Monitoring the progress of BK virus associated nephropathy in renal transplant recipients

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Abstract

Background. Nephropathy associated with BK virus (BKVAN) has recently emerged as an important cause of allograft failure following renal transplantation. The aim of this study was to evaluate the effectiveness of laboratory markers in the follow-up of patients with BKVAN.

Methods. Serial samples from seven renal transplant recipients with biopsy proven BKVAN were studied. The median follow-up time from diagnosis was 76 weeks. Intervention after the diagnosis of BKVAN included immunosuppression dose reduction, alternative immunosuppressive agents and/or antiviral therapy with cidofovir. Serial urine samples \( n=127 \) were collected for electron microscopy (EM), decoy cell detection and quantitative urine BK viral load using real-time polymerase chain reaction. Serum BK viral load was also measured serially \( n=72 \).

Results. All patients showed a reduction in serum and urine viral load during the period of follow-up co-incident with the loss of decoy cells and negative urine EM. Urine samples that were negative for decoy cells or polyomavirus by EM had a urine viral load <10^6 copies/ml and a corresponding serum viral load <10^3 copies/ml. In paired serum/urine samples, there was a proportional relationship between serum and urine viral load with each urine viral load \( \times 1000 \)-fold higher than the corresponding serum level. Serum and urine viral loads that decreased to <200 and <10^6 copies/ml, respectively, correlated with histological improvement.

Conclusion. Negative EM and absence of decoy cells could be used as broad indicators of a response to intervention. However, measurement of BK virus DNA level provided a wider dynamic range and could be a better choice for determining the extent of viral control.

Keywords: BK virus; BK virus associated nephropathy; decoy cells; electron microscopy; kidney; transplant; viral load

Introduction

BK virus is a ubiquitous human polyomavirus typically associated with subclinical primary infection in early childhood. The seroprevalence of BK virus infection in adults ranges from 60 to 100% worldwide [1]. In the United Kingdom, seroprevalence peaks at 91% at 5–9 years of age with an overall seropositivity within the population of 81% [2].

Nephropathy associated with BK virus (BKVAN) has recently emerged as an important cause of allograft failure following renal transplantation. The relatively recent recognition of this condition [3,4] probably reflects an increase in reactivation of latent BK virus infection as a consequence of the use of newer and more potent immunosuppressive agents such as tacrolimus or mycophenolate mofetil (MMF) [5,6]. Currently, the prevalence of BKVAN is estimated at between 1 and 5% of allograft biopsies [7,8]. A recent review of the current knowledge of BK virus indicated an incomplete understanding of BK virus disease and that further research is required to improve patient care [9].

Apart from histopathological examination of renal biopsy tissues, laboratory markers of BKVAN include detection of polyomavirus in urine by electron microscopy (EM), cytological examination of urine for decoy cells and the detection of BK virus DNA in blood or urine [7,10]. In a recent study, both urine decoy cell and plasma BK virus DNA detection were found to be 100% sensitive for the diagnosis of BKVAN,
with specificities of 71 and 88%, respectively. Mean viral load in plasma was found to be significantly higher in patients with biopsy-proven BKVAN [11]. As a result, it was suggested that quantitation of BK virus DNA should be evaluated as a marker for monitoring BKVAN [12,13]. In this present study, serial samples from seven patients with biopsy-proven BKVAN were monitored for the presence of BK virus using urine EM, urine decoy cell detection, urine and serum BK viral load.

Subjects and methods

Subjects

Serial samples from seven renal transplant patients (all male; median age 45, range 19–61 years; two cadaveric, three live related and two live unrelated donors) with biopsy-proven BKVAN were studied in a single centre between August 2001 and October 2003 (112 weeks). Baseline immunosuppressive therapy before the diagnosis of BKVAN included prednisolone (n = 7), tacrolimus (n = 6), MMF (n = 4), azathioprine (n = 3) and ciclosporin (n = 1). The median interval between transplantation and diagnosis of BKVAN was 53 weeks (range 11–141 weeks) and the median follow-up time from diagnosis was 76 weeks (mean 71 weeks, range 16–112). Interventions after the diagnosis of BKVAN included immunosuppression dose reduction, principally by cessation of tacrolimus and/or MMF and substitution with ciclosporin or azathioprine (Table 1). Five patients received intravenous low-dose cidofovir (0.25 mg/kg/dose) without probenecid fortnightly for between 6 and 30 weeks. Three patients had follow-up graft biopsies after intervention.

Serial urine samples (n = 127, range 7–31 per patient) were collected for EM and decoy cell detection at intervals between 2 and 4 weeks. During the first 6 months of the study, urine samples were often discarded after completion of microscopy and not available for further tests. Subsequently, most urine samples (n = 97, range 5–22 per patient) were saved and tested for urine BK viral load. Serum BK viral load was measured serially at intervals between 2 and 6 weeks (n = 72, range 8–20 per patient). Of the serum and urine samples, 46 sets were taken on the same day; and therefore could be analysed as paired serum/urine specimens.

Histology of renal biopsies

Renal biopsies were fixed in formal saline and routinely processed to paraffin wax. Antigen was retrieved by pressure cooking at 102 kPa for 2 min in 0.01 M citrate buffer. A standard streptavidin–biotin–peroxidase complex immunohistochemistry technique was used with anti-SV40 T-Ag antibody (Oncogene Research, San Diego, CA, USA) as the primary antibody at a dilution of 1:500 together with appropriate positive and negative controls. This antibody reacts with BK, JC and SV40 polyomavirus T antigens. BKVAN was diagnosed by the presence of tubulointerstitial nephritis with ‘ground glass’ nuclear inclusion-bearing tubular epithelial cells (Fig. 1c). The presence of polyomavirus antigen was confirmed by positive staining of nuclear inclusions with anti-SV 40 T-Ag antibody (Fig. 1d).

Electron microscopy of urine

Five millilitres of urine was ultracentrifuged at 100 000 × g for 60 min. The resultant pellet was resuspended in a drop of sterile distilled water and examined by negative staining EM (Philips EM 201C, Eindhoven, The Netherlands) using 3% sodium phosphotungstate, pH 6.0. Polyomavirus particles were identified by their characteristic morphology (Fig. 1a).

Urine decoy cells

Decoy cells were identified by their typical ground-glass intranuclear inclusions with a peripheral rim of hyperchromatic chromatin in cytological preparations of urine stained by the Papanicolaou method (Fig. 1b).

Real-time quantitative BK viral load

Nucleic acid was extracted from 200 μl of urine or serum using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) and eluted with 200 μl of buffer. Quantitation of BK virus DNA was carried out by real-time polymerase chain reaction (PCR)

Table 1. Patients with BKVAN—immunosuppression therapy at the time of diagnosis and subsequent interventions

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. of urine samples no. tested for viral load</th>
<th>No. of serum samples</th>
<th>Immuno-suppression therapy at the time of diagnosis*</th>
<th>Interventions</th>
<th>Duration of follow-up from diagnosis of BKVAN (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14(5)</td>
<td>11</td>
<td>MMF, Tac</td>
<td>Switch from Tac to Csa</td>
<td>112</td>
</tr>
<tr>
<td>2</td>
<td>20(6)</td>
<td>14</td>
<td>Aza, Tac</td>
<td>Switch from Tac to Csa; cidofovir</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>31(14)</td>
<td>20</td>
<td>Aza, Tac</td>
<td>Switch to Csa and MMF and then from MMF to Aza; cidofovir</td>
<td>102</td>
</tr>
<tr>
<td>4</td>
<td>18(12)</td>
<td>13</td>
<td>MMF, Tac</td>
<td>Switch from Tac to Csa; reduced and eventually stopped MMF; cidofovir</td>
<td>81</td>
</tr>
<tr>
<td>5</td>
<td>14(8)</td>
<td>11</td>
<td>Csa, Aza</td>
<td>Stop Aza</td>
<td>76</td>
</tr>
<tr>
<td>6</td>
<td>7(5)</td>
<td>8</td>
<td>MMF, Tac</td>
<td>Switch from Tac to Csa; stopped MMF; cidofovir</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>23(22)</td>
<td>20</td>
<td>MMF, Tac</td>
<td>Switch from Tac to Csa; stopped MMF; cidofovir</td>
<td>36</td>
</tr>
</tbody>
</table>

* All patients received prednisolone. Tac, tacrolimus; MMF, mycophenolate mofetil; Aza, azathioprine; Csa, ciclosporin.
using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Warrington, UK) according to the method described by Leung et al. [14]. Standard curves were constructed by plotting the threshold cycle (Ct) values against the logarithm of a serial dilution of the plasmid standard pB-VP1. In contrast to histology, EM and cytological examination, this real-time PCR was specific for BK virus [15]. Local evaluation confirmed that the probe did not cross-react with JC virus amplicon generated by the same primer set and achieved a detection limit of 200 genome copies/ml.

**Statistical analysis**

The detection of polyomavirus in urine by EM and the presence of decoy cells in urine were correlated with viral load in urine and serum. The difference in viral load between samples with positive or negative EM or decoy cells were compared using the Epi Info software version 3.01 (Center for Disease Control, Atlanta, GA, USA). The non-parametric Kruskal–Wallis test was used in the analysis and a $P$-value of <0.05 was considered as statistically significant. The relationship between urine and serum viral load was established using linear regression. Pearson correlation coefficient and 95% confidence interval were calculated using Excel software (Microsoft, CA, USA).

**Results**

BKVAN was diagnosed in seven renal transplant patients in a single unit during the period of this study. A total of 372 renal transplantations were performed between the transplantation of the first BKVAN patient and the seventh case, thus a crude incidence of 1.9% without any adjustment for duration of transplantation. During the period of
study, 803 renal transplant recipients were followed-up in the unit. The prevalence of BKVAN was therefore 0.9%.

All patients showed a reduction in serum and urine viral load over the period of follow-up. The decrease in serum viral load ranged from 1.7 log to more than 5.6 log and in urine, the decrease in viral load ranged from 2.1 log to more than 8 log (Fig. 2). Five of the seven patients had a serum viral load which fell below the detection threshold of 200 copies/ml: four of whom had detectable BK viral load in urine throughout the period of follow-up; one patient had transient negative urine BK virus DNA on two samples, but a low level BK viral load was detected again in subsequent samples.

The viral load of urine samples with and without detectable decoy cells was compared. Viral loads of urine samples with decoy cells were significantly higher than those without decoy cells ($P < 0.001$). All urine samples with viral loads $< 10^6$ copies/ml were negative for decoy cells. Likewise, the viral load of urine samples that were EM positive was significantly higher than those that were negative ($P < 0.001$). All urine samples with viral loads $< 10^6$ copies/ml were negative for polyomavirus on EM. Thus, the relationship between urine viral load and decoy cell shedding was similar to that between urine viral load and EM (Fig. 3).

A similar relationship was found when serum viral load was compared with decoy cell shedding and EM detection of polyomavirus on paired urine samples taken on the same day (Fig. 4). The serum viral load in samples where the paired urine contained decoy cells or polyomavirus by EM was significantly higher than in those with negative cytology or EM ($P < 0.001$). With only one exception, all urine samples that were negative for decoy cells or EM had a corresponding serum viral load $< 10^3$ copies/ml.

Of the 46 paired urine/serum samples where BK viral loads were measured on both samples, 24 had detectable serum viral loads, of which 22 (91.6%) were $> 10^3$ copies/ml. All serum samples with a detectable BK viral load had detectable viral load in the corresponding urine sample. A scatter plot of the serum viral load with the corresponding urine viral load is shown in Fig. 5. Regression analysis shows a constant relationship between urine and serum viral load, the urine viral load being 3-log higher than its corresponding serum level $[\log(\text{urine viral load}) = \log(\text{serum viral load}) + 3; \text{coefficient of correlation} = 0.82; 95\% \text{ confidence interval} = 0.62–0.92]$. Consistent with the above finding, of the 22 serum samples with a viral load below detection limit, 19 (86.4%) of the paired urine samples had viral load below $10^6$ copies/ml.

This 3-log relationship between urine and serum viral load correlates with the viral load limit whereby decoy cells and polyomavirus by EM become undetectable ($10^6$ copies/ml in urine and $10^3$ in serum).

None of the seven patients lost their graft. The median peak creatinine level was 290 $\mu$mol/l (mean 348; range 253–602 $\mu$mol/l). All patients showed stabilization of creatinine level with a median decrease from the peak level by 13% (range 2–43%). The decrease in creatinine level occurred in association with the decrease in viral load and negative EM and decoy cells (Fig. 2). Three patients (patients 4, 5 and 6) had follow-up graft biopsies after stabilization of renal function. In patients 4 and 5, no histological evidence of BKVAN was found which correlated with a serum BK viral load $< 10^3$ copies/ml.

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viral load below the detection limit and a urine viral load below $10^6$ copies/ml ($1.6 \times 10^5$ and $1.6 \times 10^3$ copies/ml, respectively). In patient 6, only a few BK inclusions were found. In this patient, serum viral load had just fallen to below the detection limit and the corresponding urine viral load was $2.5 \times 10^6$ copies/ml. This subsequently fell to $6.9 \times 10^4$ copies/ml over the next 10 weeks.

**Discussion**

The management of BKVAN after renal transplantation presents a major challenge. BKVAN often co-exists with acute rejection and both conditions may cause graft dysfunction. Whilst rejection requires escalation of immunosuppression, BKVAN is the consequence of an opportunistic infection/reactivation which can only be effectively controlled by reducing the level of immunosuppression. *In vitro*, BK virus infection is susceptible to the antiviral agent cidofovir [16]. Cidofovir, however, is highly nephrotoxic and may potentiate further allograft damage. Recently, a low-dose approach has been suggested [17] which was employed in some of the patients in this series. It is clearly of paramount importance to have a means of monitoring virological response closely and accurately in order to minimize the duration of exposure to this nephrotoxic agent.

Six of the seven patients in this study were on an immunosuppressive regimen containing tacrolimus and four of these were also on MMF (Table 1). This is consistent with the current trend of the usage of immunosuppressive therapy in renal transplant recipients. However, the number of patients in this study was too small to determine the risk factor of a particular immunosuppressive drug or regimen.

A number of studies have examined the use of laboratory markers to help in the diagnosis of BKVAN [7,10]. Based on success in the quantification of BK virus DNA in blood and urine at diagnosis, it has been suggested that viral load could also be used to monitor the progress of disease [12,13]. However, it is not clear how viral loads compare with other...
Fig. 4. Serum BK viral load compared to decoy cell and EM detection of polyomavirus in urine. The black bar in each series represents the median viral load.

Fig. 5. Urine BK viral load compared to serum BK viral load. The straight line represents the regression line with the formula of log (urine viral load) = log(blood viral load) + 3. Correlation coefficient was 0.82 (95% confidence interval 0.62–0.92).
markers such as urine EM or cytology when used in such a context. The aim of this study was to identify the effectiveness of laboratory markers to monitor viral control and to determine the relationship between urine and serum BK viral load in patients with BKVAN in comparison with urine EM and cytology.

Serial samples from this study demonstrated a decline in all four markers during follow-up. Urine viral load of $<10^6$ and a serum viral load of $<10^3$ copies/ml were consistently associated with negative EM and the absence of decoy cells in urine. The greatest dynamic range of viral load was seen in urine samples. The relationship between urine and serum BK viral load is interesting in that urine viral load was consistently 3 log higher than that in serum. This is contrary to the findings of Leung et al. [15] and Merlino et al. [18], neither of whom identified any correlation between urine and serum BK viral load. However, the renal transplant recipients included in these two studies were not known to have BKVAN and the serum viral load reported was much lower. In our study, all patients had biopsy-proven BKVAN with much higher serum and urine viral loads. This proportional relationship between urine and serum viral load is corroborated by respective thresholds of $10^6$ and $10^3$ copies/ml at which polyomavirus becomes detectable by urine EM and decoy cells appear. The source of BK virus DNA in urine is not clear, but presumably represents virions or viral DNA travelling from tubular epithelial cells via denuded basement membranes to peritubular capillaries. Alternatively, it is also possible that serum BK virus DNA derives from the replication of BK virus in extra-renal tissues. The finding of a proportional relationship between urine and serum viral load in this study supports the former theory. However, further studies to compare lineages of urine and serum viruses are required.

There were a number of limitations in this study. Only patients with confirmed BKVAN were studied and no control population was included. As allograft failure could be observed for up to 240 weeks [9], the median follow-up period of 76 weeks may not be long enough. Due to the nature of the study, not all samples were tested by all the markers, especially during the first 6 months of the study. Also, the number of patients in this study is small and their management was not uniform. This paper is therefore not intended for studying outcome according to therapeutic changes. Despite these limitations, a decline in serum and urine viral load was demonstrated in each patient over time, correlating with the disappearance of polyomavirus and decoy cells from urine by EM and cytology, respectively. The three patients who had follow-up graft biopsies provided some further insight into the usefulness of the viral load measurement. Two patients (patients 4 and 5) no longer had histological evidence of BKVAN and one (patient 6) showed significant improvement. This correlated with a serum BK viral load $<200$ copies/ml and a urine viral load of $10^6$ copies/ml or below.

This study has therefore identified a relationship between the different markers that are commonly used to monitor the progress of patients with BKVAN. In transplant centres where routine quantitation of BK virus DNA is not available, negative EM and the absence of decoy cells could be used as markers to indicate viral control. However, measurement of the BK virus DNA level gives a wider dynamic range and could be a better choice for determining the extent of viral control.

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