Journal of Pathology
J Pathol 2015; **237:** 379–389
Published online 19 August 2015 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/path.4584

ORIGINAL PAPER

The oncogenic potential of BK-polyomavirus is linked to viral integration into the human genome

Daniel J Kenan,[1] Piotr A Mieczkowski,[2] Raquel Burger-Calderon,[3] Harsharan K Singh[1] and Volker Nickeleit[1]*

[1] *Division of Nephropathology, Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA*
[2] *Department of Genetics, University of North Carolina, Chapel Hill, NC, USA*
[3] *School of Dentistry, University of North Carolina, Chapel Hill, NC, USA*

**Correspondence to: V Nickeleit, UNC Division of Nephropathology, University of North Carolina Medical School, Department of Pathology and Laboratory Medicine, Brinkhous-Bullitt Building, Room 409, Campus Box 7525, Chapel Hill, NC 27599–7525, USA. E-mail: volker_nickeleit@med.unc.edu*

Abstract

It has been suggested that BK-polyomavirus is linked to oncogenesis via high expression levels of large T-antigen in some urothelial neoplasms arising following kidney transplantation. However, a causal association between BK-polyomavirus, large T-antigen expression and oncogenesis has never been demonstrated in humans. Here we describe an investigation using high-throughput sequencing of tumour DNA obtained from an urothelial carcinoma arising in a renal allograft. We show that a novel BK-polyomavirus strain, named CH-1, is integrated into exon 26 of the myosin-binding protein C1 gene (*MYBPC1*) on chromosome 12 in tumour cells but not in normal renal cells. Integration of the BK-polyomavirus results in a number of discrete alterations in viral gene expression, including: (a) disruption of VP1 protein expression and robust expression of large T-antigen; (b) preclusion of viral replication; and (c) deletions in the non-coding control region (NCCR), with presumed alterations in promoter feedback loops. Viral integration disrupts one *MYBPC1* gene copy and likely alters its expression. Circular episomal BK-polyomavirus gene sequences are not found, and the renal allograft shows no productive polyomavirus infection or polyomavirus nephropathy. These findings support the hypothesis that integration of polyomaviruses is essential to tumourigenesis. It is likely that dysregulation of large T-antigen, with persistent over-expression in non-lytic cells, promotes cell growth, genetic instability and neoplastic transformation.

Keywords: kidney transplantation; polyomavirus; polyomavirus nephropathy; virus integration; BK virus; cell transformation, neoplastic; large T Antigen; oncogenesis; carcinoma

Received 5 April 2015; Revised 22 June 2015; Accepted 3 July 2015

No conflicts of interest were declared.

Introduction

Renal transplant patients exhibit an increased incidence of viral infections, due to the immunosuppressive therapy that is required [1]. Also related to dysfunctional immune surveillance is an increased incidence of malignancy, with renal transplant patients exhibiting approximately 4.5-fold increased incidence of cancer across all sites surveyed [2], and a three- to four-fold increased incidence of urothelial carcinoma [3,4]. Although it is often difficult to prove a viral cause of oncogenesis, there is evidence that some tumours are induced by viruses. Merkel cell carcinoma, promoted by Merkel polyomavirus, is one such example [5].

Viral oncogenesis is associated with integration of viral sequences into the genome of infected cells [6]. Integration can lead to a number of different events, including both loss of function and gain of function in genes regulating the cell cycle. Viral promoter activation of flanking human genomic sequences, or expression of viral oncogenes, can induce neoplastic growth, as can acquisition of activating mutations within viral growth-promoting genes.

Several recent reports suggest an association between polyomavirus infection and urothelial or collecting duct carcinoma in renal allograft recipients, affecting either the transplanted organ or the genito-urinary tract of the recipient (summarized in Table 1). Such tumours are characteristically of high grade, express polyomavirus large T-antigen and show no evidence of productive polyomavirus infection with viral replication [7]. Despite abundant circumstantial evidence that polyomavirus is oncogenic in these cases, proof of polyomavirus oncogenesis in genito-urinary tumours remains incomplete.

BK-polyomavirus most commonly causes a productive viral infection in renal allografts [8–13]. BK and its close relative, JC-polyomavirus, are nearly ubiquitous in

Table 1. Large T antigen-positive urological malignancies in transplant recipients as reported in the literature

Site	Histology*	Year published	Reference
Allograft kidney	Adenocarcinoma, poorly differentiated	2004	[38]
Allograft kidney	RCC, poorly differentiated	2007	[39]
Allograft kidney	Collecting duct carcinoma	2008	[40]
Allograft kidney	TCC, high grade	2009	[41]
Allograft kidney	RCC, poorly differentiated	2012	[42]
Allograft kidney	TCC, high grade, micropapillary variant	2013	[43]
Allograft kidney	TCC, papillary, nested and sarcomatoid variants	2013	[44]
Bladder	UC, high grade	2002	[45]
Bladder	UC, high grade	2008	[46]
Bladder	UC, micropapillary	2009	[47]
Bladder	Adenocarcinoma	2010	[48]
Bladder	UC, high grade, nested and sarcomatoid variants	2013	[49]
Bladder	TCC grade 3	2013	[50]
Bladder	UC, high grade with glandular differentiation	2013	[51]
Bladder	UC, high grade with micropapillary differentiation	2013	[51]
Bladder	UC, micropapillary	2013	[28]
Bladder	UC, micropapillary	2013	[28]
Bladder	UC, micropapillary	2015	[54]
Native kidney	Collecting duct carcinoma	2013	[52]
Ureter	UC, high grade	2014	[53]

*Diagnostic terminology as reported: RCC, renal cell carcinoma; TCC, transitional cell carcinoma; UC, urothelial carcinoma.

human populations, with seroconversion rates approaching 90% by mid-childhood [14,15]. Primary infection with polyomaviruses is likely via the respiratory route, often leading to dissemination and establishment of a latent infection in the urinary tract, especially renal tubular cells and urothelium. Periodically, polyomavirus can reactivate and undergo transient, self-limiting and clinically insignificant cycles of replication, leading to shedding of virus in the urine and occasionally transient episodes of viraemia. Reactivation is more common in the setting of immunosuppression, and in a minority of patients can lead to the development of a productive intrarenal polyomavirus infection with organ injury, termed polyomavirus nephropathy [16,17]. In the renal transplant population, polyomavirus nephropathy has an incidence of approximately 4% [8,9,11]. In contrast, neoplasms associated with polyomaviruses, in both immunocompetent and immunosuppressed patients, are exceptionally rare.

Here we describe a patient presenting with a high-grade urothelial carcinoma arising in a renal allograft. We show for the first time in a renal allograft tumour that BK-polyomavirus has integrated into human chromosomal DNA with expression of large T-antigen but without expression of late genes and lacking viral replication. The molecular details that emerge from deep sequencing of the tumour suggest several possible mechanisms of virally mediated oncogenesis, which are discussed.

Materials and methods

Ethics

This study was performed in accordance with the principles expressed in the Helsinki Declaration (1975; as revised 1983) and was approved by the Institutional Review Board (IRB) of the University of North Carolina (Protocol No. 12–0038).

Histopathology

Histological work-up and standard diagnostic immunohistochemistry for the characterization of the neoplasm followed routine pathology procedures.

Antibodies

Immunohistochemical detection of polyomavirus-associated antigens was performed on formalin-fixed, paraffin-embedded (FFPE) tissue, with mouse monoclonal antibodies directed against the SV40 large T-antigen (Clone 416; Calbiochem, San Diego, CA, USA), the VP1 capsid protein (clone 597) and the JC-polyomavirus large T-antigen (clone Pab 416) [18], following standard protocols published previously [13,17,19,20]. Transmission electron microscopy studies on glutaraldehyde-fixed tissue were performed according to standard protocols.

Laser capture microdissection (LCM)

Tissue sections (8 µm) were cut from FFPE blocks onto Zeiss Membrane Slides (1.0 PEN, cat. no. 415190-9041-000, Carl Zeiss Microscopy, Thornwood, NY, USA). The Ambion LCM Staining Kit (Life Sciences, Grand Island, NY, USA) was employed according to the manufacturer's instructions, with modifications as reported by Cianciolo *et al.* [21]. Tissue sections were cleared by incubations in xylene, then briefly air dried before LCM. The slides were stored at 4 °C when not on the LCM stage.

All LCM was performed using a Zeiss PALM Microbeam laser microdissection system (Carl Zeiss Microscopy, Thornwood, NY) with PALMRobo v4.6 software. Tissue was captured into AdhesiveCaps

(#415190-9201-000, Carl Zeiss Microscopy, Thornwood, NY), and DNA isolated from captured tissue using the Ambion RecoverAll system as described below.

Approximately 100 cross-sections from normal kidney tubules, distant from the neoplasm, were collected by LCM. Each cross-section contained, on average, approximately 18 (range 6–37) nuclei for a total recovery of approximately 1800 nuclear equivalents/microdissected sample. Approximately equivalent numbers of tumour nuclei were also microdissected from areas of both invasive and *in situ* urothelial carcinoma.

Real-time PCR

Load levels of BK- and JC-polyomaviruses were determined by real-time TaqMan PCR assay, using the ABI PRISM 7900HT Sequence Detection System (Foster City, CA, USA), with well-characterized probes and primers specific for BK- and JC-polyomaviruses [10,17,18]. Real-time detection of PCR products was achieved using a fluorescence hydrolysis (TaqMan) probe. Primers and probes were purchased from TIB Molbiol LLC (Adelphia, NJ, USA). The primer and probe sequences for large T-antigen gene detection were as follows: BK-virus, forward 5′-AGCA GGCAAGGGTTCTATTACTAAAT-3′, reverse 5′-GAA GCAACAGCAGATTCTCAACA-3′; BK-virus TaqMan probe, 5′-6-FAM-AAGACCCTAAAGACTTTCC CTCTGATCTACACCAGTTT-TAMRA-3′; JC-virus, forward 5′-TTAGTGGTATACACAGCAAAAGAAG CA-3′, reverse 5′-AAAACACAGGATCCCAACACTC TAC-3′; and JC virus TaqMan probe, 5′-6-FAM-TC CTGTAGATCTGCATGCA-MGB-NFQ-3′. The primer and probe sequences for BK *VP1* gene detection were as follows: BK-virus, forward 5′-GCAGCTCCCAAAAA GCCAAA-3′, reverse 5′-CTGGGTTTAGGAAGCATT CTA-3′; BK-virus TaqMan probe, 5′-6-FAM-ACCCG TGCAAGTGCCAAAACTACTAATAAAAGG-TAM RA-3′ (6-FAM, 6-fluorescein amidite; TAMRA, tetramethylrhodamine; MGB-NFQ, dihydrocyclopyrroloindole tripeptide minor groove binder non-fluorescent quencher).

Quantitative linearity of the TaqMan assay exhibited a dynamic linear range of $250–2.5 \pm 10^{10}$ BKV copies/ml sample (data not shown).

DNA isolation from tissue

Using frozen tissue, total cellular nucleic acids were isolated from frozen tumour tissue samples using the Ambion MELT Total Nucleic Acid Isolation System (Life Sciences, Grand Island, NY, USA). Tissue sections were cut on a cryostat at 10 µm thickness and were processed according to the manufacturer's alternative instructions for DNA isolation, including an RNase A incubation step. Isolated DNA was evaluated using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and was determined to have a concentration of 166 ng/µl.

Using FFPE tissue, total cellular nucleic acids were additionally isolated from laser capture-microdissected samples of FFPE tissue, using the Ambion RecoverAll Total Nucleic Acid Isolation Kit (Life Sciences). Microdissected samples were processed according to the manufacturer's alternative instructions for DNA isolation, including an RNase A incubation step.

Deep sequencing and sequence analysis

Isolated frozen tumour DNA was fragmented by ultrasonication and libraries prepared prior to high-throughput sequencing, using an Illumina HiSeq Sequencing System (Illumina, San Diego, CA, USA). Approximately 166 million genomic DNA fragments were sequenced. The fragments were assembled using the CLC Genomics Workbench 6.5.1 (CLC bio, Boston, MA, USA), with mappings onto the human genome and the NCBI database of all viral genomes (http://www.ncbi.nlm.nih.gov/genome/viruses/).

Of the fragments, 93% mapped onto human chromosomes and BK-polyomavirus sequences, with a coverage of approximately 10-fold, indicating that each nucleotide in the haploid genome was sequenced 10 times on average. True coverage varies from position to position, due primarily to significant aneuploidy in the tumour. The remaining 7% of fragments that did not map to human and polyomavirus sequences represent mostly repetitive human sequences that are not mapped in the database. The only viruses that mapped with the tumour DNA dataset were BK-polyomavirus and PhIX174, which is a sequencing control.

Confirmatory PCR reactions were performed using primers specific for *MYBPC1* and BK-polyomavirus sequences located on both sides of the breakpoints of viral integration into the tumour genome. The *MYBPC1* primer sequences used were: 5′-GACTG GGAAAGAAAGTGACCTTGC-3′, 5′-CCTTCTGAAT GGTATAGCCTGTG-3′ and 5′-GCTGGGGTTTAAGA CCAGCTCTG-3′. The BK-polyomavirus primer sequences used were: 5′-TGCTGATATTTGTGGCCT GTTTACTA-3′, 5′-CATGTGACCAACACAGCTAC C-3′, 5′-CCCAACAGCCCAGTC-3′, 5′-GAAAATCCT TACAAAACTCAGAG-3′ and 5′-CTCCACATAGAC ATAGAGTTTCTGC-3′.

For BK genotype analysis, a multiple sequence alignment was calculated from *VP1* gene DNA sequences obtained from BK-polyomavirus isolates, representing the major genotype subgroups as defined previously [22–24]. The specific accession numbers used were as follows: BK-polyomavirus isolate WW (canonical), GenBank No. AB211371.1, genotype group VI; BK-polyomavirus isolate Dik, GenBank No. AB211369.1, genotype group VI; BK-polyomavirus isolate PittNP1, GenBank No. DQ989813.1, genotype group VI; BK-polyomavirus isolate PittVR8, GenBank No. DQ989806.1, genotype group VI; BK-polyomavirus isolate PittVM3, GenBank No. DQ989798.1, genotype group V; BK-polyomavirus isolate PittVR2, GenBank No. DQ989796.1, genotype

group V; BK-polyomavirus isolate PittNP5, GenBank No. DQ989804.1, genotype group Ia; BK-polyomavirus isolate PittVR4, GenBank No. DQ989812.1, genotype group Ia; BK-polyomavirus isolate UT, GenBank No. DQ305492.1, genotype group Ia; BK-polyomavirus isolate PittNP4, GenBank No. DQ989802.1, genotype group 1a; BK-polyomavirus isolate THK-9a, GenBank No. AB217921.1, genotype group 1c; BK-polyomavirus isolate TW-8a, GenBank No. AB217920.1, genotype group 1c; BK-polyomavirus AS, GenBank No. M23122.1, genotype group III; BK-polyomavirus isolate TW-3a, GenBank No. AB217919.1, genotype group IV; JC-polyomavirus, NCBI Reference Sequence NC_001699.1. VP1 DNA sequences were aligned using ClustalW2 via the European Bioinformatics Institute (EMBL–EBI) [25]. The neighbour-joining phylogenetic tree calculated by ClustalW2 was then visualized as a radial tree, using FigTree v 1.4.2 (see Figure 4; software developed and provided by Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh: http://tree.bio.ed.ac.uk/).

Results

Clinical history and presentation

A 54 year-old African-American male presented to his local emergency department in 2012 with severe suprapubic and left flank pain. In 2003 he had received a renal transplant of cadaveric origin for hypertensive nephropathy. His post-transplant course was uneventful, with a baseline serum creatinine of 1.5–2.1 mg/dl and no clinical evidence of polyomavirus nephropathy or episodes of rejection. His recent maintenance immunosuppression regimen included mycophenolate mofetil (250 mg twice daily), tacrolimus (3 mg three times daily) and methylprednisolone (6 mg once daily).

On presentation to the emergency department, the patient showed acute allograft failure (serum creatinine 4.3 mg/dl) and an active urine sediment (3+ haematuria, 2+ proteinuria). Imaging studies demonstrated severe hydronephrosis of the transplanted kidney and a poorly defined heterogeneous mass in the renal pelvis. The diagnosis of a malignant neoplasm was confirmed by urine cytology showing markedly atypical urothelial cells. No polyomavirus inclusion-bearing decoy cells were seen. The patient underwent transplant nephrectomy.

Pathological findings

Gross examination of the explanted renal allograft demonstrated a completely excised white-tan tumour, 5.0 cm in greatest diameter, that was based in the renal pelvis, invaded deeply into the medulla and markedly narrowed the ureteral outlet (Figure 1). The majority of the renal pelvis revealed urothelial thickening and papillary nodularity suggestive of an *in situ* tumour component.

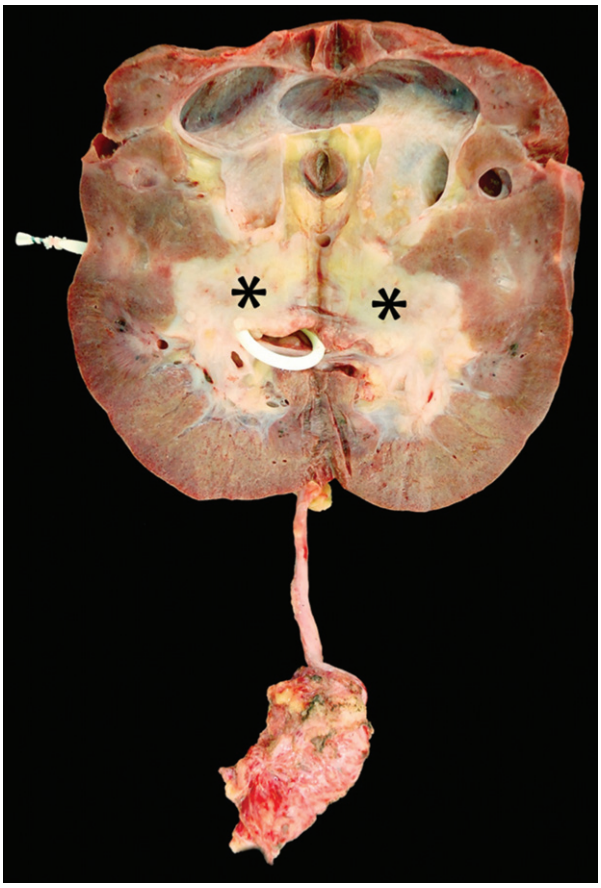

Figure 1. Allograft nephrectomy specimen, bivalved. The invasive tumour (*) infiltrates the renal medulla and extends into the inner cortex; the pelvis shows hydronephrosis; a nephrostomy drain is seen *in situ*; the ureter and a portion of the urinary bladder extend downwards.

Histomorphology and immunohistochemistry

Histological examination of the tumour revealed a high-grade malignant transitional cell carcinoma deeply invading the renal medulla and cortex (Figure 2A, B). Tumour cells showed moderate to severe nuclear pleomorphism and were arranged in solid nests or cords with focal glandular and papillary differentiation. Mucicarmine stain highlighted a few foci of intraluminal mucin (not shown). Many areas of the tumour displayed comedonecrosis. Tumour nests were surrounded by a dense desmoplastic stroma. Viral inclusion bodies were not present. The pelvic mucosa displayed areas of papillary urothelial carcinoma and flat urothelial carcinoma *in situ*. Adjacent to the tumour, the renal parenchyma was without diagnostic abnormalities, and there was no evidence of polyomavirus nephropathy.

Electron microscopy of tumour cells showed highly pleomorphic epithelial cell elements with vesicular and partially clumped chromatin. In many tumour nuclei, so-called 'nuclear granules' [7] were noted (Figure 3). Virus particles were not present.

By immunohistochemistry, the tumour displayed diffuse strong staining for GATA3 and diffuse staining for CK7 and CAM5.2, with a subset of tumour cells staining for P63 and CD10 (not shown). No diagnostic staining

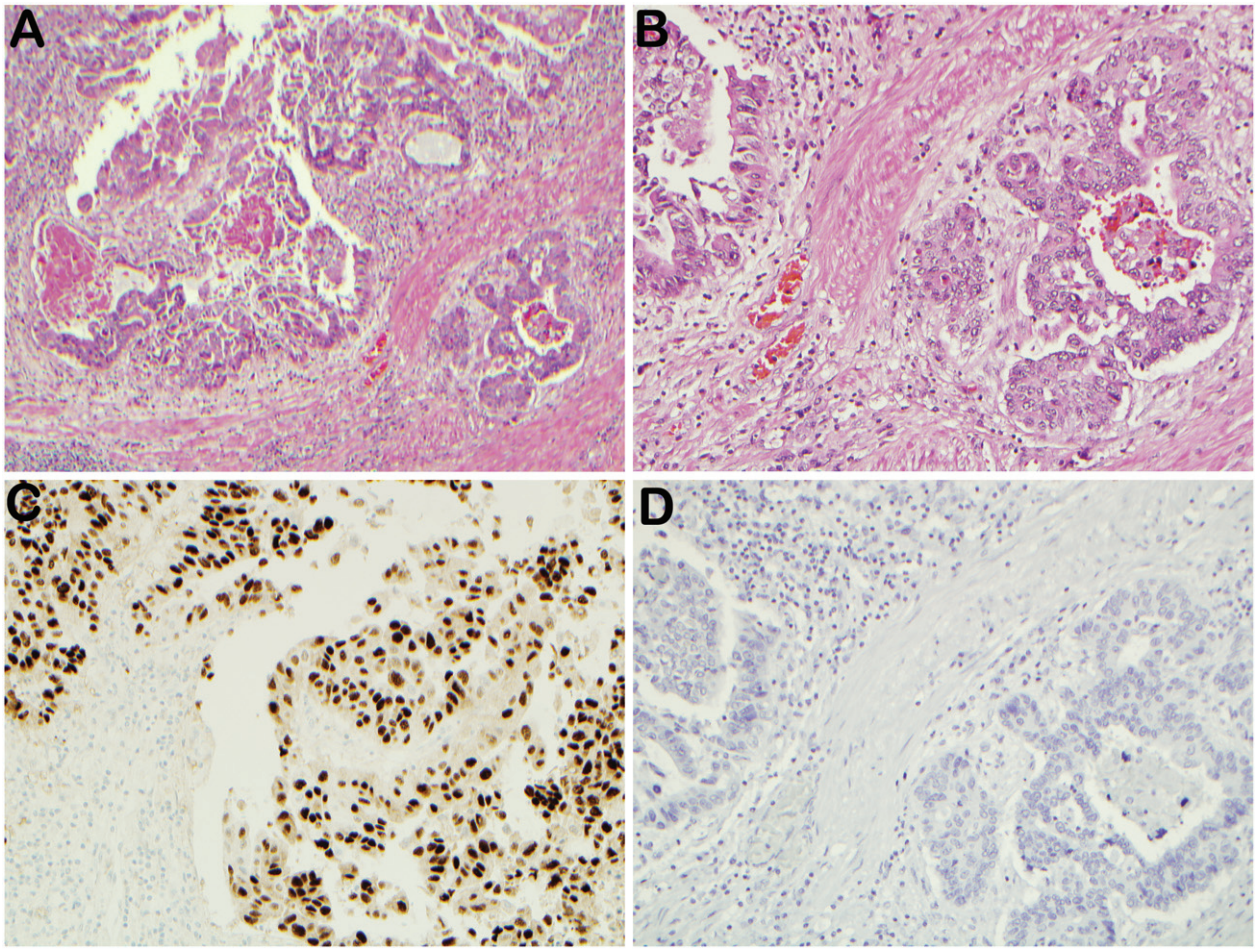

Figure 2. Histology of renal allograft tumour (FFPE tissue). (A) Top of image displays an *in situ* component and the bottom displays an invasive component; H&E stain; magnification = ×100. (B) The invasive carcinoma shows glandular differentiation with central foci of debris and moderate nuclear atypia; H&E stain; magnification = ×200. (C) Immunohistochemistry with an antibody against SV40 large T-antigen shows strong intranuclear staining; magnification = ×200. (D) Immunohistochemistry with an antibody against polyomavirus capsid protein VP1 shows no expression of VP1 in the tumour; magnification = ×200.

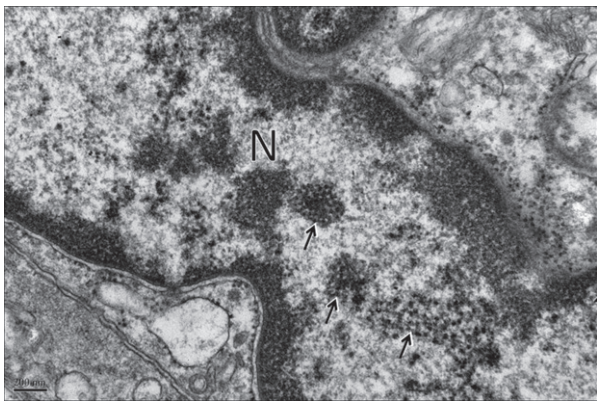

Figure 3. Urothelial carcinoma cell nucleus with so-called 'nuclear granules' (arrows; N, tumour cell nucleus). There is no evidence of viral particles. EM magnification = ×25 000.

was noted in tumour cells for PAX8, PAX2, Uroplakin III, CD20, S100, vimentin, racemase or RCC. CK5/6 highlighted only rare tumour cells. This staining pattern supported a transitional cell origin of the neoplasm. The tumour was classified as a poorly differentiated invasive transitional cell carcinoma and was staged as pT3 pNx, clinical stage III [26].

Special studies to detect polyomaviruses

Immunohistochemical staining was performed with antibodies against SV40 polyomavirus large T-antigen (the so-called 'pan-polyomavirus antigen'), JC-polyomavirus-specific large T-antigen, and polyomavirus VP1 capsid protein. Diffuse and strong staining limited to the nuclei of invasive and *in situ* tumour cells was only noted with the SV40 large T-antigen incubation (Figure 2C), indicating high levels of large T-antigen expression in tumour nuclei. No staining with antibodies specific for VP1 capsid protein (Figure 2D) or JC T-antigen (data not shown) was detected in tumour cells. Non-tumour cells did not stain with any antibodies (data not shown).

Microdissection and real-time PCR analysis

PCR studies on laser-microdissected FFPE tissue were performed in order to determine which elements within the allograft nephrectomy contained polyomavirus gene

sequences. Approximately comparable numbers of cells were microdissected from tumour tissue and from adjacent normal cortical renal tubules, and genomic DNA was extracted. Microdissected samples of neoplastic cells contained DNA sequences specific for segments of BK-polyomavirus large T-antigen and *VP1* genes. In tumour cells, no PCR signals were detected with primers specific for JC-polyomavirus sequences. No BK or JC-polyomavirus DNA sequences were detected in microdissected normal renal parenchyma (data not shown).

To assess whether full-length BK-polyomavirus sequences were present in the tumour, additional PCR reactions were performed on the microdissected DNA samples, using a set of primer sequences spanning multiple segments of the BK viral genome. DNA from tumour tissue yielded amplified bands of expected sizes, using primer sets spanning all regions of the polyomavirus genome, with the exception of PCR reactions using primer sets flanking the 3′ end of the *VP1* gene (data not shown). DNA from adjacent normal kidney tissue yielded no amplification with any primers specific for BK-polyomavirus. These findings led us to suspect that the circular viral genome was linearized and/or partially deleted in tumour cells, with a break point near the 3′ end of the *VP1* gene. Linearization would also suggest that the virus was integrated into the host genome. To further study the possibility of BK-polyomavirus integration into tumour genomic DNA, we performed high-throughput DNA sequencing from fresh-frozen tumour tissue.

Tumour genome sequencing

Genomic DNA sequencing revealed a novel integrated 5039 base pair (bp) BK-polyomavirus, with sequence phylogenetic features of a previously undescribed variant of genotype 1A (Figure 4; designated BK-polyomavirus isolate CH-1; GenBank Accession No. KP984526). The assembled sequence data showed that the virus was linearized with a breakpoint between codons 309 and 310 of the *VP1* gene. The virus was inserted into exon 26 of the human *MYBPC1* gene at position 102 069 082 on chromosome 12. Insertion occurred within codon 890 of the *MYBPC1* open reading frame (Figure 5). Every BK-polyomavirus sequence fragment that spanned the mapped breakpoint was interrupted between *VP1* codons 309 and 310 (the 5′ and 3′ termini in the linearized BK-polyomavirus isolate genome), thereby excluding the presence of circular episomal BK-polyomavirus in the tumour. No other chromosomal integration sites were detected.

Targeted PCR assays with specific BK-polyomavirus and *MYBPC1* primers on either side of the BK breakpoint and *MYBPC1* integration sites yielded bands of expected sizes with tumour DNA (data not shown; primer sets listed in Materials and methods). Control PCR assays with normal human DNA and BK-polyomavirus DNA templates did not show amplification products for the breakpoint PCR reactions.

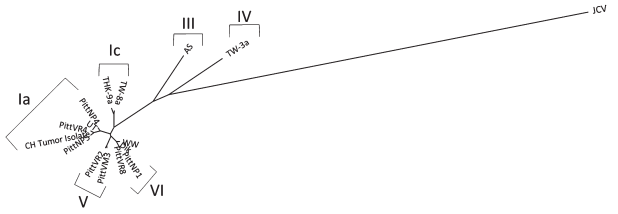

Figure 4. Radial phylogenetic tree of VP1 capsid protein DNA sequences using ClustalW2 and FigTree v 1.4.2, comparing the Chapel Hill tumour-associated polyomavirus-1 (termed CH-1) sequence to 14 polyomavirus sequences representing the major genotype groups of BK and JC-polyomaviruses, as indicated.

These additional PCR assays confirm that the joined human–viral sequences are not artefacts of library construction and that a single copy of BK-polyomavirus is integrated into the tumour genome, as determined by deep sequencing.

At the integration site within *MYBPC1*, approximately half of the human DNA sequencing reads showed normal uninterrupted *MYBPC1* sequences, suggesting that integration occurred in only one of the two copies of chromosome 12. The insertion resulted in fusion of the *VP1* coding sequences with *MYBPC1* exonic sequences, predicting a fusion protein consisting of the first 309 codons of VP1, followed by six out-of-frame codons in MYBPC1 exon 26, followed by a stop codon.

The viral genes encoding small and large T-antigens were intact. Mutation analysis revealed 41 single nucleotide differences within the large-T antigen gene, as compared to canonical BK-polyomavirus sequences (eg GenBank Accession No. AB211371.1), mainly representing silent sequence variations that do not change amino acid sequences (data not shown).

The non-coding control region (NCCR) of the integrated BK-polyomavirus demonstrated deletions of sequence blocks, as compared to archetypal BK-polyomavirus sequences (annotated in GenBank sequence of the BK-polyomavirus CH-1, Accession No. KP984526). As compared to the canonical so-called 'OPQRS' NCCR architecture (where each letter refers to a short DNA sequence motif that binds cellular proteins regulating viral gene expression and replication), the tumour variant BK-virus displayed an OPS block architecture with deletion of the Q and R blocks.

No other polyomaviruses of any strain, or any other viral gene sequences, were seen in the dataset, confirming that a single polyomavirus was associated with the tumour. The human tumour genomic DNA demonstrated marked aneuploidy that was suggested by variations in sequencing read densities and was confirmed by cytogenetic microarray analysis (data not shown).

Clinical follow-up

The patient tolerated the transplant nephrectomy well and was discharged on postoperative day 14. Immunosuppression was stopped and haemodialysis started. No aggressive interventions were initiated for treatment of the neoplasm of donor origin. The patient was last seen

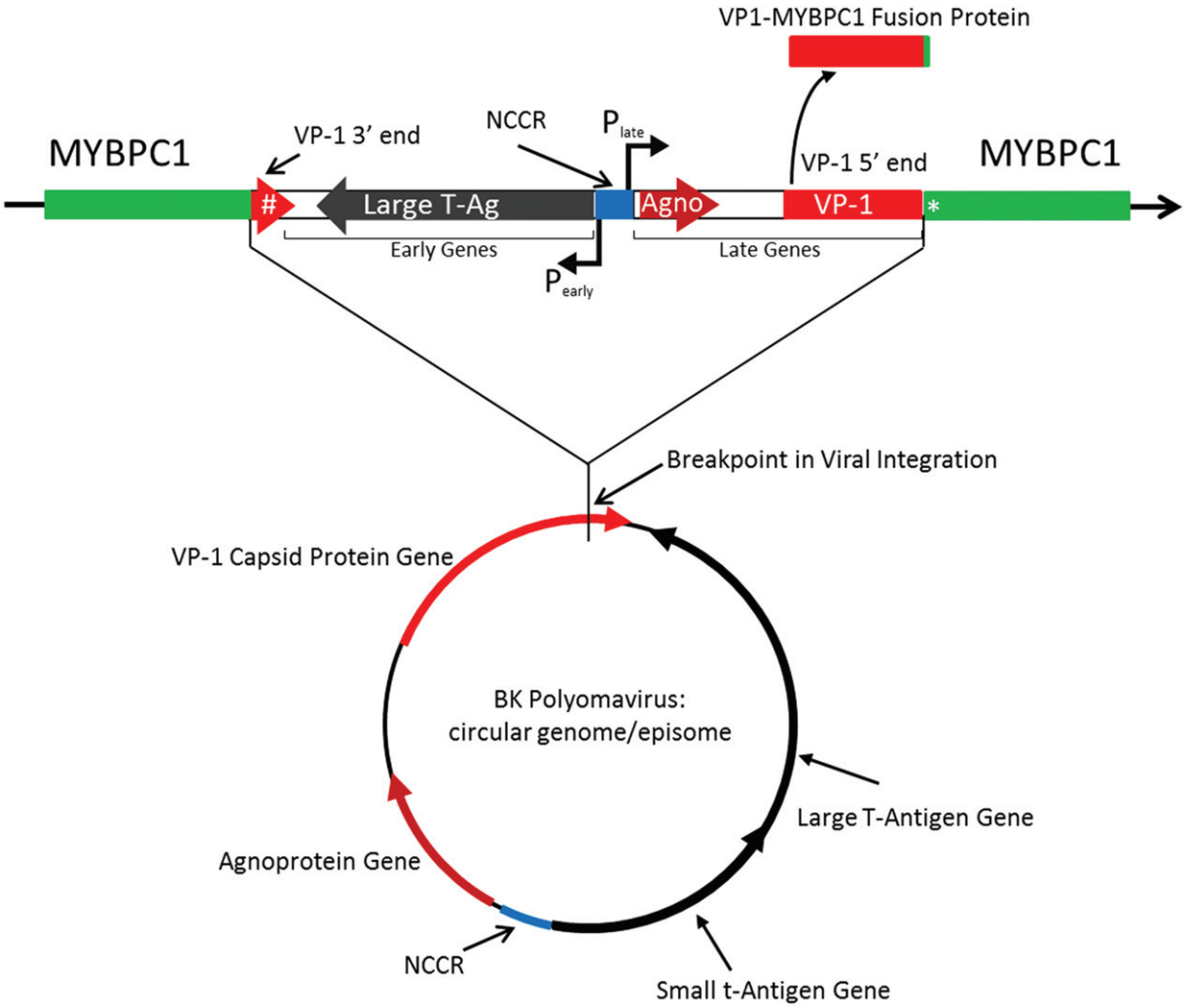

Figure 5. Schematic of the BKV integration site and molecular details of the virus–host junctions. Human chromosomal integration of the circular BK-polyomavirus genome results in linearization of the virus, with a breakpoint between codons 309 and 310 of the *VP1* gene, and integration into exon 26 of the *MYBVC1* gene at position 102 069 082 on chromosome 12. The coding sequences for capsid protein VP1 are indicated by a light red arrow in the circular genome. Linearization and integration disrupts the *VP1* gene, with the C-terminus encoded at the left end of the integrated fusion (indicated by # in the small red arrow) and the N-terminus encoded at the right end of the integrated fusion (larger red rectangle). Integration further results in the generation of a putative fusion protein between the N-terminus of VP1 and a short six-amino acid segment within out-of-frame MYBPC1 exonic sequences before a stop codon is encountered (indicated by * in the right green MYBPC1 exon). The blue NCCR (non-coding control region) in the virus is located towards the middle of the integrated virus, where both the early and late promoters (P-early and P-late, respectively) are expected to remain intact. Likewise, large T-antigen and small t-antigen sequences remain intact (black arrows), as well as the late gene product agnoprotein (dark red arrow). Transcription of the *MYBPC1* gene proceeds from left to right, and the direction of transcription of the early and late BK-polyomavirus gene products is indicated by the direction of the arrows representing these genes.

20 months after the nephrectomy and continued to do well, with no evidence of recurrent tumour or metastasis.

Discussion

Our study shows for the first time that a high-grade urothelial tumour arising in a renal allograft is associated with BK-polyomavirus fully integrated into the tumour genome at a single location. The integrated BK-polyomavirus differs from known strains and is named 'Chapel Hill tumour associated polyomavirus-1' (or CH-1 in brief). Linearization and integration of CH-1 disrupts the late viral gene encoding the major capsid protein VP1, while the viral gene for large T-antigen remains intact, with elevated protein expression. No other viruses or polyomavirus strains were found within the tumour. Taken together, these findings suggest that integration of BK-polyomavirus and sustained expression of large T-antigen are important steps in tumourigenesis in this patient, who lacks any clinical history of 'productive' polyomavirus nephropathy.

Integration appears to be an important feature of tumour-causing polyomaviruses. In recent studies of Merkel cell polyomavirus isolated from tumoural skin lesions, 19 of 27 isolates showed viral integration into the cancer genome [27]. It is possible that technical factors limited detection in the eight cases where integration

could not be demonstrated. In Merkel cell carcinoma, the integration site breakpoints are widely distributed in both the tumour genome and the viral genome, with no preferred sites. Only a minority of Merkel cell polyomavirus genome integration breakpoints occurred in the *VP1* gene [27]. In a study investigating BK-polyomavirus integration in bladder carcinoma, one of two cases was shown by nested PCR to have integrated BK-polyomavirus [28]. Technical limitations may have prevented detection of viral integration in the second patient. Integration of other polyomaviruses, including SV40, BK and JC, has also been reported in experimental cell culture models [29], but there is no compelling evidence that polyomaviruses other than Merkel cell polyomavirus commonly integrate into host genomes and cause disease in humans [30].

Viral integration could lead to oncogenesis through a number of mechanisms, including: (a) insertional inactivation; (b) insertional activation; (c) creation of fusion proteins with novel properties; (d) mutation of viral sequences; and (e) increased expression of viral growth-regulatory proteins. In the case reported here, insertional inactivation at the integration site could be caused by disruption of *MYBPC1* sequences. MYBPC1, also known as myosin binding protein C, slow type, is a structural protein located in the A-bands of vertebrate striated muscle [31]. Its deletion or mutation is associated with disorders of muscle contraction, including distal arthrogryposis and lethal congenital contracture syndrome [32]. There is no established oncogenic link with MYBPC1. Thus, insertional inactivation is unlikely to play a role in this tumour.

Insertional activation could be caused by fortuitous expression of cellular genes via juxtaposition of integrated viral promoters. The orientation of the integrated BK-polyomavirus is such that the early viral promoter is directed towards genes upstream of the *MYBPC1* gene on the non-coding strand, and the late promoter is directed towards genes downstream on the *MYBPC1*-coding strand (Figure 5). Analysis of known genes both upstream and downstream of *MYBPC1* reveals no likely candidates for oncogenic proteins that could be up-regulated. Thus, insertional activation is unlikely to play a role in this tumour.

Creation of a novel fusion protein is possible, given the fusion of DNA sequences of *VP1* and 'nonsense' out-of-frame sequences from an exon of *MYBPC1*. However, there is no evidence that the VP1 fusion protein is expressed, despite multiple attempts at detection by western blot analysis (data not shown). It is possible that the VP1 fusion protein is expressed but is too unstable to be detected. It is also possible that the late promoter is not activated due to rearrangements in the NCCR region (discussed below). Taken together, we infer that a gain of function from a novel fusion protein is unlikely to play a role in this tumour.

The integrated BK-polyomavirus isolate shows mutations, including deletion of the Q- and R-blocks within the NCCR region. We hypothesize that these changes may be important for oncogenesis. Viral replication and early and late promoter activity are controlled by NCCR blocks O, P and S, which are intact in our isolate [33]. Blocks Q and R, deleted in our isolate, contain binding sites for a number of transcriptional regulatory factors, including NF-1 (nuclear factor-1) and SP-1 (specificity protein-1) [33]. Thus, deletion of the Q- and R-blocks could alter viral promoter activity and could contribute to the increased expression of viral growth regulatory proteins, such as small t- and large T-antigens. Small t-antigen is known to bind to and perturb the function of the cellular Ser/Thr phosphatase PP2A [34]. This perturbation has been shown to work in concert with activities of the large T-antigen to promote cell transformation in cell culture [35].

In productive polyomavirus infections, such as polyomavirus nephropathy, the circular episomal virus undergoes phased gene expression in permissive host cells, first with early promoter expression of the T antigens that drive viral DNA replication [36]. After viral DNA replication begins, the late promoter becomes more active and the late gene products contribute to down-regulation of large T-antigen expression by a variety of mechanisms. One mechanism of T antigen down-regulation is through late promoter-directed expression of a viral microRNA (miRNA) that is complementary to the 3′ coding end of the T antigen mRNA [37]. Such down-regulation is not possible in the integrated BK-virus, due to the fact that integration separates the late promoter from the region encoding BK miRNA. This interruption of miRNA-mediated down-regulation likely contributes to the high level of expression of large T-antigen.

Our data suggest that viral integration impedes viral replication within the tumour. Although large T-antigen expression is up-regulated and the NCCR O-block is intact, both supporting viral DNA replication, any viral replication that is initiated would proceed bidirectionally from the origin of replication into human sequences flanking the site of integration. Due to linearization, BK-VP1 sequences cannot be completely replicated and transcribed to produce daughter virions, and these were undetectable in the tumour.

The findings of this study lead us to propose a mechanism of oncogenesis in this BKV-associated tumour, illustrated in Figure 6. In general, polyomavirus infection can either lead to a productive infection, with viral replication, host cell lysis and release of mature daughter virions, or it can lead to a latent infection. Both types of infection involve a number of virus–host interactions, including promoter activation from the NCCR, increased expression of large T-antigen, small t-antigen, inhibition of p53 and Rb growth regulatory proteins by binding of large T-antigen, and perturbation of PP2A function by small t-antigen binding. Since cells with productive polyomavirus infections are lysed, neoplastic growth is not possible.

In the case of Merkel cell carcinoma, integration of the MC polyomavirus has been described as a 'biological accident' [5], in some cases with dire consequences. The integration of BK-polyomavirus can be thought

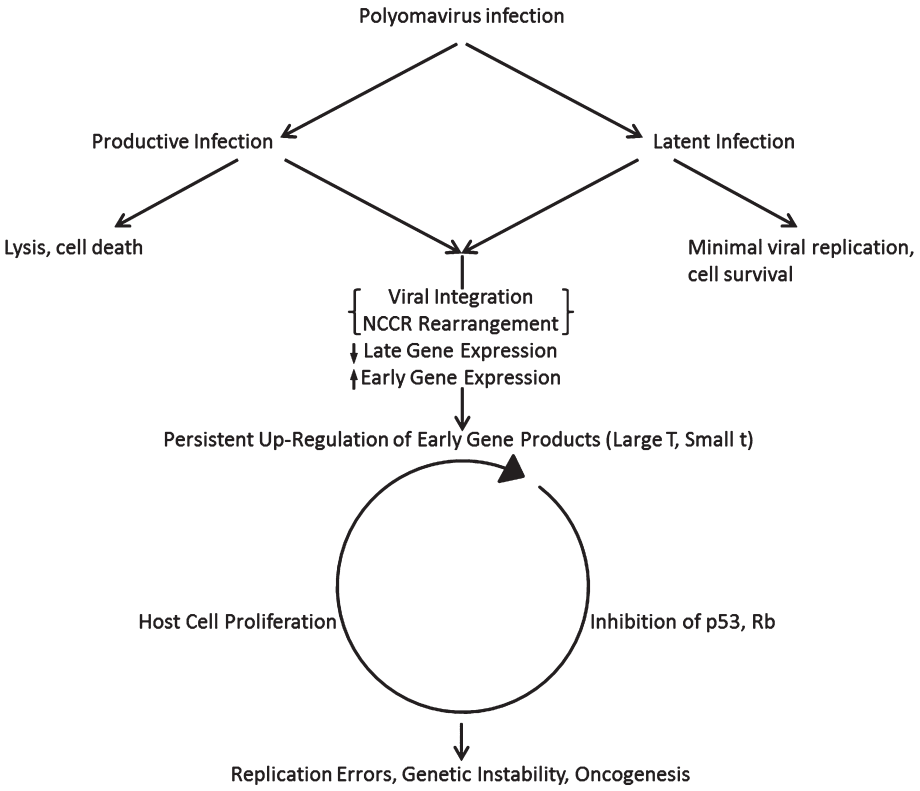

Figure 6. Proposed mechanism of oncogenesis by integration of BK-polyomavirus. Viral infection leads to either productive infection, resulting in cell death, or latency, resulting in cell survival but with minimal viral replication. Either mechanism may lead to sporadic viral integration through unknown mechanisms. Once integrated, the virus is no longer replication-competent and cannot produce new virions. Integration with linearization may occur before or after rearrangement of the NCCR, which can influence BK-polyomavirus gene expression and transcription of the large T-antigen gene sequence. Large T-antigen or an active fragment thereof is typically expressed and up-regulated via alteration of the NCCR region and attenuation of negative feedback loops from late gene products. Dysregulated large T-antigen expression is known to inhibit cellular p53 and Rb function and can thereby promote oncogenesis.

of in similar terms. If the early promoter is active and the large T-antigen gene is intact, as is the case in our isolate, then large-T antigen protein expression ensues. In normal circular polyomaviruses early gene expression is transient, due to down-regulation by various mechanisms, including miRNA expression. However, in the case of rearranged integrated viruses, miRNA may not be expressed due to uncoupling of the late promoter. If large T- and small t-antigen expression is not down-regulated, then sustained expression can drive host cell proliferation, as well as sustained perturbation of p53, Rb and PP2A, ultimately leading to a breakdown in surveillance mechanisms and allowing variant, genetically unstable, neoplastic cell populations to emerge. Given the prevalence of BK-polyomavirus in the genitourinary system, especially in the transplant setting, rare biological accidents of viral DNA integration may account for oncogenesis in kidney transplant recipients.

Acknowledgements

We thank Vincent Moylan for skillful dissection and photography of the nephrectomy specimen, Scott Smith MD for information on the diagnosis of this tumour, and Bruna Brylawski and Lauraine Rivier PhD for expert technical assistance with the immunohistochemistry and PCR assays. We acknowledge the High-throughput sequencing Facility at the University of North Carolina for generating the sequence data, Robert Bagnell and the Microscopy Service Laboratory at the University of North Carolina for help with the laser microdissection, and the Division of Nephropathology, Department of Pathology and Laboratory Medicine, at the University of North Carolina for financial support of this project.

Author contributions

DJK and VN designed experiments, analysed data and wrote the manuscript; DJK, RBC and PAM conducted experiments; and VN and HKS interpreted the clinical data. All authors were involved in writing the paper and approved the submitted and published version. VN is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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