Reactivation and Mutation of Newly Discovered WU, KI, and Merkel Cell Carcinoma Polyomaviruses in Immunosuppressed Individuals

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Background. Infection with the human polyomaviruses BK (BKV) and JC (JCV) is almost ubiquitous, asymptomatic, and lifelong. However, reactivation during immunosuppression, associated with mutations in the transcriptional control region (TCR) that up-regulates viral replication, can cause life-threatening disease. In this study, we investigated whether the recently discovered WU and KI polyomaviruses (WUPyV and KIPyV) and Merkel cell polyomavirus (MCPyV) could, like BKV and JCV, persist, mutate, and reactivate in immunodeficient subjects.

Methods. Autopsy samples of lymphoid tissue from 42 AIDS-immunosuppressed subjects and 55 control samples were screened by polymerase chain reaction for all 5 polyomaviruses. TCR sequences from KIPyV and WUPyV recovered from both immunosuppressed and nonimmunosuppressed subjects were compared.

Results. Combined polyomavirus detection frequencies were much higher for the immunosuppressed group, compared with the nonimmunosuppressed group (35.7% vs. 3.6%), with viral loads in lymphoid tissues ranging from \(10^8\) to \(10^5\) viral genome copies per 10^6 cells. MCPyV was recovered from only 1 HIV-negative study subject. TCR sequences from reactivated WUPyV and KIPyV variants showed a number of point mutations and insertions that were absent in viruses recovered from respiratory tract specimens obtained from nonimmunosuppressed subjects.

Conclusions. KIPyV and WUPyV show reactivation frequencies comparable to those of BKV and JCV during immunosuppression. TCR changes that potentially lead to transcriptional dysregulation may have pathogenic consequences equivalent in severity to those observed for JCV and BKV.

Members of the Polyomaviridae are small, circular, double-stranded DNA viruses. The 2 human polyomaviruses BK (BKV) and JC (JCV) were discovered in 1971; BKV was recovered from the urine of a renal allograft recipient [1] and JCV from the brain of a patient with progressive multifocal leukoencephalopathy (PML) [2]. Subsequent serological surveys have shown that both viruses are acquired during childhood, probably by the respiratory route, and generally reach worldwide seroprevalence rates in adults of \(75\%\)–\(80\%\) [3–6].

Primary BKV and JCV infections are normally asymptomatic and followed by the establishment of latency, predominantly in the kidney [7, 8] and the central nervous system [9, 10]. Although primary infections are generally asymptomatic, there are a number of diseases associated with reactivation of JCV, such as PML [11]. BKV reactivation typically occurs in immunosuppressed individuals, often after kidney transplantation, and leads to several renal disorders, including polyomavirus-associated nephropathy (PAN) [12–14]. The transcriptional control region (TCR) of BKV and JCV contains promoter and enhancer sequences that control early and late gene expression. Rearrangements and mutations occurring in the TCR are commonly found in sequenced JCV samples obtained from patients with PML [15–23], although no clear correlation between these mutations and the prognosis or severity of disease has yet been made. TCR mutations have also been seen in sequenced BKV samples obtained from patients with PAN [24, 25].
Recently, virus researchers have identified additional human polyomaviruses. The related WU and KI polyomaviruses (WUPyV and KIPyV) were cloned from respiratory tract specimens [26, 27] and little is known about their tissue tropism, transmission, and disease associations. Although frequently detected in respiratory tract specimens, particularly those from children [26–28], no association with respiratory disease has been shown [29]. A third new human polyomavirus, Merkel cell carcinoma polyomavirus (MCPyV), was discovered by digital transcriptome subtraction from a rare human skin cancer, Merkel cell carcinoma, [30]. Clonal integration of the MCPyV genome within the tumor genome was demonstrated, suggesting a direct oncogenic role in the tumor.

Merkel cell carcinomas occur more frequently than expected among immunosuppressed transplant patients and patients with AIDS [31], and reactivation associated with immunosuppression is a common feature of BKV and JCV infections. To investigate whether reactivation of WUPyV, KIPyV, and MCPyV infections was also associated with immunosuppression, we screened lymphoid tissue autopsy samples from a large cohort of immunocompromised patients with AIDS and control subjects for the presence of WUPyV, KIPyV, and MCPyV by use of polymerase chain reaction (PCR). Although none of the study subjects were diagnosed with PAN or PML, samples were also screened for BKV and JCV to compare the frequencies of reactivation in the absence of these diseases. TCR sequences were obtained from samples with positive PCR results and compared with sequences from respiratory tract samples to assess the association of reactivation with mutations in this region.

**MATERIALS AND METHODS**

**Test specimens.** Samples from immunocompetent and immunosuppressed subjects were obtained from the Edinburgh Medical Research Council HIV Brain and Tissue Bank. Consent for the use of archival postmortem tissue samples was obtained from the Lothian Research Ethics Committee ([LREC] number 2002/4/36). Samples of lymphoid tissue (11 lymph node samples and 86 spleen samples) were obtained from 42 HIV-positive individuals with AIDS and control subjects for the presence of WUPyV, KIPyV, and MCPyV from a large cohort of immunocompromised patients with AIDS and control subjects for the presence of WUPyV, KIPyV, and MCPyV by use of polymerase chain reaction (PCR). Although none of the study subjects were diagnosed with PAN or PML, samples were also screened for BKV and JCV to compare the frequencies of reactivation in the absence of these diseases. TCR sequences were obtained from samples with positive PCR results and compared with sequences from respiratory tract samples to assess the association of reactivation with mutations in this region.

The respiratory tract specimen study group was a subset, selected on the basis of sample availability, of 727 respiratory tract specimens (nasopharyngeal swab samples, nasopharyngeal aspirate samples, bronchoalveolar lavage fluid samples, tracheal swab samples, tracheal aspirate samples, and sputum samples) obtained from 499 different individuals in a larger sample archive that had previously been screened for WUPyV and KIPyV [29]. All samples were taken from immunocompetent patients who had been referred to the Specialist Virology Centre (SVC), Royal Infirmary of Edinburgh, for virus testing. Prior to use, all samples were anonymized and deposited in the SVC respiratory tract sample archive, using a procedure approved by the LREC to maintain confidentiality, as described elsewhere [32].

**Detection of human polyomavirus DNA by PCR.** Total nucleic acid was extracted from clinical or autopsy specimens and respiratory tract samples, as described elsewhere [32]. For WUPyV and KIPyV DNA detection, nested PCR was performed with nested primers that hybridized to regions of sequence conservation between the 2 virus groups [29]. For detection of BKV and JCV, a combined PCR was developed that used nested primers (table 1, which appears only in the electronic version of the *Journal*) that matched highly conserved sequences in the non-structural gene region. For detection of MCPyV, nested primers were designed that hybridized to both published isolates [30] in the small T-antigen coding sequence (table 1, which appears only in the electronic version of the *Journal*). All reactions were performed under the amplification conditions described elsewhere for detection of WUPyV-KIPyV [29].

DNA samples from respiratory tract specimens were pooled for assay with each set of screening primers. Each assay was performed on 1 μL of pooled DNA containing 0.1 μL from each of 10 individual samples, representing approximately 0.8 μL of original secretion. For each autopsy tissue sample, 0.5 μg of extracted DNA was assayed with each set of primers, corresponding to approximately 65,000 cells. The viral loads in PCR-positive autopsy samples were semiquantified by assay of serial 10-fold dilutions of template DNA in duplicate (starting with 0.5 μg) by PCR. Dilutions at which 50% PCR positivity was expected were calculated by using the Reed-Münch formula [33].

To enhance the ability of the screening PCR to detect WUPyV-KIPyV and BKV-JCV coinfections, new inner antisense primers that were specific to a single viral genotype were designed to be used in conjunction with the originally used conserved inner sense WUPyV-KIPyV or BKV-JCV primers. Separate, second-round PCR amplification reactions with the originally used conserved inner sense primers were employed for type-specific amplification of the 2 viruses in samples with positive results.

The TCRs of polyomaviruses recovered from samples with positive results were amplified with primers recognizing both BKV and JCV or WUPyV and KIPyV (positions of the 5′ base in the published complete genome sequences of the prototype BKV
and JCV sequences [NC_001538 and J02226] or the prototype WUPyV and KIPyV sequences [NC_009539 and NC_009238], respectively) (table 1, which appears only in the electronic version of the Journal). PCR was performed under the amplification reaction conditions described elsewhere for WUPyV and KIPyV detection [29]. Positive second-round PCR amplicons were sequenced in both directions by using the inner sense and inner antisense primers, as described elsewhere [29].

**RESULTS**

**Assay sensitivity** The sensitivity of PCR for the detection of BKV-JCV and WUPyV-KIPyV was tested by using standards of plasmid DNA containing cloned viral sequences, except for KIPyV, for which cloned DNA was not available and primary PCR amplicons of known concentration were used instead. Standards were diluted to 100, 10, 1, and 0.1 copies/μL carrier DNA. Replicate PCR was performed by using 1 μL of target DNA at each of the 4 dilutions (table 2). The input amount corresponding to 50% positivity in PCR was calculated using the Reed-Münch formula. PCR assays for both BKV-JCV and WUPyV-KIPyV showed sensitivities for single copies of target sequences, consistent with previously developed nested PCR assays (e.g., [32]). The exception was the KIPyV PCR, in which a 50% PCR detection threshold of 10 copies was observed. However, the PCR product used in the DNA titration contained a substantial amount of nontarget DNA that was amplified nonspecifically during the PCR, and its presence likely affected the copy-number calculation.

Because 0.5-μg quantities of DNA were assayed for the autopsy test samples (see next section), single-copy sensitivity for polyomavirus DNA equates to an assay sensitivity of approximately 15 copies per 10^6 cells for autopsy tissues, and approximately 125 copies/100 μL for respiratory tract samples (tested by using DNA equivalent to 0.8 μL of original secretion per sample in pools).

**Detection of human polyomavirus DNA in autopsy samples.** This study investigated the frequency of detection and viral loads for human polyomaviruses in lymphoid tissue samples from 97 immunocompetent and immunosuppressed individuals. All screening was carried out by nested PCR with 3 sets of conserved primers: 1 set for combined detection of WUPyV and KIPyV, 1 for BKV and JCV, and a third set for MCPyV. Virus-positive samples were sequenced to identify the infecting virus by comparison with published sequences from each group.

Samples from 20 individuals were virus-positive on initial screening. A single sample was identified as virus-positive by use of the MCPyV primer set, 5 were identified as positive with only the WUPyV-KIPyV primer set, 11 were positive with only the BKV-JCV primer set, and 3 were positive with both the WUPyV-KIPyV and BKV-JCV primer sets. Sequence analysis of the 22 BKV-JCV–positive and WUPyV-KIPyV–positive amplicons showed that 4 were positive for WUPyV (1 from an IDU with AIDS and 3 from MSM with AIDS), 4 were positive for KIPyV (1 from a control sample and 3 from MSM with AIDS), 10 were positive for BKV (1 from an IDU with AIDS and 9 from MSM with AIDS), and 4 were positive for JCV (1 from an IDU without AIDS, 1 from an MSM with AIDS, and 2 from IDU with AIDS). The lone MCPyV-positive PCR result came from samples obtained from an HIV-negative (nonimmunosuppressed) IDU. In the amplified region, all sequences were identical to published sequences for these viruses (EF444549–444554, EF127906–127908, AY628224–628238, AB262404–262412, and EU375803–375804 for WUPyV, KIPyV, BKV, JCV, and MCPyV, respectively), with the exception of a single point-mutation in the JCV sequence obtained from the IDU without AIDS. All virus-positive samples were also tested by using type-specific primers for WUPyV and KIPyV or BKV and JCV to identify any occurrence of WUPyV-KIPyV or BKV-JCV coinfections. These reactions confirmed previous sequence analysis and demonstrated that there were no cases of WUPyV-KIPyV or BKV-JCV coinfection.

The viral loads in virus-positive samples were semiquantified by sample DNA titrations prior to duplicate PCR (figure 1). Approximate viral loads were determined by calculating the dilution at which a 50% PCR positivity would be expected by using the Reed-Münch formula and multiplying this result by 15 genome copies per 10^6 cells. Three samples previously identified as

<p>| Table 2. Determination of sensitivity of screening assays for polyomavirus detection. |
|---------------------------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Target virus</th>
<th>DNA copies added per reaction</th>
<th>Dilution corresponding to 50% PCR positivity, copies/μL¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>WUPyV</td>
<td>4/4 4/4 4/8 1/8</td>
<td>1</td>
</tr>
<tr>
<td>KIPyV</td>
<td>4/4 2/4 1/4 1/8</td>
<td>10</td>
</tr>
<tr>
<td>BKV</td>
<td>4/4 4/4 4/8 2/8</td>
<td>1</td>
</tr>
<tr>
<td>JCV</td>
<td>4/4 4/4 6/8 2/8</td>
<td>0.316</td>
</tr>
</tbody>
</table>

¹ Calculated by use of the Reed-Münch formula (see Methods).
Virus positive were found to be negative in the repeated high-concentration (0.5 μg) PCRs and were marked as falling below the limit of detection for reproducible detection in a single screening reaction and considered “negative or low” in further analysis. When immunosuppressed individuals were compared with control subjects, immunosuppressed individuals showed a higher frequency of polyomavirus detection, and higher viral titers were often seen in immunosuppressed individuals, particularly MSM with AIDS. The detection frequencies for BKV and WUPyV, as well as for combined WUPyV-KIPyV and BKV-JCV and total polyomavirus groups were significantly increased among immunosuppressed individuals (table 3).

**Polyomavirus detection in respiratory tract samples.** A total of 727 respiratory tract samples from 499 individuals were screened by use of MCPyV PCR and the combined BKV-JCV PCR on pools of 10 specimens. Initially reactive pools were split into individual components, yielding a total of 6 virus-positive samples, each of which was obtained from a different individual; there were 5 samples positive for BKV-JCV and 1 positive for MCPyV (1.0% and 0.2% study subject prevalences, respectively). Sequencing the amplicons with positive PCR results for combined BKV-JCV PCR revealed that all were JCV positive and were identical in the amplified region to published JCV sequences, except for a single sequence with 2 point mutations. Of the 5 individuals with samples positive for JCV, 4 were immunocompromised, and these samples may represent reactivated virus rather than primary infection. A previous screening of this respiratory tract sample cohort for WUPyV and KIPyV found 8 samples positive for each of these viruses in the subset analyzed in the current study, each from a single individual, with prevalences for both viruses of 1.6% [29].

**Analysis of TCR sequences.** TCR sequences from WUPyV-positive and KIPyV-positive autopsy samples were amplified and compared with those found in respiratory tract samples from subjects who were not immunosuppressed. The latter should represent the nonmutated genotype of wild-type, horizontally transmitted viruses. Mutations, including substitutions,

![Figure 1. Approximate viral titers calculated from endpoint dilutions of DNA extracted from autopsy samples of lymphoid tissue. Each detected virus is represented by a single point; matching symbols (+, *, or #) are assigned to the multiple viruses detected in each of 3 individuals in whom >1 virus was detected. Dashed line, limit of detection for reproducible detection in a single screening reaction. Three samples previously identified as virus positive were found to be negative in the repeated high-concentration (0.5 μg) PCRs and are shown as falling below this limit. The no. of individuals per group and the frequency with which any of the 4 polyomaviruses were detected are listed below the group name. BKV, BK polyomavirus; IDU, injection drug user; JCV, polyomavirus virus; KIPyV, KI polyomavirus; MSM, men who had had sex with men; WUPyV, WU polyomavirus.](image)

**Table 3. Polyomavirus detection in autopsy tissue samples from immunosuppressed individuals and control subjects.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control subjects</th>
<th>Immunosuppressed subjects</th>
<th>$P^a$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKV</td>
<td>0/55 (0)</td>
<td>8/42 (19)</td>
<td>&lt;.001</td>
<td>14.4</td>
</tr>
<tr>
<td>JCV</td>
<td>1/55 (1.8)</td>
<td>3/42 (7.1)</td>
<td>.19</td>
<td>1.69</td>
</tr>
<tr>
<td>WUPyV</td>
<td>0/55 (0)</td>
<td>3/42 (7.1)</td>
<td>.045</td>
<td>4.0</td>
</tr>
<tr>
<td>KIPyV</td>
<td>1/55 (1.8)</td>
<td>3/42 (7.1)</td>
<td>.085</td>
<td>2.96</td>
</tr>
<tr>
<td>MCPyV</td>
<td>1/55 (1.8)</td>
<td>0/42 (0)</td>
<td>.99</td>
<td>NA</td>
</tr>
<tr>
<td>BKV-JCV</td>
<td>1/55 (1.8)</td>
<td>11/42 (26.2)</td>
<td>&lt;.001</td>
<td>15</td>
</tr>
<tr>
<td>WUPyV-KIPyV</td>
<td>1/55 (1.8)</td>
<td>6/42 (14.3)</td>
<td>.01</td>
<td>6.6</td>
</tr>
<tr>
<td>Total$^b$</td>
<td>2/55 (3.6)</td>
<td>15/42 (35.7)</td>
<td>&lt;.001</td>
<td>21.6</td>
</tr>
</tbody>
</table>

$^a$ Data are proportion (%) of subjects. Bold type indicates significant $P$ values (<.05); the probability calculation for MCPyV used Fisher's exact test in the absence of viral load data. NA, not available.

$^b$ Excluding MCPyV.
deletions, or insertions, were seen at 10 sites in the WUPyV TCR sequences. The 4 WUPyV TCR sequences from viruses recovered from autopsy samples contained a relatively large number of mutations, with 4–9 mutations per sequence at these 10 identified polymorphic sites (figure 2). The majority of mutations, including a 10-nucleotide deletion, were seen in ≥2 of the autopsy samples. This frequency was significantly higher than that of the control samples (1 mutation in 6 sequences) \((P < .001)\).

Mutations were less common in the KIPyV TCR sequences analyzed; however, of the 8 identified polymorphic sites, sequences from the immunosuppressed study subjects showed a mean of 3 substitutions compared to only 0.24 in sequences from respiratory tract sample controls \((P < .001)\).

**DISCUSSION**

This study has demonstrated a significant correlation between AIDS-related immunosuppression and reactivation of the BKV-JCV and WUPyV-KIPyV groups of human polyomaviruses but not the more distantly related MCPyV. These findings support the hypothesis of similar persistence and similar underlying immune control mechanisms that sup-

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**Figure 2.** Transcriptional control region mutations from viruses detected in autopsy tissue samples and respiratory tract samples. Central line, published sequences for each of the 4 viruses (EF444549–444554 and EF127906–127908 for WUPyV and KIPyV, respectively) with appropriate nucleotide numbering. Ovals and dashes along this line, insertion or deletion areas and nucleotides, respectively, variable among the published sequences listed. Boxes along this line, mutations found in sequences from this study; each horizontal row represents a sequence derived from an individual patient belonging to the group listed at left. Filled boxes, sequences in which a given mutation is present. ATG, translational initiation codon; control, control subject; IDU, injection drug user; ori, origin of viral replication; MSM, men who had had sex with men; VP2, viral protein 2.
press the replication of each of the human polyomaviruses, which are disrupted in AIDS.

It is more difficult to interpret the significance of the results obtained for MCPyV; the infrequency with which it was detected may result from an absence of immune-related reactivation and, most problematic, a lack of knowledge about its normal epidemiology and prevalence. It is known that infections with BKV and JCV occur early in life and affect the majority of individuals, as well as that on the basis of respiratory tract sample testing [26, 28, 29], it seems likely that WUPyV and KIPyV are equally prevalent, yet we cannot assume that past and/or persistent infection with MCPyV occurs with the same high frequency. It is possible that it is detected relatively infrequently in autopsy samples because the study subjects have not been exposed to the virus in the past.

To our knowledge, this is the first study to demonstrate a correlation between immunosuppression and the reactivation of the newly discovered WU and KI polyomaviruses. Previous studies investigating the reactivation of BKV and JCV in immunosuppressed individuals have been performed but have generally focused on detection in the brain or urine, rather than in lymphoid tissue, and have often been undertaken in the context of known clinical associations (PML and PAN in the cases of JCV and BKV, respectively) [22, 34–38]. In our investigation of individuals without polyomavirus-related diseases, we have shown that BKV and JCV were detected far more frequently in the lymphoid tissue of immunosuppressed individuals occurred than in that of immunocompetent individuals.

TCR sequences from the polyomavirus-infected study subjects were identified to investigate possible correlations between mutations in this region and reactivation. The increased frequency with which rearranged TCR sequences are observed in virus recovered from patients with PML is well established [15–23], and a similarly high frequency of mutated BKV TCR sequences has been observed in renal biopsy samples obtained from PAN patients [24, 25]. However, the role of polyomavirus TCR rearrangements as a factor contributing to viral reactivation, as opposed to a secondary effect arising from reactivation, is still unclear.

Consistent with these observations for BKV and JCV, the frequency of mutations in the TCR was much higher than in the coding region amplified by the screening PCR and also in large-product PCR amplicons sequenced from WUPyV-KIPyV positive samples obtained from immunosuppressed patients (data not shown). However, our study design cannot exclude the possible influence on TCR diversity of different durations of infection and differences in tissue origin between study subjects who provided respiratory tract and autopsy samples. Furthermore, within the immunosuppressed study group, no clear correlations were found between mutation frequency and viral titer or clinical outcomes. Finally, the observed mutations in WUPyV and KIPyV avoided likely transcriptional promoters, findings that contrast with observations of frequent mutations in these sites, particularly duplication and/or insertion in JCV-associated cases of PML [21, 39].

Although the study design precluded a detailed examination of the possible consequences of reactivation of WUPyV and KIPyV (a task made particularly difficult by the multisystemic nature of AIDS-related disease), the findings warrant further investigations of the viruses’ replication in patients experiencing other immunodeficient states and their potential harmful effects. Differences between the newly discovered polyomaviruses and BKV and JCV with respect to their cellular tropisms, and the potential for polyomaviruses to transform cells and induce malignant change, greatly increase the range of human diseases that might be caused by members of this virus family.

**Acknowledgments**

We would like to acknowledge the assistance of Frances Carnie in providing access to the autopsy samples for analysis.

**References**


