	−0.004 (−0.100–0.091)	—
Mycophenolate mofetil/ mycophenolic acid (yes/no)	0.032 (−0.017–0.081)	—
Rapamycin (yes/no)	−0.016 (−0.080–0.048)	—

[a]$p = 0.097$; [b]$p = 0.086$; [c]$p = 0.078$; [d]$p = 0.086$.
After analysis with multivariate linear regression model adjusted for age, the presence of BK viruria ($p = 0.086$) and female patient ($p = 0.086$) were found tendency to correlate with NFATc1 DNA binding activity in renal transplant patients.

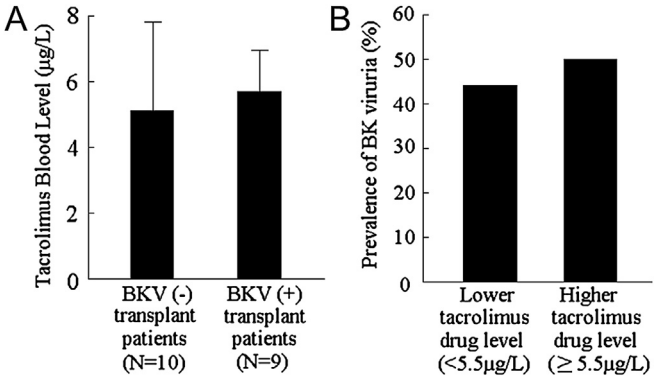

Fig. 2. Comparison of tacrolimus blood levels and BK virus (BKV) viruria, (A) Tacrolimus blood levels in BKV (+) and BKV (−) renal transplant patients. (B) The presence of BKV viruria in renal transplant patients with lower (<5.5 μg/L) and higher tacrolimus drug levels (≥ 5.5 μg/L). Renal transplant patients who were taking cyclosporine or rapamycin were excluded.

genes of the biotransformation enzyme (CYP3A5) and the transporter protein ABCB1 [15]. Therefore several authors have attempted to determine the level of tacrolimus-induced immunosuppression by measuring calcineurin phosphatase activity [16,17] or the inhibition of NFAT-regulated gene expression [7] in PBMCs from transplant patients. Although calcineurin activity in PBMCs from transplant patients has been associated with acute rejection [18,19], the measurement of calcineurin activity is complicated and not clinically practical because it uses ^{32}P [20,21]. The quantitative analysis of NFAT-dependent gene expression before and after treatment has been shown to be specific and is correlated with the incidence of infection and malignancy [8,22]. In this study, NFATc1 DNA binding activity was measured in PBMCs from renal transplant patients by an enzyme-linked immunosorbent assay-based method [23]. This assay was easy to use, nonradioactive, highly reproducible, specific and sensitive for NFATc1, and suitable for high-throughput screening, and is widely used in hematological and immunological studies [24,25].

We found that the NFAT DNA binding activity tended to be lower in PBMCs from renal transplant patients than those from healthy controls (0.22 ± 0.06 vs. 0.23 ± 0.03; $p = 0.133$). A possible reason for this not reaching statistical significance is that BKV reactivation in renal transplant patients may elicit inflammation and

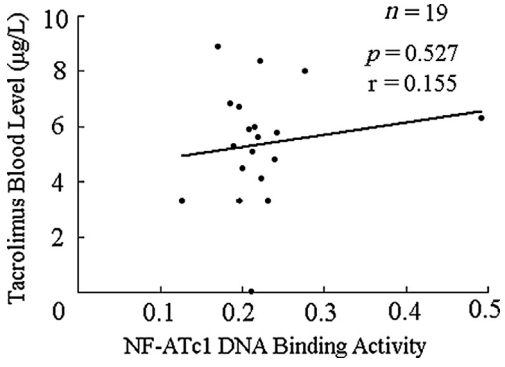

Fig. 3. Correlation of tacrolimus blood levels with nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) DNA binding activity in peripheral blood mononuclear cells from renal transplant patients receiving tacrolimus. Renal transplant patients who were taking cyclosporine or rapamycin were excluded.

nephropathy, which in turn increase the NFAT activity in PBMCs. This increased NFAT activity would counterbalance the tacrolimus-induced suppression of NFAT. BKV viruria was found in nearly half (42.3%) of the renal transplant patients in our study, which was attributed to the use of various immunosuppressive drugs [26]. Thus, we divided the renal transplant patients into BKV (+) and BKV (−) subgroups for further analysis. We found that NFAT DNA binding activity was higher in BKV (+) than BKV (−) renal transplant patients. We did not divide the healthy controls into subgroups according to their BKV reactivation status because BKV reactivation was relatively infrequent (15.4%) and the NFAT activity in healthy controls did not differ based on the BKV viruria status (0.21 ± 0.03 vs. 0.23 ± 0.03; $p = 0.20$). Furthermore, while BKV reactivation is usually asymptomatic and transient in healthy individuals, our data concerning viral loads appear to indicate that reactivation is more severe and persistent in renal transplant patients (Table 1).

We did not find that the use of immunosuppressants could lower the DNA binding activity of NFAT in our analysis (Table 2). This may show that the DNA binding activities of NFAT in renal transplant patients are affected by complex interactions between various factors including the host immunity, rejection reaction, different immunosuppressive drugs at different dosages, and infections such as from the BK virus. Because of the small case number, it was impossible to investigate these complex interactions in our study. However it is quite interesting that we found a statistical trend of elevated NFAT DNA binding activity correlated with BKV viruria. It is conceivable that BKV viral infection may trigger the expression of inflammatory genes. However, it has been shown that NFAT and the urine levels of cytokines downstream of it, such as interleukin-2, interferon-γ and granulocyte macrophage colony-stimulating factor, do not seem to be increased in BKV viruria [27,28]. In contrast, Jordan et al [29] reported that increased NFAT activity may promote BKV viral transcription through binding directly to the BKV promoter. Recently, Li et al [30] showed that overexpression of NFAT could promote BKV replication and cyclosporine could inhibit BKV replication. Clinically, our study showed that elevated NFAT activity also tended to associate with BKV viruria. It is quite possible that increased NFAT activity may be a potential risk factor for BKV reactivation in renal transplant patients.

We note at least three drawbacks in the present study. The first is that we monitored the BKV viral load using urine, but not sera samples. BKV viremia has a better predictive role than BKV viruria in the development of BK nephropathy. Second, because this study was a cross-sectional study and had a relatively small case population, we could not show any association between NFAT activity and the development of rejection or malignancy, and we only found a statistical trend between NFAT activity and the presence of BKV viruria. A longitudinal observational study enrolling more renal transplant patients should be conducted to clarify this issue in the future. The third drawback is that we did not elucidate the molecular basis showing how the elevated DNA binding activity of NFAT leads to BKV reactivation in renal transplant patients. We noted that one transplant patient had biopsy-proven BKV nephropathy and his NFAT activity was 0.21, but it is difficult to draw any conclusions from a single case.

Our studies revealed no association between NFATc1 DNA binding activity and the blood level of tacrolimus in transplant patients. This may be because most of the patients were receiving a low daily dosage of tacrolimus, which would correspond to the low tacrolimus blood levels (5.4 ± 2.1 μg/L) observed in the patients in the study. Several studies have also shown great variability in the expression of NFAT-regulated genes [31] and calcineurin phosphatase activity [32] in patients with low blood levels of tacrolimus

(<11.4 μg/L). In a clinical setting, it is important to evaluate the immunosuppressive status of transplant patients with low tacrolimus blood levels to avoid tacrolimus-related toxicity and allograft rejection.

In conclusion, NFATc1 DNA binding activity was lower in renal transplant patients without BKV viruria and did not correlate with tacrolimus blood levels.

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