Quantification of polyoma BK viruria in hemorrhagic cystitis complicating bone marrow transplantation

Anskar Y. H. Leung, Christine K. M. Suen, Albert K. W. Lie, Raymond H. S. Liang, Kwok Y. Yuen, and Yok L. Kwong

Polyoma BK virus (BKV) is frequently identified in the urine of bone marrow transplantation (BMT) patients with hemorrhagic cystitis (HC). However, viruria is common even in asymptomatic patients, making a direct causative role of BKV difficult to establish. This study prospectively quantified BKV viruria and viremia in 50 BMT patients to define the quantitative relationship of BKV reactivation with HC. Adenovirus (ADV) was similarly quantified as a control. More than 800 patient samples were quantified for BKV VP1 gene with a real-time quantitative polymerase chain reaction. Twenty patients (40%) developed HC, 6 with gross hematuria (HC grade 2 or higher) and 14 with microscopic hematuria (HC grade 1). When compared with asymptomatic patients, patients with HC had significantly higher peak BK viruria \((6 \times 10^3 \text{ copies/d})\) and larger total amounts of BK excreted during BMT \((4.9 \times 10^5 \text{ copies/d})\) than asymptomatic patients \((7.7 \times 10^5 \text{ copies/d})\). There was no detectable increase in BK viremia. Binary logistic regression analysis showed that BK viruria was the only risk factor, with HC not related to age, conditioning regimen, type of BMT, and graft-versus-host disease. Furthermore, the levels of ADV viruria in patients with or without HC were similar and comparable with those of BK viruria in patients without HC, suggesting that the significant increase in BK viruria in patients with HC was not due to background viral reactivation or damage to the urothelium. BK viruria was quantitatively related to the occurrence of HC after BMT. (Blood. 2001;98:1971-1978) 

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Introduction

Hemorrhagic cystitis (HC) is an important cause of morbidity and occasional mortality in patients undergoing bone marrow transplantation (BMT).\(^1\) The manifestations vary from microscopic hematuria to severe bladder hemorrhage leading to clot retention and renal failure. Its incidence has varied from 7% to 68% of BMT cases.\(^1,5\) Although mild HC usually subsides with supportive treatment including blood and platelet transfusion, severe HC may require bladder irrigation, cystoscopy, and cauterization.\(^5\)

Hemorrhagic cystitis has been ascribed to the toxic effects of drugs in the BMT conditioning regimen. Cyclophosphamide (Cy) is the most important one, owing to its conversion to acrolein that is highly toxic to uroepithelium.\(^1,6\) This can be countered by the use of 2-mercaptoethane sulfonate (MESNA),\(^1,7\) which is now included routinely in conditioning regimens containing Cy. However, despite the use of MESNA, HC still remains a clinical problem, implying that Cy is not the only cause.\(^4\) Other risks have been implicated, including the use of busulfan and pelvic irradiation during conditioning, older age at transplantation, allogeneic BMT, and graft-versus-host disease (GVHD).\(^1,5,11\) Most of these risk factors have not been observed consistently, so that their roles in causing HC remain undefined.

The polyoma BK virus (BKV) was observed in early studies to be associated with the development of HC during BMT.\(^12\) Several studies have used virologic methods to document BKV as a risk factor.\(^1,12-14\) However, subsequent studies with the highly sensitive polymerase chain reaction (PCR) showed that BKV could be detected in the urine of BMT patients with or without HC.\(^15-18\) This is owing to the fact that, after primary infection, BKV remains dormant in the uroepithelium.\(^19\) During BMT, the intense immunosuppression leads to increased viral replication that results in viruria. Because over 90% of adults are seropositive for BKV, it is not unexpected that many patients may develop viruria, particularly when PCR is used to detect the virus.\(^20\) Therefore, the role of BKV in HC remains contentious. Furthermore, the lack of conclusive evidence to implicate BKV in the pathogenesis of HC has hampered the development of therapeutic measures based on inhibition of the virus.

In this study, we tested the hypothesis that although BKV could be detected nonquantitatively by PCR in most BMT patients, a quantitative PCR (Q-PCR) method might show a discriminating difference in the level of BK viruria in HC patients. We further evaluated the temporal relationship between BKV reactivation and HC, which might be important in the design of preventive strategies. Because adenovirus (ADV) infection has also been proposed to contribute to HC, mainly in pediatric BMT,\(^21\) we also quantified ADV viruria/viremia to determine if it might be a confounding factor, as well as to control for the possibility of background viral reactivation or regimen-related toxic damage to the urothelium after BMT.

Patients, materials, and methods

Patients

Fifty consecutive unselected patients undergoing BMT at Queen Mary Hospital, Hong Kong, were studied prospectively (Table 1). None had a
Table 1. Clinical characteristics of 50 BMT patients evaluated for HC

<table>
<thead>
<tr>
<th>Condition</th>
<th>No HC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Male/Female</td>
<td>16:14</td>
<td>10:10</td>
</tr>
<tr>
<td>Age</td>
<td>35 ± 2 (17-67)</td>
<td>39 ± 2 (22-50)</td>
</tr>
</tbody>
</table>

Diagnosis
- CML: 12
- AML: 8
- NHL: 4
- Others: 6
- MUDs: 7

Conditioning regimens
- Autologous: 9
- Related donors*: 14
- MUD: 7

<table>
<thead>
<tr>
<th>Conditioning regimen</th>
<th>No HC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu-Cy</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Cy-TBI</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Bu-Cy-TBI</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CBV</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>CarboPEC</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Melphalan</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>BEAM</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fludarabine-TBI</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Acute GVHD
- Nil: 21
- Grade 1: 8
- Grade 2 or above: 5
- Grade 3: 4
- Grade 4: 3

HC
- Grade 1: 14
- Grade 2: 2
- Grade 3: 1
- Grade 4: 3

CML indicates chronic myelogenous leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; Bu-Cy, busulfan/cyclophosphamide; CBV, cyclophosphamide, BCNU, etoposide, carboplatin, etoposide, cyclophosphamide; BEAM, BCNU, etoposide, cytarabine arabinoside, melphalan.
*HLA-identical sibling (n = 24); one HLA-mismatched parent (n = 2); syngeneic donor (n = 1).

For BMT patients, 24-hour urine specimens and 1 mL plasma were collected a day before the commencement of conditioning, on the day of marrow infusion (day 0), and weekly thereafter until discharge from the hospital. Urinary collection might be temporarily suspended during bladder irrigation in cases of severe HC. The 24-hour urine sample was collected for the quantification of viruria to obviate potential problems related to variation of viruria during the day. When HC developed, specimen collection was repeated every 3 days. The volume of urine was recorded and 50 mL was aliquoted and centrifuged at 3200 rpm for 30 minutes. The urinary sediment so obtained was washed with saline and resuspended in 200 µL water. DNA was extracted by the QIAamp DNA Blood MiniKit (Qiagen, Basel, Switzerland) and eluted with 200 µL buffer. DNA was also extracted from each of 200 µL of urine and plasma collected at the similar time points with the same protocol. For the healthy controls, only a spot 50-mL urine sample was collected and processed as for BMT patients. Plasma (but not whole blood) was used for quantification of viremia to avoid interference due to the presence of BKV in the nucleated cells.

Conventional PCR for BKV

Conventional PCR for the BKV VP1 gene (GenBank accession number V01109) was performed the forward primer VP (5'-AGT GGA TGG GCA GCC TAT GTA-3') and the reverse primer VP (5'-TCA TCT CTG GGT CCC CTG GA-3'), which gave a 95-base pair (bp) product.

Q-PCR

Quantitative PCR was performed by a real-time PCR assay, a technique that allows simple and rapid quantification of a target sequence to be made during the exponential phase of the PCR, using the ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA). Briefly, PCR primers for the BKV VP1 gene (VP1 and VP2), and the TaqMan BKV probe (5'-AGG TAG AAG AGG TTA GGG TGT TATG A GC A CAG-3') were designed by the Primer Express software (PE Biosystems). Q-PCR amplification reactions were set up in a reaction volume of 50 µL using the TaqMan Universal PCR
Master Mix (PE Biosystems), containing 10 μL purified DNA, 200 and 400 nM of VPf and VPr, and 50 nM TaqMan probe. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). Real-time PCR amplification data were collected continuously and analyzed with the Sequence Detection System (PE Biosystems). Quantification of ADV viruria was performed similarly. A 72-bp sequence in the ADV hexon gene with homology shared by serotypes 11, 34, and 35 (GenBank accession number AB018424) was amplified by the primers HEXf (5' -ACT ACA TGA ACG GCC GGG T-3') and HEXr (5'-GAG ACC ACC TGG CAC CAA TG-3') and detected by the TaqMan ADV probe 5'-FAM (TGC CGC 9'-TAMRA-3') and detected by the fluorescence signal of the passive reference dye (ROX), to obtain a ratio (by boxes next to the corresponding curves). Gray curves denote no template control reactions with known starting amounts of pB-VP1 (0.5-500 fg plasmid DNA, denoted $x$), where $x$ is the amount in picograms, $6.023 \times 10^{23}$ is the number of copies in 1 mole of plasmid, and 660 Np is the molar weight of the plasmid (660 is the average molecular weight of a nucleotide pair, and Np is the number of nucleotide pairs in the plasmid standard, being 3 367 for pB-VP1, and 3 176 for pB-Hex). To calculate the daily viruria excretion, results from 10 μL extracted DNA obtained by Q-PCR were multiplied by the following correction factors: $20 \times 24$-hour urine volume (mL)/50 for urinary sediment, and $20 \times 5$ 24-hour urine volume (mL) for free urine. To calculate the total viral excretion during the BMT period (both sediments and free urine), the daily viral excretion (in genome copies) was integrated over time during BMT (ie, area under the curve in a plot of viral excretion versus time) using computer software (PRISM, Graphpad Software, San Diego, CA).

Patient samples were tested in triplicate, their respective CtS were determined, and the initial starting sequence amount calculated from the standard curve (Figure 1). The calculations for daily viruria for BKV and ADV were identical. To convert to viral genome equivalent copies, the following formula was used: copy number = $x / 10^{23} \times 6.023 \times 10^{23} / 660 Np$, where $x$ is the amount in picograms, $6.023 \times 10^{23}$ is the number of copies in 1 mole of plasmid, and 660 Np is the molar weight of the plasmid (660 is the average molecular weight of a nucleotide pair, and Np is the number of nucleotide pairs in the plasmid standard, being 3 367 for pB-VP1, and 3 176 for pB-Hex). To calculate the daily viruria excretion, results from 10 μL extracted DNA obtained by Q-PCR were multiplied by the following correction factors: $20 \times 24$-hour urine volume (mL)/50 for urinary sediment, and $20 \times 5$ 24-hour urine volume (mL) for free urine. To calculate the total viral excretion during the BMT period (both sediments and free urine), the daily viral excretion (in genome copies) was integrated over time during BMT (ie, area under the curve in a plot of viral excretion versus time) using computer software (PRISM, Graphpad Software, San Diego, CA).

PCR precautions
All precautions preventing contamination were rigorously adhered to. DNA extraction, cloning of pB-VP1/pB-Hex, and Q-PCR were performed in 3 different laboratories. All samples were handled with positive displacement pipettes with aerosol-resistant tips. Negative blanks included in each PCR did not give positive results.

Quality assurance of Q-PCR
To control for plate-to-plate variation in PCR efficiencies, so that all results were comparable, a standard curve was constructed in each Q-PCR experiment with serial dilutions of the same stock solution of pB-VP1/pB-Hex, which was prepared at the start of the project and frozen at $-20^\circ$C in small aliquots for single use only.

Statistical analysis
Comparison between groups of data were performed with the Mann-Whitney test or the Kruskal-Wallis test. The occurrence of HC was evaluated by binary logistic regression (SPSS, Chicago, IL). The occurrence of HC was the dependent variable and the following factors were entered as covariates: age of the patients, the source of stem cells (autologous versus siblings versus matched unrelated donors), the conditioning regimens (non-Cy/total body irradiation [TBI] versus Cy), time between transplantation and peaks in BK viruria during BMT, and the presence of GVHD (grade $\geq 2$) prior to HC, the lowest platelet count and the highest serum creatinine concentration during BMT (ie, area under the curve in a plot of viral excretion versus time) using computer software (PRISM, Graphpad Software, San Diego, CA).

Statistical analysis
Comparison between groups of data were performed with the Mann-Whitney test or the Kruskal-Wallis test. To define the relationship between BK viruria and HC, the following measurements of BK viruria were evaluated: peak viruria at the day of peaking of BK viruria; total viruria during BMT; viruria prior to HC; total viruria prior to the onset of HC in HC patients, and total viruria during BMT in non-HC patients; mean daily viruria prior to HC; total viruria during BMT in non-HC patients; mean daily viruria prior to HC; total viruria/number of samples collected prior to HC in HC patients, and total viruria/number of samples collected during BMT in non-HC patients; mean daily viruria during BMT in non-HC patients; and a composite score of potential risk factors to the occurrence of HC was evaluated by binary logistic regression (SPSS, Chicago, IL). The occurrence of HC was the dependent variable and the following factors were entered as covariates: age of the patients, the source of stem cells (autologous versus siblings versus matched unrelated donors), the conditioning regimens (non-Cy/total body irradiation [TBI] versus Cy), time between transplantation and peaks in BK viruria during BMT, and the presence of GVHD (grade $\geq 2$) prior to HC, the lowest platelet count and the highest serum creatinine concentration during BMT (ie, area under the curve in a plot of viral excretion versus time) using computer software (PRISM, Graphpad Software, San Diego, CA).

Figure 1. Standard curves for quantification of BKV and ADV. (A) Amplification plots of pB-VP1 for the construction of a standard curve. Amplification plot for reactions with known starting amounts of pB-VP1 (0.5-5000 fg plasmid DNA, denoted by boxes next to the corresponding curves). Gray curves denote no template control reactions. Cycle number is plotted against change in normalized reporter signal (ΔRn). For each reaction, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reference dye (ROX), to obtain a ratio defined as the normalized reporter signal (Rn). (B) Plot of standard curve of starting pB-VP1 amount against Ct. Black circles represent pB-VP1 standards as demonstrated in panel A. Gray circles represent patient samples. A standard curve is constructed for every assay.
Results

Patients and clinical course

The clinical characteristics of the patients were shown in Tables 1 and 2. Twenty patients developed HC (Tables 3 and 4). Two patterns of HC can be defined. In patients 1 through 6, HC presented as gross hematuria (≥ grade 2) and occurred after hematopoietic reconstitution, with median onset at day 37 (range, 29–72). A combination of medical and surgical treatment in these patients did not result in immediate cessation of bleeding, which lasted a median of 18 (range, 8–110) days. On the other hand, in patients 7 through 20, HC presented with only microscopic hematuria (grade 1), with median onset at day 4 (range, 1–25). The onset of high-grade HC (≥ grade 2) was significantly later than that of grade 1 HC (day 37 versus day 4, P < .01).

PCR

Conventional PCR for BKV was positive in the urinary sediments of all 50 patients at 2 or more time points during the BMT period. However, it was not demonstrable in any of the plasma specimens tested. As leukopenia developed during BMT, making the number of white cells in the urine small, BKV detection in urinary sediments was unlikely to be explained by the presence of blood in the urine. In the normal controls, 16 of 40 (40%) showed weakly positive PCR results.

Q-PCR of BKV

The BK viruria was quantifiable serially in all 50 patients (median: 7 time point, about 800 samples analyzed). Quantification of BKV in free urine and urinary sediment showed results comparable in both magnitude and timing (Table 2). For simplicity of data presentation, only results of urinary sediments will subsequently be shown. A detectable rise in viruria (>10 times the baseline) occurred in 47 patients. Three patients had no significant increase in viruria. The time from BMT to peak viruria varied from 2 to 57 days (median, 15 days). There was no significant difference between the timing of peak viruria in patients with or without HC (day 21 versus day 14, P = .2). However, the peak viruria in patients with HC was significantly higher than those without HC (median peak value 6 × 10^12 versus 5.7 × 10^7 genome copies, P < .001) (Figure 2). Furthermore, the total amount of virus excreted during the BMT period was also significantly higher in patients with HC as compared to those without (4.9 × 10^13 versus 7.7 × 10^8, P < .001; Figure 3). This difference was not related to a difference in the total number of samples collected, because the median numbers of urine samples collected for HC and non-HC patients were 9 and 7, respectively, and the median durations of hospitalization were 40 and 41 days (P > .05 for both parameters.

Table 2. Clinical and virologic characteristics of 50 BMT patients evaluated for HC

<table>
<thead>
<tr>
<th>Virologic and laboratory parameters</th>
<th>No HC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BK viruria (median genome copies/d)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak†</td>
<td>2.2 × 10^4</td>
<td>2 × 10^12</td>
</tr>
<tr>
<td>Total</td>
<td>3.5 × 10^3</td>
<td>3.9 × 10^13</td>
</tr>
<tr>
<td>Prior to HC‡</td>
<td>3.5 × 10^4</td>
<td>1.2 × 10^13</td>
</tr>
<tr>
<td>Mean daily prior to HC§</td>
<td>8.0 × 10^7</td>
<td>5.5 × 10^11</td>
</tr>
<tr>
<td>Urinary sediment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak†</td>
<td>5.7 × 10^12</td>
<td>6 × 10^12</td>
</tr>
<tr>
<td>Total</td>
<td>7.7 × 10^8</td>
<td>4.9 × 10^13</td>
</tr>
<tr>
<td>Prior to HC</td>
<td>7.7 × 10^12</td>
<td>8.6 × 10^12</td>
</tr>
<tr>
<td>Mean daily prior to HC</td>
<td>2.3 × 10^7</td>
<td>5.3 × 10^10</td>
</tr>
<tr>
<td><strong>ADV viruria (median genome copies/d)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free urine</td>
<td>2.2 × 10^7</td>
<td>7.5 × 10^7</td>
</tr>
<tr>
<td>Urinary sediments</td>
<td>8.3 × 10^5</td>
<td>8.6 × 10^9</td>
</tr>
<tr>
<td><strong>ADV viremia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median genome copies/mL</td>
<td>2.7 × 10^7</td>
<td>3.3 × 10^4</td>
</tr>
<tr>
<td><strong>Serum creatinine (μmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>81 (50-129)</td>
<td>73 (53-171)</td>
</tr>
<tr>
<td><strong>Table 3. Virologic and laboratory parameters</strong></td>
<td></td>
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<tr>
<td><strong>BKV viruria (median genome copies/d)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>5.7 × 10^7</td>
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<tr>
<td>Total</td>
<td>7.7 × 10^8</td>
<td>3.9 × 10^12</td>
</tr>
<tr>
<td>Viruria prior to HC</td>
<td>7.7 × 10^8</td>
<td>1.4 × 10^11</td>
</tr>
<tr>
<td><strong>Number of patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily viruria &gt; 10^10/d</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Daily viruria &lt; 10^10/d</td>
<td>24</td>
<td>5</td>
</tr>
</tbody>
</table>

†At the day of peaking of BK viruria; total is total viruria during BMT period.
‡In HC patients, total viruria prior to HC; in non-HC patients, total viruria during BMT.
§In non-HC patients, calculated by total viruria/total number of samples collected.
P ＊Highest creatinine during the period of HC or BMT.
P †Mann-Whitney test, with NS indicating not significant.
P ¶Kruskal-Wallis test.
was in the order of $10^3$ mL/d, the controls had comparable levels of mL). Assuming that the average urine output in normal subjects

there were some overlaps. As shown in Figure 2, taking more than $10^{10}$ BKV copies/d as a cutoff, of 21 patients considered to have significant viruria, 15 developed HC (grade 1-4). In the remaining 29 patients with viruria less than $10^{10}$ copies/d, only 5 of them had grade 1 HC. Therefore, BKV excretion of more than $10^{10}$ copies/d occurred in all the cases with severe HC (grade $>2$) and 9 of 14 patients with mild HC (grade 1). On the other hand, 6 of 30 patients without HC also had significant viral shedding (Table 5). When the amount of viruria was analyzed according to the severity of HC, patients with HC of grade 2 or higher had levels of BK viruria comparable to patients with HC of grade 1 (peak viruria $1.4 \times 10^{13}$/d versus $1.1 \times 10^{12}$/d; total viruria: $2.5 \times 10^{14}$ versus $3.9 \times 10^{12}$, respectively, $P < .05$, Mann-Whitney test).

Q-PCR of ADV

The ADV viruria was quantifiable in all patients. A detectable rise in viruria in either urinary sediment or free urine (> 10 times the baseline level) occurred in 28 patients during the course of BMT. In the remaining 22 patients, ADV viruria remained at a steady level throughout the BMT (the highest level during BMT was taken as the peak). There was no association of the occurrence of HC with a rise in viruria (15 patients in HC group had an increase in ADV viruria versus 13 patients in non-HC group), or with the median time of peak viruria (in both HC and non-HC group, ADV viruria in the urinary sediments peaked at 21 days after BMT, and in free urine at 16 and 14 days, respectively). Furthermore, the magnitude of ADV viruria was also comparable in patients with or without HC (Table 2). This was shown both in the free urine and sediment, although for reasons not defined in this study, the levels of ADV in the free urine consistently exceeded those of the sediment. Interestingly, the median level of ADV viruria in the free urine in patients with or without HC were comparable with that of BKV in patients without HC ($2.2 \times 10^2$ versus $7.5 \times 10^2$ versus $2.2 \times 10^3$, $P = \text{not significant}$); but all 3 were 4 to 5 logs lower that the median level of

Mann-Whitney test). Finally, both the total and the mean daily viruria prior to the onset of HC were significantly higher in HC patients as compared with non-HC patients ($8.6 \times 10^3$ versus $7.7 \times 10^3, P < .001$, and $5.3 \times 10^3$ versus $2.3 \times 10^3, P < .001$).

In the control subjects where BKV could be detected by conventional PCR, Q-PCR of spot urine samples showed a median genome copy of $5.3 \times 10^3$/mL (range, $3.1 \times 10^3$-$1.5 \times 10^4$ copies/mL). Assuming that the average urine output in normal subjects was in the order of $10^3$ mL/d, the controls had comparable levels of BK viruria as patients without HC.

Parallel with the 2 clinical types of HC, 2 patterns of viruria were observed. In patients 1 through 6 with HC of grade 2 or higher, peaking of viruria preceded the onset of HC (Figure 4). On the other hand, in patients 7 through 20 with grade 1 HC, peaking of viruria coincided or occurred later than the onset of HC in most of the patients.

Quantitative PCR was also performed in 144 plasma specimens from 46 patients. Although BKV was not detected by conventional PCR in any of the cases, it was quantifiable in 42 cases (Table 2). There was no detectable increase in BK viremia in any of the patients during the course of BMT, including those samples that were taken before, during, or after the onset of HC. There was also no significant difference in the level of BK viremia in patients with or without HC.

HC and BK viruria

Although as a group patients with HC have significantly higher levels of viral excretion as compared to those without, there were some overlaps. As shown in Figure 2, taking more than $10^{10}$ BKV copies/d as a cutoff, of 21 patients considered to have significant viruria, 15 developed HC (grade 1-4). In the remaining 29 patients
Platelet
Peak ADV
1 and 3.
without HC, which was comparable with that of BK viremia in
viremia at the time of peak viruria was similar in patients with or
An arbitrary
cutoff at $10^{10}$ genome copies/d gives the best predictive value for the occurrence of
HC, viruria, and clinicopathologic parameters

Among the various factors entered into the time-dependent binary logistic regression, only the quantity of peak daily BK viruria and the lowest platelet count during HC (HC group) or BMT (non-HC

BKV in patients with HC ($2 \times 10^{12}$). Finally, the level of ADV
viremia at the time of peak viruria was similar in patients with or
without HC, which was comparable with that of BK viremia in
patients without HC (Table 2).

HC, viruria, and clinicopathologic parameters

Among the various factors entered into the time-dependent binary logistic regression, only the quantity of peak daily BK viruria and the lowest platelet count during HC (HC group) or BMT (non-HC

Discussion

In this study, we have addressed several important issues concerning
BK viruria and HC during BMT. We demonstrated that using

Figure 2. Peak BKV excretion per day in patients with and without HC, showing a significant difference in viral excretion between the 2 groups. An arbitrary cutoff at $10^{10}$ genome copies/d gives the best predictive value for the occurrence of HC. This also identifies all patients with HC grade 2 or higher.

Figure 3. Total BKV viral excretion during the entire BMT period plotted against the number of patients. This showed a significant difference between the median total viral excretion in patients without HC (in the range of $10^{8}-10^{10}$, panel A) as compared with patients with HC (in the range of $10^{12}-10^{14}$, panel B).
the PCR to test for BK viral sequence, viruria occurred in all BMT patients, but only 40% of them developed HC. This phenomenon has been shown previously, making the original observations of the association between BK viruria and HC controversial. To resolve this problem, our study indicated that although there was baseline viruria, Q-PCR showed that HC patients had significantly higher viral excretion. The peak viruria and total viral excretion in HC patients were approximately \(10^6\) times higher than non-HC patients, respectively. Because it is unknown whether variations of viral excretion might occur during the day, we have chosen to examine 24-hour urine to obviate possible problems associated with the study of spot urine samples. However, a previous study examining spot urine with a competitive PCR technique showed that patients with or without HC excreted \(10^6\) to \(10^7\) and \(2 \times 10^3\) to \(8.9 \times 10^3\) BKV copies/mL, respectively. Although the technique was different, the magnitudes of BK viruria in that study were similar to those in our study (assuming that the daily urine output was in the order of \(10^3\) mL). Therefore, our results showed that BK viruria was quantitatively related to the occurrence of HC.

Several confounding factors might influence the significance of the increase in BK viruria. First, this might simply be a reflection of injury to the urothelium due to the conditioning regimen. However, BK viruria in patients without HC was similar in magnitude to normal controls, arguing against a significant regimen-related damage to the bladder epithelium. Furthermore, quantification of ADV as a control showed that patients with or without HC excreted \(10^6\) to \(10^7\) and \(2 \times 10^3\) to \(8.9 \times 10^3\) BKV copies/mL, respectively. Although the technique was different, the magnitudes of BK viruria in that study were similar to those in our study (assuming that the daily urine output was in the order of \(10^3\) mL). Therefore, our results showed that BK viruria was quantitatively related to the occurrence of HC.

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The sensitivity and specificity of BK viral load were calculated using the cutoff of \(10^{10}\) copies/d. The sensitivity and specificity of BK viral load were 75% and 80%, respectively. The positive predictive value was 71%, and the negative predictive value was 83% for HC. For HC grade 2, the sensitivity and specificity were 100% and 66%, respectively. The positive predictive value was 29%, and the negative predictive value was 100%. For HC grade 4, the sensitivity and specificity were 100% and 61%, respectively. The positive predictive value was 14%, and the negative predictive value was 100%.

Table 5. Sensitivity, specificities, and predictive values of BK viruria at a cutoff of \(10^{10}\) copies/d.

<table>
<thead>
<tr>
<th>HC or Grade</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive Predictive Value (%)</th>
<th>Negative Predictive Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All HC</td>
<td>75</td>
<td>80</td>
<td>71</td>
<td>83</td>
</tr>
<tr>
<td>HC ≥ grade 2</td>
<td>100</td>
<td>66</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td>HC = grade 4</td>
<td>100</td>
<td>61</td>
<td>14</td>
<td>100</td>
</tr>
</tbody>
</table>
although plasma BKV was quantifiable, there was no significant increase in any of the patients during the clinical course, implying that plasma BKV PCR might not be a useful test in HC. This is different from renal allografting, where BKV PCR is useful in predicting the occurrence of viral nephropathy.23 Finally, there was a highly variable interval (range, 1-58 days) from peak viruria to the onset of severe HC, and whether this could be fully explainable by our proposed model or was due to other confounding factors not examined in this study remains unclear. However, the demonstration in this study of a highly significant increase in BK viruria indicates a role in the pathogenesis of HC. Hence, the above observations should probably not be seen to negate the importance of BKV in HC, but rather to provide grounds for further research.

The findings of this study are of potential therapeutic implications. Presently, there is no satisfactory treatment of HC of grade 2 or higher. Medical treatment with conjugated ureosides and uricosuric interventions have variable successes, so that HC continues to cause prolonged morbidity in BMT patients. We have shown that significant viruria invariably occurred in patients who would ultimately develop HC, particularly in those severely affected, who would benefit most from early or preventive treatment. However, the positive predictive value of BK viruria for severe HC is still low in our study, to the fact that some patients with significant BK viruria might not develop HC. The definition of possible coexisting factors that may act with BK viruria to give rise to HC may improve the predictive value of this test, possibly by studying a larger number of patients with severe HC that occurs after engraftment. Our observation that a BKV surge often precedes severe HC implies that prophylactic suppression of BKV replication may be of preventive benefit. In this study, all patients have received acyclovir prophylaxis, and most have received ganciclovir. These agents did not appear to have any impact on BK viruria. However, newer nucleoside analogues have been found to show in vivo and in vitro activities against polyoma viruses.33 Furthermore, bacterial DNA gyrase inhibitors have also been shown to suppress BKV replication and cytopathic effects in vitro.32 These agents need to be evaluated prospectively to see if they are of use in suppressing BKV replication and therefore the occurrence of HC.

In conclusion, the results of this study support that BKV plays a role in the pathogenesis of HC after BMT, based on its temporal and quantitative association with this disorder. Preventive measures based on inhibition of BK viral replication may therefore be of clinical benefit.

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References

Quantification of polyoma BK viruria in hemorrhagic cystitis complicating bone marrow transplantation

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