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BRIEF DEFINITIVE REPORT

Donor-derived, metastatic urothelial cancer after kidney transplantation associated with a potentially oncogenic BK polyomavirus

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Abstract

BK polyomavirus has been linked to urothelial carcinoma in immunosuppressed patients. Here, we performed comprehensive genomic analysis of a BK polyomavirus-associated, metachronous, multifocal and metastatic micropapillary urothelial cancer in a kidney transplant recipient. Dissecting cancer heterogeneity by sorting technologies prior to array-comparative genomic hybridization followed by short tandem repeat analysis revealed that the metastatic urothelial cancer was of donor origin (4-year-old male). The top 50 cancer-associated genes showed no key driver mutations as assessed by next-generation sequencing. Whole genome sequencing and BK polyomavirus-specific amplification provided evidence for episomal and subgenomic chromosomally integrated BK polyomavirus genomes, which carried the same unique 17-bp deletion signature in the viral non-coding control region (NCCR). Whereas no role in oncogenesis could be attributed to the host gene integration in chromosome 1, the 17-bp deletion in the NCCR increased early viral gene expression, but decreased viral replication capacity. Consequently, urothelial cells were exposed to high levels of the transforming BK polyomavirus early proteins large tumour antigen and small tumour antigen from episomal and integrated gene expression. Surgery combined with discontinuation of immunosuppression resulted in complete remission, but sacrificed the renal transplant. Thus, this report links, for the first time, BK polyomavirus NCCR rearrangements with oncogenic transformation in urothelial cancer in immunosuppressed patients.

Keywords: urothelial cancer; transplantation; kidney; bladder; polyomavirus; LTag; NCCR; mutation; genome

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No conflicts of interest were declared.

Introduction

Several studies have reported an increased incidence of malignancies after solid organ transplantation, and have indicated a role of infections in tumourigenesis [1,2]. In particular, an association between BK polyomavirus (BKPyV) infection and the risk of urothelial cancer (UC) has been discussed [3,4]. The BKPyV genome contains three different functional regions, called the early viral genome region (EVGR), the late viral genome region (LVGR), and the non-coding control region (NCCR). Indeed, EVGR proteins such as large tumour antigen (LTag) and small tumour antigen (sTag) have been linked to oncogenic cell transformation [5].

We describe the case of a kidney transplant recipient developing BKPyV-associated, multifocal and micropapillary UC. At the age of 42 years, he received a kidney transplant from a deceased 4-year-old male donor. Eight years post-transplantation (ptx), LTag-positive UC of the bladder was diagnosed and treated by endoscopic resection. Nine years ptx, local UC recurrence was diagnosed and surgically removed, but 1 year later the patient was diagnosed with a second recurrence presenting as LTag-positive, multifocal micropapillary, muscle-invasive UC of the graft kidney

pelvis, which metastasized to the bladder wall and to a pelvic lymph node (Figure 1A; supplementary material, Figure S1A and Table S1). Following surgical removal, immunosuppression was discontinued, and, despite multiple recurrences and progression to metastatic UC disease, the patient remained UC recurrence-free during 4 years of follow-up.

Materials and methods

This study was performed in accordance with the principles expressed in the Helsinki Declaration (1975; revised 1983), and was approved by the Ethical Committee Nordwest- und Zentralschweiz in Basel, Switzerland (EKNZ 2014-313).

Immunohistochemistry with an anti-simian virus 40 (SV40) LTag antibody (MRQ-4 mouse clone; Ventana Medical Systems, Tucson, AZ, USA) on the Ventana BenchMark XT platform demonstrated LTag expression. Fresh-frozen tissue obtained from the recent surgical specimens 10 years ptx was used for further analyses. Formalin-fixed paraffin-embedded tissues were available from the 8-year and 9-year ptx biopsies.

We applied DNA content-based cell nucleus sorting followed by array-comparative genomic hybridization (arrayCGH) to determine the evolutionary history of the individual cancer tissues [6]. (Array data have been submitted to the Gene Expression Omnibus repository, accession number GSE90778.) Short tandem repeat (STR) analysis was applied to identify the genealogy of the cancer tissue (supplementary material, Supplementary materials and methods). The IonAmpliseq Cancer Hotspot Panel was used for targeted next-generation sequencing (NGS) (supplementary material, Supplementary materials and methods).

Extracted DNA from tumour and unaffected tissue were tested with quantitative real-time polymerase chain reaction (PCR) for BKPyV genome loads [7], and human aspartoacylase quantitative PCR (qPCR) was used for normalization [8]. BKPyV-specific long-range PCR was performed with outward primers partially overlapping the *Eco*R1 site present in the *VP1* gene (Figure 2; supplementary material, Table S4), yielding a full-length genome of 5116 bp and a short derivative of 3270 bp [9]. The complete BKPyV genomes were determined by Sanger sequencing. Additionally, the episomal BKPyV genomes were deep-sequenced with the Illumina MiniSeq (Illumina, Carlsbad, CA, USA) (supplementary material, Supplementary materials and methods). To detect potential chromosomal integration, shallow whole genome sequencing (WGS) was performed with the Illumina NextSeq 500 (supplementary material, Supplementary materials and methods). Primers spanning the integration breakpoints were used for PCR-based confirmation of WGS results (Figure 2; supplementary material, Table S4, and Supplementary materials and methods).

For functional analysis, the NCCR of the BKPyV-UC genome was amplified and inserted into the bidirectional reporter gene plasmid pHRG1 [10,11]. The respective NCCR pHRG1 reporter constructs were sequenced and transfected into HEK293 cells, and activity was quantified by flow cytometry for the EVGR and the LVGR [11]. Recombinant full-length BKPyV genomes were generated by inserting the NCCRs derived from different BKPyV strains into the Dunlop genome [10,11]. COS7 cells were transfected with the recombinant BKPyV (rBKPyV) genomes [7], and supernatants were used to infect human primary proximal renal tubular epithelial cells (hRPTECs) with rBKPyV as described previously [10,11]. The hRPTEC culture supernatants were harvested at 1, 3, 5, 7 and 9 days post-infection, and rBKPyV loads released into the culture supernatants were compared by the use of qPCR (Figure 3D) [10,11].

Results and discussion

ArrayCGH and STR revealed that the UC originated from the donor kidney pelvis (Figure 1B; supplementary material, Figure S1B). However, no somatic key driver point mutations were detected in the UC genome (supplementary material, Table S2). Instead, the UC in the pelvis was shown to originate from the donor kidney rather than from the urinary bladder of the 52-year-old recipient, who had a much longer history of potential exposure to carcinogenic agents, such as ongoing cigarette smoking, and a lifetime accumulation of oncogenic events.

Immunohistochemistry indicated LTag expression, and WGS identified one integrated episomal BKPyV genome and two episomal BKPyV genomes (Figure 2) in the 10-year ptx cancer specimens: five read pairs aligned to the viral genome and to human chromosome 1. We identified breakpoints in chr1:16055645–16055650_*PLEKHM2* and the capsid coding sequences of Vp1 and Vp2/Vp3, accompanied by a 1355-bp deletion between Vp1 and Vp2/Vp3 (Figure 2; Figures S4 and S5). Importantly, no role in oncogenesis could be attributed to the affected host gene. Three independent primer pairs designed to match the upstream and downstream genome breakpoints amplified exclusively a single band of 3.7 kb, which, upon sequencing, yielded solely the integrated BKPyV genome, ruling out the presence of concatemers (Figure 3A; supplementary material, Table S4). To determine the relative amounts of the integrated BKPyV genome and the two episomal forms, two different PCRs were used: one targeting LTag sequences (LTag_qPCR in Figure 2; Table S4) present in all three UC-derived BKPyV genomes (i.e. the episomes of 5116 bp and 3270 bp as well as the 3761-bp integrated genome), and another one targeting Vp1 sequences exclusively present in the episomal forms (Vp1_qPCR in Figure 2; supplementary material, Table S4). The results were normalized to *ACY* as a housekeeping gene [8], and revealed an LTag target/Vp1 target ratio of 3:2,

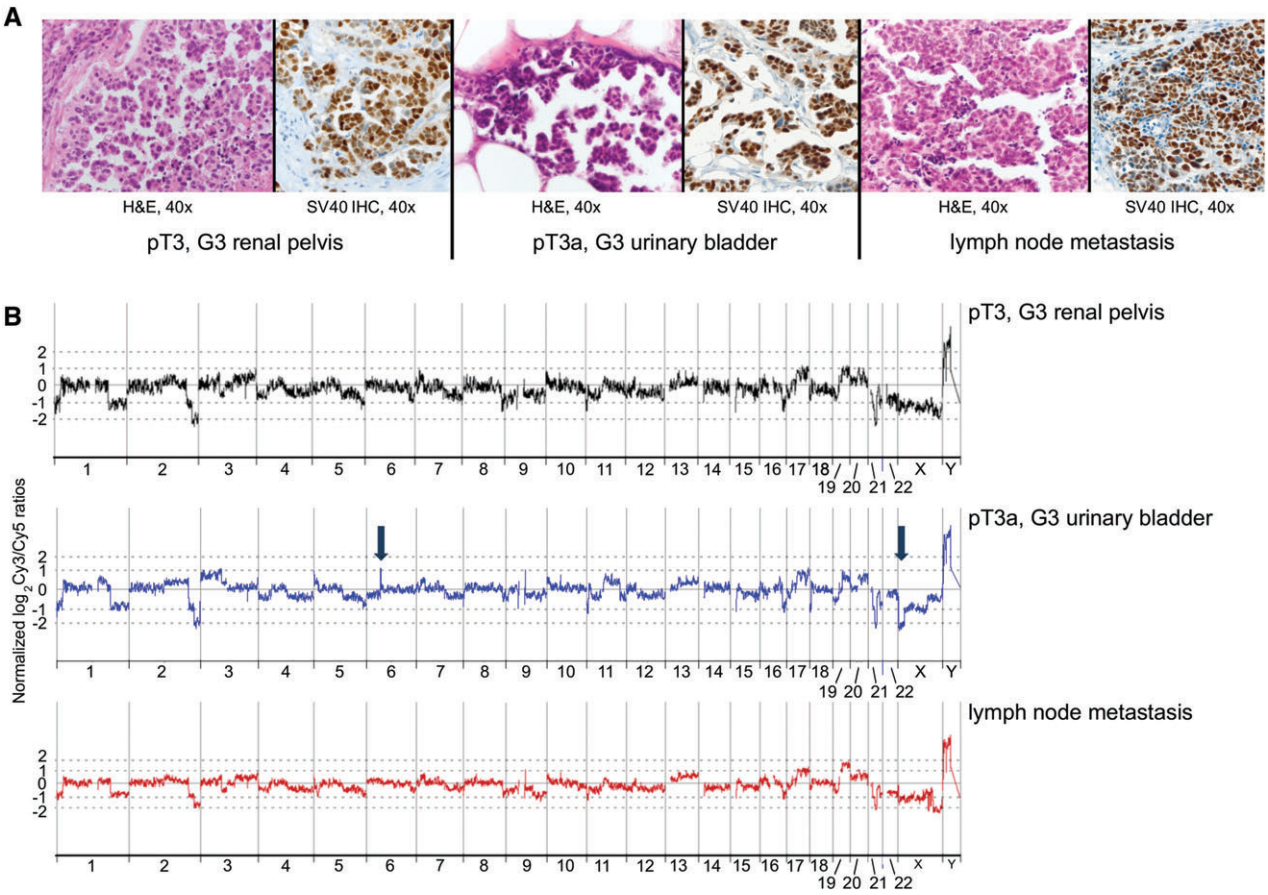

Figure 1. Morphological and genomic characterization of cancer sites 10 years ptx. (A) Haematoxylin and eosin (H&E) staining and SV40 LTag immunohistochemistry (IHC) of micropapillary UC of the kidney transplant recipient. The morphological picture indicates a close relationship of the different sites and differs from the previous tumour appearances (not shown). (B) ArrayCGH profiles with copy number alterations of aneuploid cancer populations 10 years ptx. Arrows indicate key differences. *x*-axis: chromosome number. *y*-axis: normalized $\log_2$ Cy3/Cy5 ratios (relative count of DNA probe signals). The samples appear to be highly related, suggesting that the lymph node metastasis was derived directly from the renal pelvis, as the bladder showed a unique deletion in the X chromosome (right arrow). In contrast, there was no evidence of a clonal relationship with the previous tumour appearances (supplementary material, Figure S2).

as expected for three independent LTag genome and two Vp1 targets.

Importantly, the full-length BKPyV episome and the chromosomally integrated truncated BKPyV genome carried an identical, and so far not reported, 17-bp deletion in the P-block of the UC BKPyV NCCR (Figures 2 and 3B). Functional analyses with a bidirectional reporter vector revealed that this 17-bp deletion NCCR was sufficient to activate EVGR expression, and contributed to LTag expression (Figure 3C). Unlike patient-derived NCCR rearrangements characterized previously [10], however, the UC-derived 17-bp deletion NCCR showed impaired progression into the late viral life cycle and offset efficient lytic replication (Figure 3D). The fact that the intact full-length viral episome carried the same NCCR deletion as the truncated integrated genome argues that this 17-bp deletion occurred first, and was followed by viral genome integration, both of which contribute to LTag expression. It remains challenging to dissect the relative contributions of immunosuppression and ongoing BKPyV replication during this process. It is well known that chronic immunosuppression by itself favours the development of malignancy [1]. However, one would expect exposure to carcinogens and/or hereditary susceptibility to be present before such cells can be unleashed to give rise to established cancer after loss of immune control.

Apart from surgery, postoperative termination of immunosuppression was sufficient to control metastatic disease. This suggests that aberrant LTag expression resulting from the unique BKPyV NCCR deletion conferred a strong transforming oncogenic drive, which could not be cleared immunologically without discontinuation of immunosuppression [12].

There is growing evidence that reactivation of polyomavirus infection under immunosuppression might play an important role in the development of aggressive UC after renal transplantation [4,13]. A driving role of BKPyV infection is also supported by the low mutational load in cancer-related genes in our case as compared with common UCs, which show some of the highest mutation rates among malignant human tumours [14]. Similarly, low mutational loads have been seen in Merkel cell carcinoma, which is known to be associated with Merkel cell polyomavirus infection [15]. Indeed, all UC manifestations of our patient at 8, 9 and 10 years

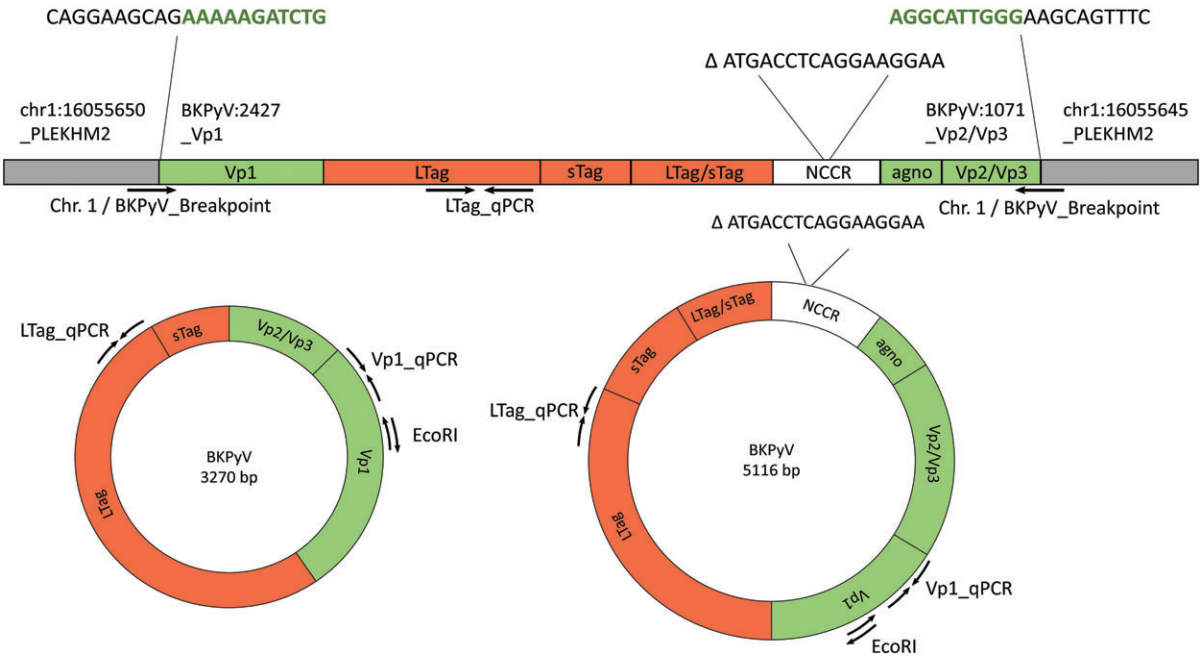

Figure 2. BKPyV genomes in the 10-year ptx UC specimen. The integrated genome and the breakpoints in chromosome 1 were located in the *PLEKHM2* gene as identified by WGS. The breakpoint sequences are shown in black for the human chromosome 1 *PLEKHM2* gene and green for the BKPyV genome. The locations and directions of primers used for amplification of the episomal BKPyV genome at the *Eco*RI site or the qPCRs targeting LTag (LTag_qPCR) and Vp1 (Vp1_qPCR) are depicted by arrows. The integrated BKPyV genome is shortened by a 1355-bp deletion between Vp2/Vp3 and Vp1, and by a 17-bp deletion in the NCCR P-block. In addition, an episomal full-length BKPyV genome of 5116 bp and a short genome of 3270 bp were identified. Whereas the larger episomal genome on the right-hand side is the only genome with a complete EVGR and a complete LVGR, the shorter episomal genome has a deletion of 1847 bp removing parts of the common LTag/sTag sequence, the NCCR, agno (agnoprotein-gene), and the Vp2/Vp3.

ptx were LTag-positive, and eventually turned into a metastasizing micropapillary variant at the final, most advanced stage. Thus, the aggressive micropapillary morphology strongly suggests that BKPyV acts as an oncogenic driver, as discussed previously [4,13]. Although high-level urinary BKPyV replication occurs in 20–40% of patients after kidney transplantation, most UCs diagnosed to date have been LTag-negative [16]. The factors that unleash BKPyV oncogenicity in individual patients are presently unknown, but appear to involve constitutive EVGR expression of LTag and sTag [5]. Viral integration into human DNA has been proposed as an important feature of oncogenic polyomaviruses disrupting progression from constitutive EVGR to lytic LVGR expression by removing relevant viral gene sequences [17]. This can occur as a result of breakpoints in the viral DNA followed either by host DNA [18] or by multiple copies of viral DNA [19]. Such integration is supposed to support an imbalance between increased LTag expression and reduced virus-induced cell-lysis resulting in sustained perturbation of pRb and p53, both of which bind to LTag. A rearranged NCCR in the context of BKPyV-associated cancer has been reported [18], in which the Q-block and R-block are deleted from the NCCR of the integrated BKPyV genome. Similar deletions have been detected in kidney transplant patients, e.g. del(5.3), del(3.2), and del(15.10) (supplementary material, Figure S1 of [10]), all of which increased EVGR expression comparably to the 17-bp deletion reported here, but, unlike the 17-bp deletion described here, also conferred increased replicative capacity and cytopathology [10]. Although our analyses provide evidence of both NCCR rearrangement and genomic integration, our functional studies challenge the view that integration of polyomaviruses is a necessary condition for tumourigenesis. The 17-bp deletion in the NCCR P-block in the non-integrated BKPyV genome may lead to the same early versus late gene expression imbalance as an LVGR genomic disruption [18,19]. We have demonstrated that the 17-bp deletion NCCR constitutively activated LTag and sTag EVGR expression, while impairing LVGR expression and the replicative capacity of isogenic derivatives. Thus, viral replication with accumulation of viral particles and subsequent (onco)cytolytic cell death was impaired. Importantly, all stages of the UC in our patient were LTag-positive, suggesting that expression of this virus-encoded transforming function contributed to malignant progression. The now relapse-free time of 4 years after diagnosis of this metastasizing UC also points to the role of regaining anti-tumour immunity following cessation of immunosuppression, and questions the need for additional aggressive therapeutic interventions in such situations [12].

Taking the above findings together, the occurrence of aggressive LTag-positive UC can be regarded as a rare 'biological accident' in the light of the high prevalence of high-level BKPyV replication in immunosuppressed patients, e.g. after kidney transplantation [18]. However, given the huge number of viral generations in an

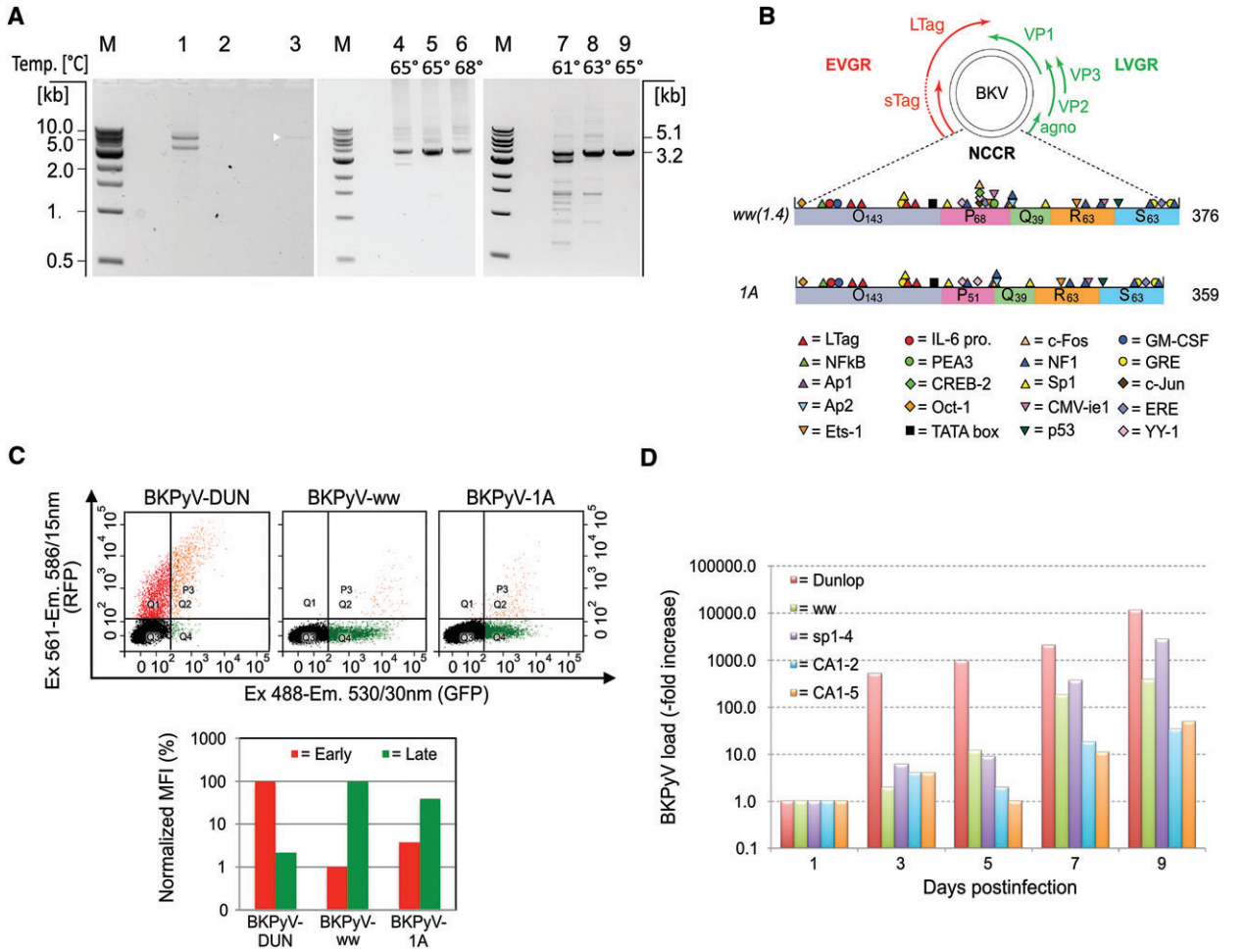

Figure 3. Characterization of the BKPyV genome in the 10-year ptx UC tissue specimen. (A) Gel electrophoresis of PCR products after amplification with outward primers from the *Eco*R1 site of the *VP1* gene (supplementary material, Table S4) yields a full-length BKPyV DNA genome (5116 bp) bearing a small deletion in the NCCR and a smaller subgenomic fragment (3270 bp) lacking the N-terminal Vp2, the NCCR and N-terminal LTag sequences. M, DNA marker in kilobase pairs. Lane 1: 50 ng of UC tissue DNA. Lane 2: negative template control. Lane 3: control BKPyV-positive urine specimen, highlighted by the white triangle. Lanes 4–9: amplification of chromosome 1 integrated BKPyV genome of 3761 bp with breakpoint-specific primers. Lanes 4–6: UC tissue DNA amplified with three different pairs of breakpoint-specific primers (supplementary material, Table S4). Lanes 7–9: UC tissue DNA amplified with pair 1 of the breakpoint-specific primers (supplementary material, Table S4), with an annealing temperature gradient PCR for increasing stringency (61 °C, 63 °C, and 65 °C). (B) Schematic representation of transcription factor binding sites in the BKPyV archetype NCCR (ww1.4) of 375 bp, and the BKPyV-1A NCCR (359 bp) carrying a 17-bp deletion in the P-block P41–P57. Red: EVGR. Green: LVGR. (C) NCCR-driven reporter gene expression of the EVGR (red; dsRed) and the LVGR (green; enhanced green fluorescent protein) in the bidirectional reporter vector pHRG1 following transfection into HEK293 cells. Top panels: flow cytometry. Bottom panels: normalized mean fluorescence intensity (MFI). Positive control: BKPyV-DUN (Dunlop strain). BKPyV-ww: archetype strain. BKPyV-1A: 17-bp deletion NCCR identified in episomal and integrated BKPyV genomes of the UC. (D) Comparison of viral replication in Vero cells after infection of the indicated BKPyV NCCR variants: Dunlop strain, archetype ww(1.4); ww(sp1–4) mutant; and two independent recombinant clones bearing the patient-derived NCCR CA1-2 and CA1-5. Em, emission; Ex, excitation; GFP, green fluorescent protein; RFP, red fluorescent protein.

immunosuppressed host, a rare clinical manifestation can become a relevant complication in the long-term perspective of otherwise successful kidney transplantation. Further studies are needed to explore the relative incidence and clinical importance of such alternative events.

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Author contributions statement

The authors contributed in the following way: CAR, CR, LB, HHH: designed the study; MR, KN, DCM, VP, TV, BB, ETA: carried out the experiments; DCM, MR, KN, SR, LQ, CR, CAR, BB, R. Gosert: performed data analyses and interpretation; CW, R. Grobholz: acquired samples; DCM, MR, KN: wrote the manuscript; CAR, LB, HHH: reviewed the manuscript. All authors approved the submitted version.

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*Cited only in supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Table S1. Summary of histopathological results

Table S2. NGS-results of 10 year post-transplantation UC sites

Table S3. Tabular summary of STR results

Table S4. Primer and probe sequences

Figure S1. Overview over disease progression and cancer distribution 10 years post-transplantation

Figure S2. Sorting and arrayCGH profiles of cancer sites 8 years and 9 years post-transplantation

Figure S3. Sorting profiles of UC sites 10 years post-transplantation

Figure S4. Detection of BKPyV integration site in chromosome 1 using WGS

Figure S5. Coverage of whole genome and targeted amplicon sequencing of BKPyV

Figure S6. Comparison of NCCR rearrangements