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Research Article

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Urinary donor-derived cell-free DNA as a non-invasive biomarker for BK polyomavirus-associated nephropathy

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Abstract: BK polyomavirus-associated nephropathy (BKPyVAN) is a common cause of allograft failure. However, differentiation between BKPyVAN and type I T cell-mediated rejection (TCMR) is challenging when simian virus 40 (SV40) staining is negative, because of the similarities in histopathology. This study investigated whether donor-derived cell-free DNA (ddcfDNA) can be used to differentiate BKPyVAN. Target region capture sequencing was applied to detect the ddcfDNAs of 12 recipients with stable graft function, 22 with type I TCMR, 21 with proven BKPyVAN, and 5 with possible PyVAN. We found that urinary ddcfDNA levels were upregulated in recipients with graft injury, whereas plasma ddcfDNA levels were comparable for all groups. The median urinary concentrations and fractions of ddcfDNA in proven BKPyVAN recipients were significantly higher than those in type I TCMR recipients (10.4 vs. 6.1 ng/mL, $P<0.001$ and 68.4% vs. 55.3%, $P=0.013$, respectively). Urinary ddcfDNA fractions (not concentrations) were higher in the BKPyVAN-pure subgroup than in the BKPyVAN-rejection-like subgroup (81.30% vs. 56.64%, $P=0.025$). With a cut-off value of 7.81 ng/mL, urinary ddcfDNA concentrations distinguished proven BKPyVAN from type I TCMR (area under the curve (AUC)=0.848, 95% confidence interval (95% CI): 0.734 to 0.963). These findings suggest that urinary ddcfDNA is a non-invasive biomarker which can reliably differentiate BKPyVAN from type I TCMR.

Key words: Donor-derived cell-free DNA (ddcfDNA); BK polyomavirus-associated nephropathy (BKPyVAN); T cell-mediated rejection (TCMR); Urine; Differential diagnosis

1 Introduction

The occurrence of viral infections due to the use of highly potent immunosuppressants is a leading cause of allograft dysfunction in kidney transplantation. Infection with the BK polyomavirus (BKPyV) is a prevalent complication of kidney transplantation (Hirsch and Randhawa, 2019), and it is estimated that 1%–10% of kidney transplant patients develop polyomavirus-associated nephropathy (PyVAN) (Hirsch et al., 2002, 2005; Dharnidharka et al., 2009). Screening modalities of BKPyVAN after kidney transplantation include BKPyV-DNAemia, urine BKPyV load, and urine cytology for decoy cells (Hirsch and Randhawa, 2019). Meanwhile, the standard diagnosis of BKPyVAN is obtained by histopathological detection of simian virus 40 (SV40) in renal biopsy tissues (Knowles et al., 2003). Because of the focal nature of BKPyVAN, however, the false-negative rate of biopsy is high, varying from 10% to 30% (Drachenberg et al., 2004; Pang et al., 2007). Similar to type I T cell-mediated rejection (TCMR), the histopathology of BKPyVAN also manifests interstitial inflammation and/or tubulitis (Ahuja et al., 2001). Consequently, diagnosis of BKPyVAN or type I TCMR should be handled cautiously when SV40 staining is negative, since BKPyVAN and TCMR

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are treated differently. About 28% of BKPyVAN cases are complicated by subsequent development of clinical rejection (Drachenberg et al., 2017). When BKPyVAN is complicated with allograft rejection (such as type I TCMR), making a proper diagnosis is particularly challenging but also important for treatment decision-making.

Previous biopsy studies have examined the types of inflammatory cell infiltration in BKPyVAN and TCMR. The results showed that high numbers of infiltrated B lymphocytes, plasma cells, and blood dendritic cell antigen-1^+ (BDCA-1^+) myeloid dendritic cells could differentiate BKPyVAN from TCMR (Mannon et al., 2005; Buettner et al., 2012; Yapici et al., 2016). However, the biomarkers used in these investigations lack specificity, which has limited their clinical application. Cell-free DNA (cfDNA) is a novel non-invasive method of monitoring disease. Donor-derived cell-free DNA (ddcfDNA) generated from allografts is a specific biomarker of graft injury. The diagnostic value of plasma ddcfDNA in allograft rejection and injury has been proved in nearly all types of solid organ transplantation (de Vlaminck et al., 2015; Agbor-Enoh et al., 2017; Bloom et al., 2017; Schütz et al., 2017). Unlike injuries to other solid organs, kidney injuries can also be directly reflected in the properties of urine, which makes it possible to detect allograft injury through a urinary ddcfDNA assay. Urinary levels of ddcfDNA have been proposed as a marker of allograft rejection in kidney transplantation (Sigdel et al., 2013). Chen et al. (2020) recently reported that urinary ddcfDNA levels are capable of differentiating BKPyVAN from BKPyV viruria in kidney transplant recipients. However, the use of ddcfDNA in discriminating BKPyVAN from type I TCMR has not been fully discerned.

To pursue this avenue, we conducted a single-center pilot study on the value of urinary ddcfDNA levels in kidney transplant recipients. The results of our study provide a novel non-invasive method for distinguishing between BKPyVAN and type I TCMR.

2 Materials and methods

2.1 Patients and study design

We prospectively enrolled kidney transplant recipients in the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China) with matched biopsies from June 2017 to September 2019. The inclusion criteria were: (1) age of >18 years; (2) patients showing decline of graft function from baseline to more than 20% (Shen et al., 2020). Additionally, we recruited patients with graft function of <20% from baseline, proteinuria of <0.5 g/24 h, and without evidence of BKPyV infection, for a stable renal function (STA; control) group. The exclusion criteria were: (1) graft dysfunction caused by antibody-mediated rejection (ABMR), borderline changes, Banff type II rejection, interstitial fibrosis and tubular atrophy (IFTA), post-transplant nephritis, and other infections; (2) TCMR complicated with urinary BKPyV logarithm value of <5 lg(copies/mL); (3) biopsy-proven subclinical rejection; (4) multiorgan transplantation or repeated kidney transplantation. Blood and urine samples were collected from 147 patients before biopsy and treatment. Among those who did not meet the inclusion criteria were 19 patients with ABMR, 21 with Banff type II rejection, 18 with IFTA, 12 with recurrent/de novo glomerulonephritis, 9 with borderline changes, 2 protocol biopsy patients with subclinical rejection (1 Banff IA and 1 Banff IIA), and 6 with Banff type I rejection along with urinary BKPyV between 3 and 4 lg(copies/mL). A total of 60 kidney-transplant recipients were enrolled, including 22 biopsy-proven type I TCMR patients, 21 biopsy-proven BKPyVAN patients, 5 possible PyVAN patients, and 12 patients with no pathological results (as an STA group).

2.2 BKPyV DNA detection

Blood samples were collected in ethylene diamine tetraacetic acid (EDTA) tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged to separate the plasma. Urina sanguinis samples were collected via the conventional midstream clean-catch method. BKPyV DNA was extracted and quantified using a BK virus polymerase chain reaction (PCR) kit (Sinomdgene Company, Beijing, China) following the manufacturer's protocol. Data are presented as copies of viral DNA per milliliter. The lower limit of quantification of this assay for BKPyV was 1.0×10^3 copies/mL and the lower limit of detection was 1.0×10^2 copies/mL.

2.3 Clinical and histological definition

Proven BKPyVAN was defined as positive staining of SV40 on the biopsy tissue, which excluded patients

with endoarteritis complications or with humoral rejection (Drachenberg et al., 2017). Possible PyVAN was defined as logarithm value of urinary BKPyV loads of >5 lg(copies/mL), with/without serum BKPyV-DNAemia, but with negative SV40 staining. BKPyVAN-rejection-like subgroup indicates patients with proven BKPyVAN whose histopathology suggested rejection according to the Banff 2017 criteria for type IA or type IB TCMR. BKPyVAN-pure subgroup indicates patients with proven BKPyVAN who had no histological evidence of rejection according to the Banff 2017 criteria.

2.4 Sample collection

Peripheral blood and conventional midstream clean-catch urina sanguinis samples (8 mL each) were collected into cfDNA Collecting Tubes (Streck, NE, USA) within 24 h before renal biopsy and stored at 4–37 ℃ for less than 24 h before centrifugation. Each sample was centrifuged at 1600*g* for 10 min at 4 ℃. The supernatant was then subjected to a second centrifugation at 16 000*g* for 10 min at 4 ℃ and stored at −80 ℃ for further use; or cfDNA was immediately extracted using a Circulating Nucleic Acid kit (Qiagen, Germantown, MD, Germany; Cat. No. 55114) according to the manufacturer's instructions.

2.5 Detection of blood and urine ddcfDNA

2.5.1 Library construction and target region capture sequencing

We quantified the purified cfDNA with a Qubit fluorometer (Qubit 3.0; Life Technologies, Carlsbad, CA, USA). We then constructed the DNA sequence library from >30 ng of cfDNA, using a KAPA LTP Library Preparation kit (KK8235; KAPA Biosystems, Boston, MA, USA) for each sample and eight PCR cycles for library amplification. A total of 6200 human single nucleotide polymorphic (SNP) loci were enriched with a custom TruGrade® DNA Oligos pool (IDT, California, USA) by liquid hybridization, according to the manufacturer's instruction (Zhou et al., 2019). We selected candidate SNPs based on the following standards: (1) high polymorphism in the Chinese population (minor allele frequency (MAF)=0.4–0.6); (2) single copy in the genome; and (3) even distribution across the whole genome. The captured libraries were characterized using an Agilent 2100 Bioanalyzer (high-sensitivity DNA kit; CA, USA), then pooled and sequenced for 5 million paired-end 150 clusters with the HiSeq X Ten Sequencing System (Illumina, San Diego, CA, USA) (Zhou et al., 2019).

2.5.2 Quantification of ddcfDNA

Raw data for sequencing were trimmed by removing low-quality reads, adapter contamination reads, and PCR duplicates. We then aligned the clean data against the human reference (GRCh38; https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.38) using BWA (http://bio-bwa.sourceforge.net). All polymorphic alleles were identified by base calling using Samtools (http://samtools.sourceforge.net). For every 6200 SNPs, we counted the read numbers for each allele and calculated the minor allele ratio (MAR) value for the informative SNP locus (homozygous SNP loci with at least one alternative allele read) for ddcfDNA quantification. For each informative SNP, we employed the binomial model to estimate the donor-derived allele frequency, and we determined donor genotype from the donor-specific allele frequency in the population. The Bayes approach was applied to quantify the levels of ddcfDNA. The absolute quantification of haploid ddcfDNA (ng/mL) of urine was calculated by the formula as follows: absolute quantification of urine ddcfDNA (ng/mL)=total cfDNA concentration (ng/mL)×ddcfDNA fraction (%) (Zhou et al., 2019).

2.6 Statistical analysis

All statistical analyses were performed using R software (Version 3.6.2, 64-bit). Differences in patient demographics, serum creatinine, and ddcfDNA among subgroups were analyzed with the Kruskal-Wallis rank-sum test (continuous data) and the Chi-square test (binary and ordinal data). A multiple comparison Wilcoxon Rank Sum test (http://www.statmethods.net/RiA/wmc.txt) was performed as a post-test of the Kruskal-Wallis test. We evaluated comparisons between the two subgroups with the Wilcoxon Rank Sum test. Analysis of receiver operating characteristics (ROCs) was performed with the pROC package (Version 1.16.1). The ggplot2 package (Version 3.3.0) was applied to visualize the analyzed data. P values of <0.05 were considered statistically significant.

3 Results

3.1 Patients and samples

This study enrolled 60 kidney transplant recipients who visited our hospital from June 2017 to September 2019. The patients included 22 biopsy-proven type I TCMRs, 21 proven BKPyVANs, 5 possible PyVANs, and 12 STAs (Fig. 1). The demographics of recipients in each group were as shown in Table 1. The donor- and recipient-associated parameters were comparable among the four groups. As shown in Fig. 2, the serum creatinine levels at the biopsy time point in the type I TCMR, possible PyVAN, and proven BKPyVAN groups were significantly higher than those in the STA cohort. However, no significant differences were found among type I TCMR, possible PyVAN, and proven BKPyVAN groups.

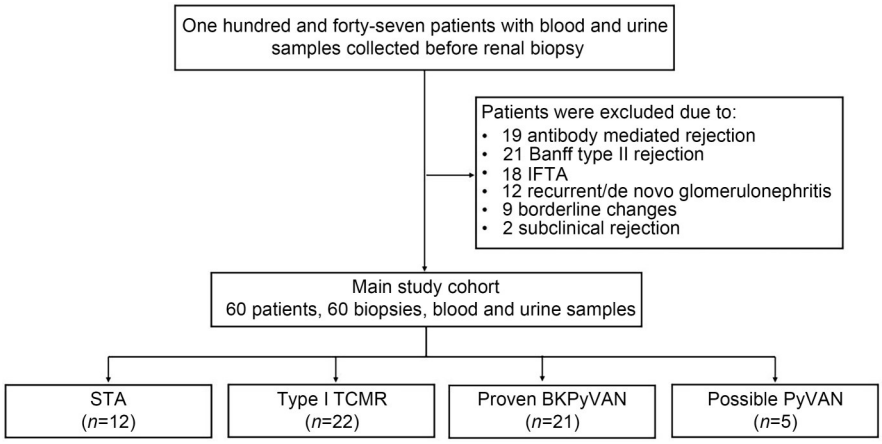

Fig. 1 Consort diagram of patient flow through the study. IFTA: interstitial fibrosis and tubular atrophy; STA: stable renal function; TCMR: T cell-mediated rejection; BKPyVAN: BK polyomavirus-associated nephropathy.

Table 1 Patient demographics in this study

Characteristics	STA (*n*=12)	Type I TCMR (*n*=22)	Proven BKPyVAN (*n*=21)	Possible PyVAN (*n*=5)	*P*
Donor-associated parameters					
Age (years)	38.67±15.36	48.09±10.62	47.14±9.35	46.40±2.51	0.276
Donor type (DCD/LD)	5/7	11/11	17/4	4/1	0.063
Sex (M/F)	5/7	15/7	13/8	4/1	0.371
Serum creatinine (μmol/L)	71.58±14.52	77.09±18.92	102.05±76.53	98.40±29.53	0.305
Recipient-associated parameters					
Age (years)	37.50±9.22	37.09±11.90	45.19±14.51	41.80±9.98	0.096
Sex (M/F)	10/2	16/6	13/8	5/0	0.279
Dialysis					0.203
Hemodialysis	10	15	17	2	
Peritoneal dialysis	1	6	2	3	
None	1	1	2	0	
Induction					0.688
ATG	4	7	6	2	
Simulect	8	13	15	3	
None	0	2	0	0	
HLA-MM	4.00±0.74	2.91±1.27	3.33±0.97	3.60±0.55	0.049
Duration post RT (d)	250 (92, 580)	360 (230, 1089)	306 (136, 554)	104 (62, 923)	0.095

STA: stable graft function; TCMR: T cell-mediated rejection; BKPyVAN: BK polyomavirus-associated nephropathy; DCD: donation after cardiac death; LD: living donor; M: male; F: female; ATG: anti-thymocyte globulin; HLA-MM: human leukocyte antigen (HLA)-mismatch; RT: renal transplantation. *P* value represents the difference among STA, type I TCMR, proven BKPyVAN, and possible PyVAN, using one-way analysis of variance (ANOVA). Data are expressed as mean±standard deviation (SD), median (interquartile range), number/number, or number.

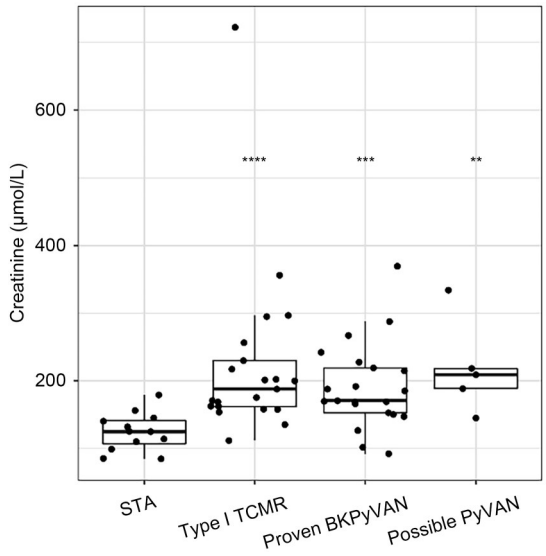

Fig. 2 Elevated serum creatinine in recipients with allograft injury. Boxplot with bold line represents the median serum level in each subgroup. ** *P*<0.01, * *P*<0.001, and **** *P*<0.0001, compared with the STA cohort. STA: stable graft function; TCMR: T cell-mediated rejection; BKPyVAN: BK polyomavirus-associated nephropathy.**

The BKPyV loads in urine and plasma samples of all subjects in the proven BKPyVAN and possible PyVAN cohorts are presented in Table 2. Three of the five subjects in the possible PyVAN group (60.0%) and 15 of the 21 subjects in the proven BKPyVAN group (71.4%) developed high-level viruria, with urinary BKPyV loads of >7 lg(copies/mL). A low level of BKPyV-DNAemia (plasma BKPyV loads of <3 lg(copies/mL)). was detected in four subjects of the possible PyVAN group and 12 (57.1%) subjects in the proven BKPyVAN group.

Table 2 BK polyomavirus (BKPyV) levels of each subject with proven BKPyVAN or possible PyVAN

Subject	BKPyV (copies/mL)	
	Urine	Plasma
Possible PyVAN		
Case 1	2.1×10^{8}	$<1.0\times10^{2}$
Case 2	3.4×10^{6}	0.5×10^{3}
Case 3	2.5×10^{8}	$<1.0\times10^{2}$
Case 4	3.0×10^{6}	0.3×10^{3}
Case 5	1.6×10^{8}	2.6×10^{4}
Proven BKPyVAN		
Case 1	2.4×10^{7}	2.6×10^{3}
Case 2	9.7×10^{8}	1.1×10^{4}
Case 3	9.9×10^{8}	1.5×10^{4}
Case 4	1.6×10^{6}	$<1.0\times10^{2}$
Case 5	7.0×10^{8}	8.0×10^{5}
Case 6	4.3×10^{8}	2.1×10^{4}
Case 7	3.4×10^{8}	4.8×10^{4}
Case 8	2.1×10^{7}	$<1.0\times10^{2}$
Case 9	3.0×10^{5}	$<1.0\times10^{2}$
Case 10	6.1×10^{5}	$<1.0\times10^{2}$
Case 11	2.0×10^{7}	$<1.0\times10^{2}$
Case 12	5.1×10^{8}	3.2×10^{4}
Case 13	1.3×10^{6}	$<1.0\times10^{2}$
Case 14	5.2×10^{8}	$<1.0\times10^{2}$
Case 15	6.3×10^{7}	$<1.0\times10^{2}$
Case 16	4.8×10^{7}	$<1.0\times10^{2}$
Case 17	5.7×10^{8}	3.3×10^{4}
Case 18	9.6×10^{8}	5.6×10^{3}
Case 19	7.5×10^{7}	$<1.0\times10^{2}$
Case 20	8.0×10^{6}	$<1.0\times10^{2}$
Case 21	3.9×10^{6}	$<1.0\times10^{2}$

The lower limit of quantification of this assay for BKPyV was 1.0×10^{3} copies/mL, and the lower limit of detection was 1.0×10^{2} copies/mL. BKPyVAN: BK polyomavirus-associated nephropathy.

3.2 Concentration and fraction of ddcfDNA in kidney transplant recipients

We analyzed the concentration and fraction of ddcfDNA levels in blood and urine samples of kidney transplant recipients with type I TCMR, possible PyVAN, proven BKPyVAN, and STA. Relative to the STA group, the absolute quantities of ddcfDNA in the blood and urine of the type I TCMR, possible PyVAN, and proven BKPyVAN groups were significantly higher (Figs. 3a and 3b). No significant differences in plasma ddcfDNA concentration were observed between the type I TCMR, possible PyVAN, and proven BKPyVAN groups. Meanwhile, the median levels of ddcfDNA in the urine of possible PyVAN and proven BKPyVAN groups were 11.1 (P=0.013) and 10.4 ng/mL (P<0.001), significantly higher than that of the type I TCMR group, which had a median concentration of 6.10 ng/mL (Fig. 3b). Notably, the fractions of ddcfDNA in blood were comparable among the four groups (P=0.341; Fig. 3c), whereas the fractions of ddcfDNA in urine differed significantly (P<0.001; Fig. 3d). The median urinary ddcfDNA fractions varied from 55.3% in the type I TCMR group to 68.4% in the proven BKPyVAN group, and we observed a high value of 81.9% in the possible PyVAN group. So, the median urinary ddcfDNA fraction level was significantly lower in the type I TCMR group than in the proven BKPyVAN and possible

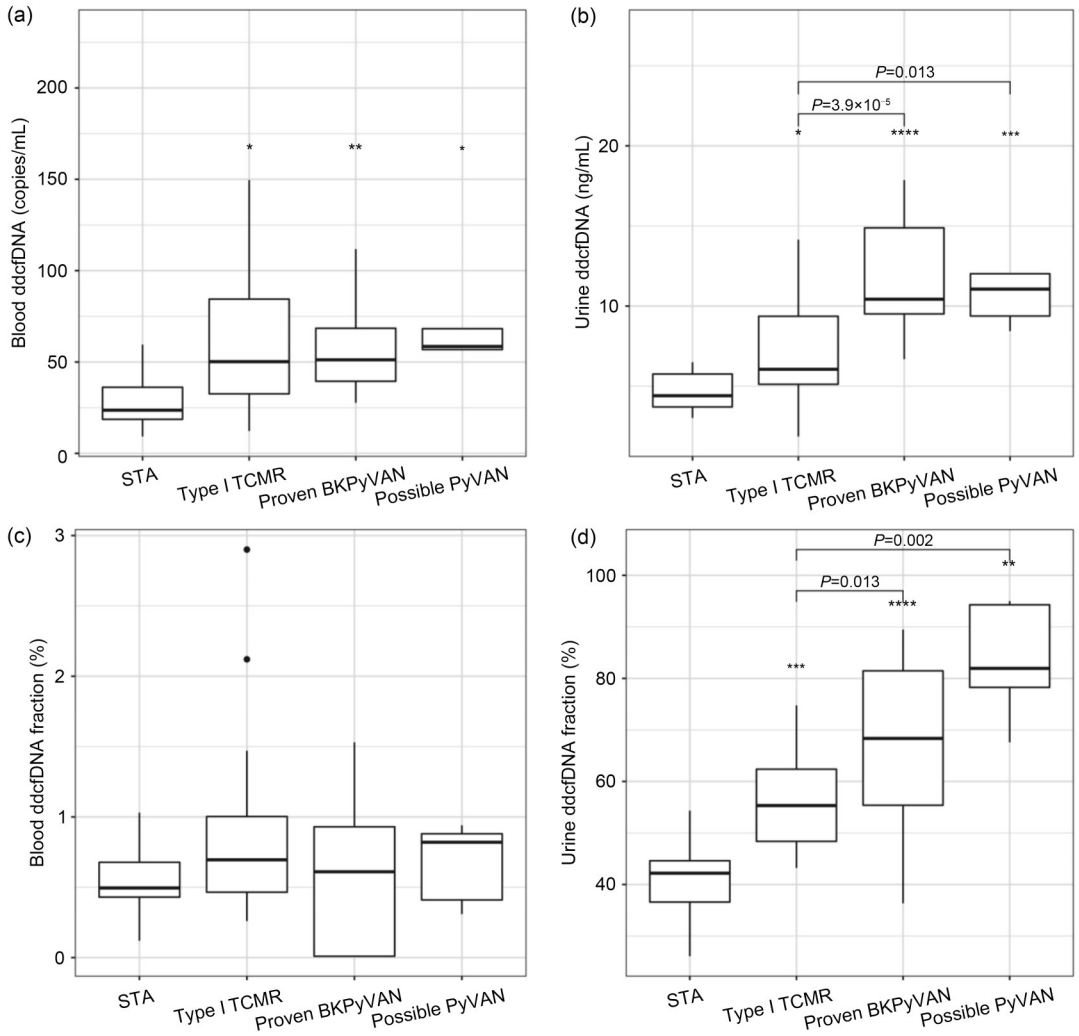

Fig. 3 Concentration and fraction of ddcfDNA in kidney transplant recipients. (a) Blood ddcfDNA concentration; (b) Urinary ddcfDNA concentration; (c) Blood ddcfDNA fraction; (d) Urinary ddcfDNA fraction. Boxplot with bold line represents the median level. * *P*<0.05, ** *P*<0.01, * *P*<0.001, and **** *P*<0.0001, compared with the STA biopsy cohort. ddcfDNA: donor-derived cell-free DNA; TCMR: T cell-mediated rejection; STA: stable graft function; BKPyVAN: BK polyomavirus-associated nephropathy.**

PyVAN groups (*P*=0.013 and *P*=0.002, respectively; Fig. 3d).

We divided the 21 recipients in the proven BKPyVAN group into two subgroups, BKPyVAN-rejection-like (*n*=13) and BKPyVAN-pure (*n*=8), based on histological evidence of rejection. The median levels of urinary ddcfDNA concentration in the BKPyVAN-rejection-like and BKPyVAN-pure subgroups were 10.59 ng/mL (*P*<0.001) and 10.27 ng/mL (*P*=0.004), respectively, significantly higher than that in the type I TCMR group (6.06 ng/mL). However, no significant difference in the median levels of urinary ddcfDNA concentration was found between the proven BKPyVAN subgroups (Fig. 4a). The median urinary ddcfDNA fraction in the BKPyVAN-pure subgroup was significantly higher than that in either the type I TCMR group (81.30% vs. 55.33%, *P*<0.001) or the BKPyVAN-rejection-like subgroup (81.30% vs. 56.64%, *P*=0.025). However, no significant difference was observed between the type I TCMR group and the BKPyVAN-rejection-like subgroup (Fig. 4b).

3.3 Diagnostic performance of urinary ddcfDNA concentration vs. fraction in BKPyVAN

The ROC curve showed the diagnostic performance of both urinary ddcfDNA concentration (area

under the curve (AUC)=0.848, 95% confidence interval (95% CI): 0.734 to 0.963) and fraction (AUC=0.722, 95% CI: 0.565 to 0.879) in distinguishing proven BKPyVAN from type I TCMR. Absolute urinary ddcfDNA had a sensitivity and specificity of 95.2% and 68.2%, respectively, at a cut-off value of 7.81 ng/mL (Fig. 5a), to discriminate proven BKPyVAN from type I TCMR. Additionally, results showed that urinary ddcfDNA fraction had a cut-off value of 77.02%, with 42.9% sensitivity and 100.0% specificity (Fig. 5b). Further, we explored the diagnostic performance of urinary ddcfDNA in discriminating between proven BKPyVAN and STA. We found that urinary ddcfDNA was in fact effective in this task (Fig. S1).

4 Discussion

Our findings demonstrated that kidney transplant recipients with graft injury (including type I TCMR, proven BKPyVAN, and possible PyVAN) had elevated levels of urinary ddcfDNA. Both the concentrations and fractions of urinary ddcfDNA in biopsy-proven BKPyVANs were higher than those in type I TCMRs. Urinary ddcfDNA fractions, but not concentrations, were significantly higher in the BKPyVAN-pure subgroup than in the BKPyVAN-rejection-like subgroup. Further, analysis of ROC curves revealed that urinary ddcfDNA levels were capable of distinguishing proven BKPyVAN from type I TCMR.

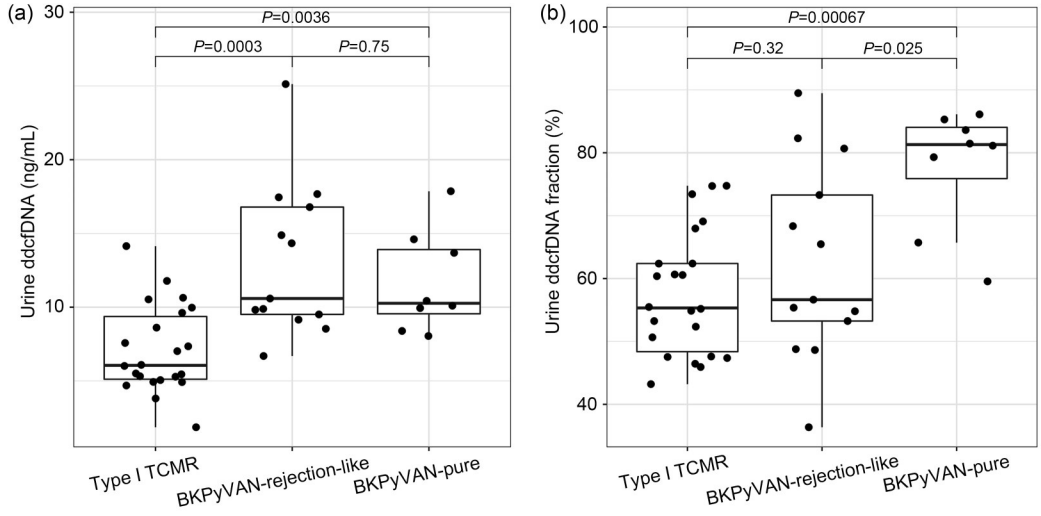

Fig. 4 Urinary ddcfDNA concentration (a) and fraction (b) in TCMR group and proven-BKPyVAN subgroups. Boxplot with bold line represents the median level. ddcfDNA: donor-derived cell-free DNA; TCMR: T cell-mediated rejection; BKPyVAN: BK polyomavirus-associated nephropathy.

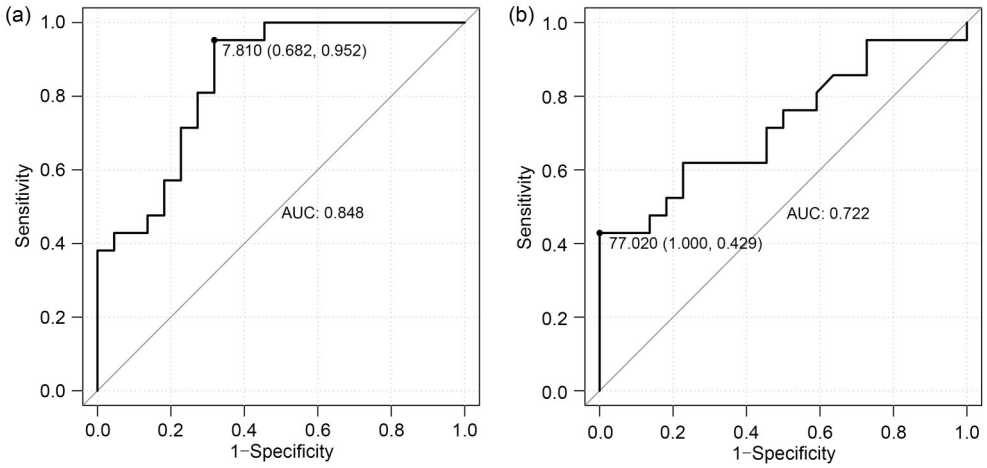

Fig. 5 ROC curve showing the concentration (a) and fraction (b) of ddcfDNA in distinguishing BKPyVAN from type I TCMR. ROC: receiver operating characteristic; AUC: area under the curve.

Since the clinical manifestations and morphological characteristics of PyVAN and type I TCMR are similar, mainly when SV40 is negative, the differential diagnosis of the two conditions is challenging. Previous studies have suggested several biomarkers as predictors of BKPyVAN, including specific gene expression profiles and urine proteomes associated with BKPyVAN, interrogated by genomics and proteomic studies (Sigdel et al., 2016; Zeng et al., 2016; Christakoudi et al., 2019). Other studies showed that the levels of chemokines such as C-X-C motif chemokine ligand 10 (CXCL10), C-C motif chemokine ligand 2 (CCL2), and CCL8 increase during the development of BKPyVAN, which highlights their values in monitoring BKPyVAN (Gniewkiewicz et al., 2019; Weseslindtner et al., 2020). However, the use of chemokines to distinguish PyVAN from TCMR is limited, since elevated levels are also observed in acute rejection (Romagnani and Crescioli, 2012; Blydt-Hansen et al., 2015). According to the study of Chen et al. (2019), morning urine specific gravity with a cut-off value of 1.010 distinguishes BKPyVAN from TCMR; however, urine specific gravity varies depending on numerous factors, which makes it less accurate for the diagnosis of BKPyVAN.

ddcfDNA is a sensitive non-invasive biomarker that indicates graft injury. Previous studies revealed the promising diagnostic value of plasma ddcfDNA levels in the diagnosis of acute rejection, especially ABMR (Gielis et al., 2019; Huang et al., 2019). In our study, plasma ddcfDNA fractions in the STA, type I TCMR, proven BKPyVAN, and possible PyVAN groups are comparable, an observation that concurs with the findings from previous studies which reported that plasma ddcfDNA was unable to discriminate graft injury caused by BKPyVAN or type I TCMR from protocol biopsies (Bloom et al., 2017; Gielis et al., 2019). This could be linked to the different histopathology changes in various types of rejection. ABMR and type II TCMR are associated with microvascular injury (Haas et al., 2018; Bloom, 2019), which is related to a significant elevation of ddcfDNA fractions in blood. In contrast, type I TCMR and BKPyVAN manifest tubulitis and interstitial inflammation that damage tubular epithelial cells, which might result in elevated urinary ddcfDNA levels. In this regard, we propose the monitoring of urinary ddcfDNA levels in such patients as being of particular importance.

The study by Sigdel et al. (2013) assessed the levels of urinary chromosome Y-specific ddcfDNA after kidney transplantation by digital PCR (dPCR) and reported no significant difference between BKPyVAN and TCMR. The detection of ddcfDNA in that study relied on chromosome Y, which is available only in female recipients who receive allografts from male donors, limiting the usefulness of this assay. We also investigated the impact of urinary ddcfDNA levels on discrimination of BKPyVAN in kidney transplant recipients. The urina sanguinis was collected for urinary ddcfDNA measurement (Chen et al., 2020). In a separate study, we found that urinary ddcfDNA fractions and concentrations in urina sanguinis, nocturia, and random urine were comparable (Zhou et al., 2021). As anticipated, both the concentrations and fractions of ddcfDNA in urine are capable of detecting acute graft injury.

Kant et al. (2020) measured plasma ddcfDNA levels in patients with BK viremia and BKPyVAN, and reported higher plasma ddcfDNA levels in biopsy-diagnosed BKPyVAN compared with BK viremia. Consistent with these results, Chen et al. (2020) also demonstrated that elevated urinary ddcfDNA levels could discriminate BKPyVAN in recipients with BK viruria. The progression of BK viral reactivation in the transplanted kidney is BKPyV viruria, BKPyV-DNAemia, and eventually BKPyVAN (Hirsch and Randhawa, 2019). BKPyVAN represents the most severe allograft involvement in BK infection, which leads to higher levels of ddcfDNA released by the death of allograft cells. Kant et al. (2020) have also noted higher ddcfDNA fractions in the subset of patients who had concomitant biopsy features that met Banff criteria for TCMR. Four of these patients had TCMR IA, two had TCMR IB, and one patient had high-grade rejection (TCMR IIA). The elevation of plasma ddcfDNA levels in TCMR IIA might correlate with the presence of intimal arteritis.

The plasma ddcfDNA fractions we measured ranged from 0 to 10%, much lower than the urinary ddcfDNA fractions, which were as high as 80%. Consequently, we suspected that detecting urinary ddcfDNA fractions would be of more importance in allograft function monitoring, especially for histopathological manifestations as tubular and interstitial injuries such as TCMR and BKPyVAN. Plasma ddcfDNA levels in BKPyVAN and type I TCMR were comparable, whereas the median concentrations and fractions of ddcfDNA in the urine of proven BKPyVAN and

possible PyVAN groups were significantly higher than those in the type I TCMR group. Further, we observed that urinary ddcfDNA is capable of discriminating BKPyVAN from type I TCMR. One possible explanation of differences in urinary ddcfDNA levels between BKPyVAN and type I TCMR is associated with the death of recipient immune cells. In TCMR, numerous inflammatory cells infiltrate into allografts due to immune activation; thus the cfDNA released by dead recipient immune cells would also be excreted into urine, resulting in elevated total amounts of cfDNA in such groups along with relatively lower urinary ddcfDNA levels. In BKPyVAN, on the other hand, the majority of cfDNAs are derived from the death of allograft cells, leading to higher urinary ddcfDNA concentrations and fractions. Therefore, our results suggest that urinary ddcfDNA levels could serve as a non-invasive assay for the diagnosis of BKPyVAN.

Five recipients in the possible PyVAN group received reduced immunosuppression therapy. The six-month follow-up study demonstrated gradual stabilization of graft function. Despite negative SV40 staining, high urinary ddcfDNA levels and the results of follow-up study consolidated the BKPyVAN diagnosis. Therefore, urinary ddcfDNA might contribute to overcoming the diagnostic puzzle of BKPyVAN created by the focal nature of virions in histopathology, which requires further study.

Still, it is challenging to identify whether BKPyVAN complicates allograft rejection, especially with type I TCMR, and whether cellular infiltration of allografts is induced by virions or alloantigens due to insufficient immunosuppression. Therefore, we further classified all 21 patients in the proven BKPyVAN group into two subgroups: BKPyVAN-rejection-like and BKPyVAN-pure. Our findings demonstrated that the urinary ddcfDNA concentrations of the BKPyVAN subgroups were comparable. However, the ddcfDNA fractions of the BKPyVAN-rejection-like subgroup were significantly lower than those of the BKPyVAN-pure subgroup, which were similar to those of the type I TCMR group. Additionally, we found that serum creatinine levels were comparable between BKPyVAN-rejection-like and BKPyVAN-pure subgroups ((188.0±75.1) μmol/L vs. (167.5±37.6) μmol/L, P=0.22; Fig. S2). This observation could be attributed to the fact that the total amount of cfDNA in urine increased (21.63 ng/mL in the BKPyVAN-rejection-like subgroup and 12.38 ng/mL in the BKPyVAN-pure subgroup, P<0.05) due to the elevated levels of recipient cfDNA generated by inflammatory cell infiltration of allografts. This subsequently lowered the relative levels of ddcfDNA in urine. We speculated that the death of allograft cells in the BKPyVAN-pure subgroup mainly resulted from viral infection. In the BKPyVAN-rejection-like subgroup, cellular infiltration triggered by alloantigens led to increases in total cfDNA amounts, resulting in lower urinary ddcfDNA fractions.

Reduced immunosuppression therapy was used in 21 patients with proven BKPyVAN. Three of 13 patients in the BKPyVAN-rejection-like subgroup had graft failure and returned to dialysis; two patients had increased creatinine levels; and the other eight patients had stable graft function. Only one in eight patients in the BKPyVAN-pure subgroup had increased creatinine levels, and the other seven recipients had stable graft function. The results of six-month follow-up revealed higher incidence of creatinine increase and graft loss in the BKPyVAN-rejection-like subgroup than in the BKPyVAN-pure subgroup after reduced immunosuppression therapy. Although no significant difference was found, the results demonstrated that urinary fractions of ddcfDNA were possibly beneficial to discriminate BKPyVAN complicated with rejection, and provided evidence for treatment and prognosis. However, urinary fraction of ddcfDNA did not help to distinguish the causes of cellular infiltration (virion or alloantigen).

The quantities of BKPyV in urine and plasma are closely related to BKPyVAN progression, and the definition thresholds have been widely studied. Urine BKPyV loads of >7 lg(copies/mL) are characterized as high-level viruria, and nearly half of patients with these levels developed BKPyV-DNAemia within 2–6 weeks (Hirsch and Randhawa, 2019). Persistence of plasma BKPyV DNA load of >3 lg(copies/mL) for more than three weeks, or plasma load of >4 lg(copies/mL), is considered indicators of increased incidence of biopsy-proven BKPyVAN (Hirsch and Randhawa, 2019). The development of BKPyV viruria to BKPyV-DNAemia and eventually to PyVAN, due to persistent infection, represents its progression in kidney transplantation (Hirsch et al., 2002). In the present study, 12 (57.1%) patients with proven BKPyVANs had already developed nephropathy, even though a low level of plasma BKPyV DNA load (<1.0×10^3 copies/mL) was detected. This is consistent with the findings by Agrawal et al.

(2017), who reported that three in five (60%) proven PyVAN patients had low levels of plasma BKPyV DNA load. These observations provided new evidence that low levels of plasma BKPyV DNA load may not exclude BKPyVAN. Additionally, we compared the diagnostic potentials of urine ddcfDNA and urine and plasma BKPyV-DNA loads in discriminating proven BKPyVAN. The true positive rates of cut-off values of urine and plasma BKV-DNA loads, urinary ddcfDNA concentration, and urinary ddcfDNA fraction were 71.4%, 33.3%, 95.2%, and 42.9%, respectively (Fig. S3). These results suggest urinary ddcfDNA as a promising diagnostic biomarker for BKPyVAN; it appears to be superior to urine/plasma BK viral load.

We caution that the present study was limited by the relatively small number of participants enrolled in a single center. Because of the low incidence rate of BKPyVAN, only 21 proven BKPyVAN cases were included. Therefore, multi-center studies with larger numbers of cases are desirable to define evidence-based thresholds of urinary ddcfDNA.

5 Conclusions

We observed that patients with acute graft injury have a distinctly higher level of urinary ddcfDNA, suggesting the diagnostic potential of urinary ddcfDNA from a precision medicine perspective. Levels of urinary ddcfDNA were capable of discriminating BKPyVAN from type I TCMR, whereas urinary ddcfDNA fraction was found to be correlated with BKPyVAN patients with rejection-like histopathology on the biopsy, an indicator which could guide clinical decision-making. These findings suggest that urinary ddcfDNA is a non-invasive biomarker for monitoring graft injury, especially for BKPyVAN.

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Author contributions

Jia SHEN and Rending WANG contributed to study design and data interpretation. Luying GUO and Rending WANG contributed to data analysis, data interpretation, and writing. Wenhua LEI, Shuaihui LIU, Pengpeng YAN, Jingyi ZHOU, and Qin ZHOU contributed to data collection. Haitao LIU, Feng LIU, and Tingya JIANG performed data analysis. Huiping WANG, Jianyong WU, and Jianghua CHEN contributed to manuscript-writing advice. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Jia SHEN, Luying GUO, Wenhua LEI, Shuaihui LIU, Pengpeng YAN, Haitao LIU, Jingyi ZHOU, Qin ZHOU, Feng LIU, Tingya JIANG, Huiping WANG, Jianyong WU, Jianghua CHEN, and Rending WANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

References

Agbor-Enoh S, Tunc I, de Vlaminck I, et al., 2017. Applying rigor and reproducibility standards to assay donor-derived cell-free DNA as a non-invasive method for detection of acute rejection and graft injury after heart transplantation. *J Heart Lung Transplantat*, 36(9):1004-1012. https://doi.org/10.1016/j.healun.2017.05.026

Agrawal N, Echenique IA, Meehan SM, et al., 2017. Variability in assessing for BK viremia: whole blood is not reliable and plasma is not above reproach—a retrospective analysis. *Transpl Int*, 30(7):670-678. https://doi.org/10.1111/tri.12951

Ahuja M, Cohen EP, Dayer AM, et al., 2001. Polyoma virus infection after renal transplantation: use of immunostaining as a guide to diagnosis. *Transplantation*, 71(7):896-899. https://doi.org/10.1097/00007890-200104150-00013

Bloom RD, 2019. Using (cell-free) DNA to incriminate rejection as the cause of kidney allograft dysfunction: do we have a verdict? *Am J Transplant*, 19(6):1609-1610. https://doi.org/10.1111/ajt.15338

Bloom RD, Bromberg JS, Poggio ED, et al., 2017. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*, 28(7):2221-2232. https://doi.org/10.1681/ASN.2016091034

Blydt-Hansen TD, Gibson IW, Gao A, et al., 2015. Elevated urinary CXCL10-to-creatinine ratio is associated with subclinical and clinical rejection in pediatric renal transplantation. *Transplantation*, 99(4):797-804. https://doi.org/10.1097/tp.0000000000000419

Buettner M, Xu H, Böhme R, et al., 2012. Predominance of T_H2 cells and plasma cells in polyoma virus nephropathy: a role for humoral immunity. *Hum Pathol*, 43(9):1453-1462.

https://doi.org/10.1016/j.humpath.2011.11.006

Chen XT, Wang ZY, Huang Y, et al., 2019. Combined detection of urine specific gravity and BK viruria on prediction of BK polyomavirus nephropathy in kidney transplant recipients. *Chin Med J*, 133(1):33-40. https://doi.org/10.1097/cm9.0000000000000579

Chen XT, Chen WF, Li J, et al., 2020. Urine donor-derived cell-free DNA helps discriminate BK polyomavirus-associated nephropathy in kidney transplant recipients with BK polyomavirus infection. *Front Immunol*, 11:1763. https://doi.org/10.3389/fimmu.2020.01763

Christakoudi S, Runglall M, Mobillo P, et al., 2019. Development of a multivariable gene-expression signature targeting T-cell-mediated rejection in peripheral blood of kidney transplant recipients validated in cross-sectional and longitudinal samples. *EBioMedicine*, 41:571-583. https://doi.org/10.1016/j.ebiom.2019.01.060

de Vlaminck I, Martin L, Kertesz M, et al., 2015. Noninvasive monitoring of infection and rejection after lung transplantation. *Proc Natl Acad Sci USA*, 112(43):13336-13341. https://doi.org/10.1073/pnas.1517494112

Dharnidharka VR, Cherikh WS, Abbott KC, 2009. An OPTN analysis of national registry data on treatment of BK virus allograft nephropathy in the United States. *Transplantation*, 87(7):1019-1026. https://doi.org/10.1097/TP.0b013e31819cc383

Drachenberg CB, Papadimitriou JC, Hirsch HH, et al., 2004. Histological patterns of polyomavirus nephropathy: correlation with graft outcome and viral load. *Am J Transplant*, 4(12):2082-2092. https://doi.org/10.1046/j.1600-6143.2004.00603.x

Drachenberg CB, Papadimitriou JC, Chaudhry MR, et al., 2017. Histological evolution of BK virus-associated nephropathy: importance of integrating clinical and pathological findings. *Am J Transplant*, 17(8):2078-2091. https://doi.org/10.1111/ajt.14314

Gielis EM, Ledeganck KJ, Dendooven A, et al., 2019. The use of plasma donor-derived, cell-free DNA to monitor acute rejection after kidney transplantation. *Nephrol Dial Transplant*, 35(4):714-721. https://doi.org/10.1093/ndt/gfz091

Gniewkiewicz MS, Czerwińska M, Gozdowska J, et al., 2019. Urinary levels of CCL2 and CXCL10 chemokines as potential biomarkers of ongoing pathological processes in kidney allograft: an association with BK virus nephropathy. *Pol Arch Intern Med*, 129(9):592-597. https://doi.org/10.20452/pamw.14926

Haas M, Loupy A, Lefaucheur C, et al., 2018. The Banff 2017 Kidney Meeting Report: revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant*, 18(2):293-307. https://doi.org/10.1111/ajt.14625

Hirsch HH, Randhawa PS, 2019. BK polyomavirus in solid organ transplantation—Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. *Clin Transplant*, 33(9):e13528. https://doi.org/10.1111/ctr.13528

Hirsch HH, Knowles W, Dickenmann M, et al., 2002. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med*, 347(7):488-496. https://doi.org/10.1056/NEJMoa020439

Hirsch HH, Brennan DC, Drachenberg CB, et al., 2005. Polyomavirus-associated nephropathy in renal transplantation: interdisciplinary analyses and recommendations. *Transplantation*, 79(10):1277-1286. https://doi.org/10.1097/01.tp.0000156165.83160.09

Huang E, Sethi S, Peng A, et al., 2019. Early clinical experience using donor-derived cell-free DNA to detect rejection in kidney transplant recipients. *Am J Transplant*, 19(6):1663-1670. https://doi.org/10.1111/ajt.15289

Kant S, Bromberg J, Haas M, et al., 2020. Donor-derived cell-free DNA and the prediction of BK virus-associated nephropathy. *Transplant Direct*, 6(11):e622. https://doi.org/10.1097/txd.0000000000001061

Knowles WA, Pipkin P, Andrews N, et al., 2003. Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. *J Med Virol*, 71(1):115-123. https://doi.org/10.1002/jmv.10450

Mannon RB, Hoffmann SC, Kampen RL, et al., 2005. Molecular evaluation of BK polyomavirus nephropathy. *Am J Transplant*, 5(12):2883-2893. https://doi.org/10.1111/j.1600-6143.2005.01096.x

Pang XL, Doucette K, LeBlanc B, et al., 2007. Monitoring of polyomavirus BK virus viruria and viremia in renal allograft recipients by use of a quantitative real-time PCR assay: one-year prospective study. *J Clin Microbiol*, 45(11):3568-3573. https://doi.org/10.1128/JCM.00655-07

Romagnani P, Crescioli C, 2012. CXCL10: a candidate biomarker in transplantation. *Clin Chim Acta*, 413(17-18):1364-1373. https://doi.org/10.1016/j.cca.2012.02.009

Schütz E, Fischer A, Beck J, et al., 2017. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. *PLoS Med*, 14(4):e1002286. https://doi.org/10.1371/journal.pmed.1002286

Shen J, Guo LY, Yan PP, et al., 2020. Prognostic value of the donor-derived cell-free DNA assay in acute renal rejection therapy: a prospective cohort study. *Clin Transplant*, 34(10):e14053. https://doi.org/10.1111/ctr.14053

Sigdel TK, Vitalone MJ, Tran TQ, et al., 2013. A rapid noninvasive assay for the detection of renal transplant injury. *Transplantation*, 96(1):97-101. https://doi.org/10.1097/TP.0b013e318295ee5a

Sigdel TK, Gao YQ, He JT, et al., 2016. Mining the human urine proteome for monitoring renal transplant injury. *Kidney Int*, 89(6):1244-1252. https://doi.org/10.1016/j.kint.2015.12.049

Weseslindtner L, Hedman L, Wang YL, et al., 2020. Longitudinal assessment of the CXCL10 blood and urine concentration in kidney transplant recipients with BK polyomavirus replication—a retrospective study. *Transpl Int*, 33(5):555-566. https://doi.org/10.1111/tri.13584

Yapici Ü, Kers J, Slavujevic-Letic I, et al., 2016. Intragraft blood dendritic cell antigen-1-positive myeloid dendritic cells increase during BK polyomavirus-associated nephropathy. *J Am Soc Nephrol*, 27(8):2502-2510. https://doi.org/10.1681/ASN.2015040442

Zeng G, Huang Y, Huang Y, et al., 2016. Antigen-specificity of T cell infiltrates in biopsies with T cell-mediated rejection and BK polyomavirus viremia: analysis by next generation sequencing. *Am J Transplant*, 16(11):3131-3138. https://doi.org/10.1111/ajt.13911

Zhou Q, Liu F, Guo LY, et al., 2021. A novel urine cell-free DNA preservation solution and its application in kidney transplantation. *Nephrology*, 26(8):684-691. https://doi.org/10.1111/nep.13884

Zhou Y, Yang GD, Liu HT, et al., 2019. A noninvasive and donor-independent method simultaneously monitors rejection and infection in patients with organ transplant. *Transplant Proc*, 51(6):1699-1705. https://doi.org/10.1016/j.transproceed.2019.04.051

Supplementary information

Figs. S1–S3