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BK polyomavirus microRNA expression and sequence variation in polyomavirus-associated nephropathy

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Highlights

- BKPyV miRNA expression and TCR sequence variation were analysed in PyVAN patients.
- Minor rearranged BKPyV populations were frequently detected in most patients.
- Archetype BKPyV strains were predominant in all patients and seem to associate with PyVAN.
- miRNAs were frequently detected and expressed at high levels in PyVAN patients.

- miRNA levels were also elevated in the presence of rearranged viral strains.

Abstract

Background. BK polyomavirus (BKPyV) infection is a common asymptomatic viral infection in the general population. Severe complications are seen in immunocompromised individuals, such as polyomavirus-associated nephropathy (PyVAN) in renal transplant recipients. Information on BKPyV microRNA expressions is scarce, although polyomavirus-encoded microRNAs have been shown to control viral replication and assist in immune evasion. Whereas the pathogenic role of rearrangements in JC polyomavirus has been well established, little is known about BKPyV rearrangements in PyVAN.

Objectives. To assess viral microRNA expression and transcriptional control region (TCR) sequence variation in PyVAN patients.

Study design. bkv-miR-B1-3p and bkv-miR-B1-5p microRNA expression was quantified in 55 plasma samples from 9 PyVAN patients and 2 controls using specific miRNA assays. TCR architectures among the viral populations in each patient were characterized by massive parallel sequencing.

Results. bkv-miR-B1-3p and bkv-miR-B1-5p miRNA expression was established in 85.5% and 98.2% of samples, respectively. On average, an 8.9-fold (bkv-miR-B1-3p) and 8.7-fold (bkv-miR-B1-5p) higher expression levels were detected in PyVAN patients as compared to controls.

Rearranged BKPyV strains with duplications and deletions were detected in 7/9 PyVAN patients, but 77.6–99.9% of all sequence reads in all samples represented archetype strains.

Conclusions. The frequent detection and increased expression of miRNAs suggest involvement in PyVAN pathogenesis. Despite the predominance of archetype BKPyV strains, the frequent detection of minor rearranged viral populations urges further study on their role in severe kidney

disease. Our results suggest that miRNA expression is increased in PyVAN patients, as well as in the presence of rearranged viral strains.

Keywords: BKPyV, miRNA, TCR, PyVAN, rearrangements, sequence variation

1. Background

Human BK polyomavirus (BKPyV) is a common DNA virus with overall 80% seroprevalence [1-3]. BKPyV is encountered in early childhood, after which asymptomatic lifelong persistence is established in the renourinary tract [4, 5]. In immunocompromised individuals, particularly in renal transplant recipients, reactivation of latent BKPyV may cause severe complications [4]. Because up to 10% of renal transplant recipients develop polyomavirus-associated nephropathy (PyVAN), these patients are screened for BKPyV viremia and viruria [6]. Presumptive PyVAN diagnosis can be made if high BKPyV load in plasma or urine ($>10^4$ or $>10^7$ copies/mL, respectively) for more than three weeks is observed [6], but histological examination of allograft biopsy and immunohistochemical SV40 large T antigen staining are used for definitive diagnosis [7]. Because no polyomavirus-specific treatments are currently available, the treatment of emerging PyVAN is based on reduction of immunosuppression [8, 9].

Asymptomatic primary BKPyV infections are caused by archetype viral strains, such as the BKPyV strain WW (GenBank accession number M15987.1), circulating in the population [10, 11].

Importantly, the archetype noncoding control region (NCCR) comprises blocks O (142 bp, containing viral *ori*), P (68 bp), Q (39 bp), R (63 bp) and S (63 bp). Blocks P, Q, R and S constitute the transcriptional control region (TCR) [12] containing promoters and enhancer elements for early and late viral genes [13, 14]. During BKPyV replication various NCCR rearrangements through deletions and duplications may take place [15, 16]. For the closely related JC polyomavirus (JCPyV) it has been well established, that NCCR rearrangements are associated with the

development of progressive multifocal leukoencephalopathy (PML) [17-19]. BKPyV replication is largely dependent on the activity of the NCCR, which in turn is influenced by the TCR architecture [14, 20-22]. Although rearranged BKPyV strains have been identified in PyVAN patients [20, 23, 24], the association of TCR modifications in the pathogenesis of BKPyV-associated kidney diseases is not yet clearly understood. microRNAs (miRNAs) are small noncoding RNA molecules that control gene expression by binding to mRNAs and by guiding their degradation [25, 26]. Similar to the closely related JCPyV, also BKPyV encodes two miRNAs, bkv-miR-B1-3p (3p miRNA) and bkv-miR-B1-5p (5p miRNA), that further regulate viral replication by reducing the expression of the large T antigen [27, 28]. The 3p miRNA, which shares identical sequence with JCPyV 3p miRNA [29], also assists the virus in evading the host immune system by reducing recognition of virus-infected cells [30, 31]. Studies on the presence and disease association of JCPyV miRNAs have shown biomarker potential for the JCPyV specific jcv-miR-J1-5p in the assessment of PML risk as well as for the development of colorectal cancer [32-35]. Previous studies have reported elevated BKPyV miRNA expression levels in PyVAN patients [28, 36, 37], but the role of BKPyV miRNAs in the development of PyVAN is still not well understood.

2. Objectives

To analyse BKPyV miRNA expression and TCR sequence variation in plasma of patients with definitive or presumptive PyVAN, and to assess whether these viral factors show potential as useful biomarkers in prognosis and monitoring of renal transplant recipients.

3. Study design

3.1 Patients and samples

The study population was comprised of nine 45 to 72-year-old (median age 62.1) renal transplant recipients with high-level BKPyV viremia ($> 10^4$ copies/mL) detected by routine screening performed at 3, 6, 9, and 12 months after kidney transplantation. Additional plasma samples were collected in routine follow-up of viremia after reduction of immunosuppression at two to four week intervals until viremia was cleared. Altogether 53 plasma samples (2-12 samples per patient) were included in the study. TCR architectures were determined from one sample per patient taken after transplantation. For this, samples with high viral loads (8500–217000 copies/mL) and producing a strong amplicon in PCR were selected for sequencing, based on the assumption that enhanced replication activity may lead to viral sequence variation. A written informed consent was obtained from all patients. The control group included two plasma samples from two renal transplant patients (a 62-year-old female, and a 69-year-old male) with stable graft function and no evidence of BKPyV viremia or viruria in routine screening protocol. Viral load of all samples was determined as previously described [38].

3.2 miRNA assays

miRNA expression was quantified using commercially available TaqMan miRNA assays (Thermo Fisher Scientific, Waltham, MA, USA) targeting the bkv-miR-B1-5p (assay ID: 007796_mat), bkv-miR-B1-3p (006801_mat) and cel-miR-39-3p (000200) miRNAs. The extracted samples were spiked with *Caenorhabditis elegans* cel-39-3p miRNA to control for reverse transcription and miRNA amplification. Detailed description of RNA extractions and miRNA assays are presented in Supplementary material. If amplification was detected in two or three of the replicates, the sample was interpreted as positive and a mean threshold cycle (Ct) was calculated. Fold change of miRNA expression in patients versus controls was calculated according to the $2^{-\Delta\Delta Ct}$ method [39] by comparing the results of each individual patient to the mean of all controls.

3.3 Characterization of BKPyV TCR regions

The aim was to sequence complete TCR regions (blocks P, Q, R and S, as described in [21]) in one continuous read using the MiSeq massive parallel sequencing platform (Illumina Inc., San Diego, CA). The forward primer was located within the origin of replication (5'- AGA GGC GGC CTC GGC CTC TTA T -3', nucleotides 102-123 according to BKPyV Dunlop strain; GenBank accession number V01108.1), and the reverse primer at the 5' end of the agnoprotein gene (5'- AGA AGC TTG TCG TGA CAG CTG G -3', nucleotides 399-419), yielding a 319 bp amplicon. TCR amplification and sequence characterization are presented in detail in Supplementary material. In addition to TCR architecture, binding sites for AP1, NFAT, NF1, Sp1 and p53 transcription factors were inspected, as these have been shown to bind to the BKPyV TCR region [13, 21, 40, 41].

4. Results

4.1 Patient characteristics

Of the study patients, 7/9 had definitive biopsy-confirmed BKPyVAN with positive staining for SV40 large T antigen, whereas 2/9 had presumptive BKPyVAN with negative staining for SV40 at the time of BKPyV viremia. Two of the biopsy-confirmed patients had graft dysfunction at the time of established BKPyV viremia. Viremia was successfully treated and cleared in all patients with reduction of immunosuppression. Detailed patient characteristics are presented in Table 1.

4.2 Quantification of miRNAs

Altogether 55 plasma samples were analysed for miRNAs. 5p miRNA expression was established in 54/55 samples (98.2%) with an average Ct value of 39.2, and 3p miRNA in 47/55 samples

(85.5%) with an average Ct value of 38.4. Expression of both miRNAs was also established in BKPyV DNA negative control patients, with average Ct values of 41.7 for 5p and 40.0 for 3p. BKPyV miRNA fold changes and viral loads varied between samples (Table 2). On average, an 8.7-fold higher expression of 5p miRNA was observed in PyVAN patients as compared to controls (2.9–19.1-fold in individual patients). The expression of 3p miRNA was on average 8.9-fold higher among patients as compared to controls. In 2/9 patients 3p miRNA expression was considerably high (13.6-fold in patient 2, and 51.5-fold in patient 5), whereas in 7/9 patients the fold changes were more modest at the most (0.9–5.0). Robust fold changes among biopsy-confirmed PyVAN patients as compared to presumptive PyVAN patients were also established for both miRNAs (mean fold change 2.2 for 5p miRNA and 8.0 for 3p miRNA). A negative correlation was established between viral load and normalized 5p (-0.61) and 3p (-0.62) miRNA cycle counts among all samples, indicating a positive correlation between viral load and the amount of both miRNAs. When correlations were inspected in each patient individually, a positive correlation was detected for 5p miRNA in patients 3 (1.0), 7 (0.4) and 8 (1.0), and for 3p miRNA in patient 8 (1.0), indicating a negative correlation between viral load and the amount of miRNA.

Of note, the amount of miRNA amplification cycles was increased from 40 cycles recommended by the manufacturer to 45 cycles for better assessment of late amplification. For 5p miRNA, 20/53 patient samples and 2/2 controls would have remained negative with 40 cycles of amplification. For 3p miRNA, positive signals were detected above 40 cycles in 19/53 patient samples and in 1/2 controls. All results for BKPyV and cel-39-3p miRNA detection from individual replicate wells are presented in Supplementary Table S1.

4.3 TCR sequence characteristics

The majority (77.6% to 97.6%) of all sequence reads in all samples represented archetype TCR regions (Table 2). From patients 3 and 8 exclusively (99.9%) archetype TCR regions were

characterized. Nucleotide numbering is according to the reverse complement of archetype BKPyV strain WW TCR sequence (GenBank accession number M15987.1, nucleotides 182-414). It should also be noted that sequence read proportions are only indicative and cannot be taken as a direct measure of viral strains. The archetype strains of patients 4, 5 and 8 had a 1-bp insertion between nucleotides 400...401, while the archetype strains of all other patients had identical length with WW TCR. A total of nine single nucleotide polymorphisms (SNPs, nucleotides 199, 200, 212, 292, 293, 326, 328, 369, 373) were identified when compared to the WW strain (Figure 1), and five of these (T212C, C326G, G328A, A369G, T373A) were located in the binding sites for transcription factor NF-1.

From six biopsy-confirmed BKPyVAN patients and from one presumptive BKPyVAN patient modified TCR regions were identified (Table 2, Figure 2). The proportion of modified TCR sequence reads was considerable in two BKPyVAN patients (15.9% for patient 6 and 22.4% for patient 7), but for the other patients the proportion was approximately 3.0%. Although the modifications were unique for each patient, similarities in TCR architectures were observed. From four patients, TCR regions with P-(Q)-(P)-Q-R-S architecture (brackets indicate a truncated block) containing duplications of partial P and Q blocks were identified. The modified BKPyV strains of patients 1, 4, 6 and 9 had 98-bp (P6-68 and Q1-35), 41-bp (P61-68 and Q1-33), 50-bp (P24-68 and Q1-5) and 82-bp (P23-68 and Q1-36) duplications, respectively. In two patients a P-(Q)-(R)-S architecture with partial Q and R block deletions was characterized. Patient 2 had a 58-bp deletion (Q37-39 and R1-55) and patient 7 a 41-bp (Q26-39 and R1-27) deletion. From patient 5 with graft dysfunction and biopsy-confirmed BKPyVAN three distinct viral strains with different TCR modifications were characterized. One viral strain had a P-(Q)-(P)-Q-R-S architecture with a 52-bp duplication of partial P (P22-68) and Q (Q1-5) blocks, one strain a P-Q-(R)-S architecture with partial R block (R1-51) deletion, and one strain a unique P-Q-(P)-Q-R-S architecture with 86-bp duplication of truncated P (P22-68) and a complete Q block. The proportions of modified TCR

sequence reads were 1.2%, 5.9% and 1.2%, respectively. No obvious association between the TCR sequence variation and clinical characteristics was established.

Duplications and deletions of archetypal P and Q blocks affected the number of binding sites of transcription factors AP-1, NFAT, NF-1, Sp1 and p53 (Table 3). In TCR regions with duplications, additional binding sites were observed, while deletions resulted in loss of binding sites for NF-1 or Sp1.

5. Discussion

This study assessed BKPyV microRNA expression and sequence variation of the viral regulatory region in severe BKPyV-associated disease among renal transplant recipients. Expression of viral 3p and 5p miRNAs was established in the majority of samples, and sequencing revealed the presence of both archetype and rearranged strains. The predominance of archetype TCR sequences observed in this study suggests that archetype rather than rearranged BKPyV strains are associated with the development of PyVAN. In this study, TCR regions were characterized from one sample collected at the peak viral load period, assuming that due to active viral replication possible rearrangements may have arisen. To further assess the emergence and impact of rearrangements on viral replication, miRNA expression and PyVAN development, several samples at different time points, and also before the onset of the disease should be analysed. Minor modifications such as single-nucleotide deletions or insertions seem to occur upon BKPyV reactivation in the kidneys [42], and such “archetype-like strains” are the most prevalent [10, 21]. Archetype-like strains, possessing SNPs and one-nucleotide insertions as compared to the archetypal WW strain, were the most prominent also in the present study. All except two of these (C326G, G328A) had been previously identified in immunosuppressed patients without BKPyV-related disease [14, 21, 42]. The two newly identified mutations were located in the NF-1 binding site.

In 7/9 patients, modified TCR sequences were additionally identified. Similar modifications have been identified in the Gardner strain [43], which is the prototype rearranged strain of BKPyV, as well as in rearranged strains from renal transplant patients with PyVAN [20, 23, 24, 44] and from a patient with tubulointerstitial nephritis [45]. Both patients with graft dysfunction had R block deletions, and in patient 5 with three distinct modified BKPyV strains, the strain with partial R block deletion had higher read counts than the other two modified strains. These particular deletions might be associated with more severe kidney problems, but it cannot be established based on only two patients. Furthermore, all rearranged strains had retained the archetypal S block containing hormone-response elements that may enhance productive BKPyV infection [46]. All rearrangements affected the number of AP-1, NFAT, NF-1, Sp1 or p53 binding sites, suggesting that these transcription factors may modulate BKPyV replication and presumably have a role in the development of PyVAN. AP-1, NFAT, NF-1 and Sp1 have all been shown to regulate the transcription of BKPyV early and late genes *in vitro* [40, 41, 47, 48]. Further studies, including RNASeq or ChIPSeq approaches are needed to analyse the true impact of rearrangements and modifications in transcription factor binding sites on BKPyV gene expression, and to assess the biological relevance of these alterations.

Similar to a previous study [36], we observed a positive correlation between viral loads and the amount of miRNAs. Viral miRNAs were also detected in some BKPyV-DNA-negative samples, which could be due to the 45 amplification cycles allowing the detection of very low miRNA levels. Virus-encoded miRNAs have proven to be essential in the establishment of viral persistence and development of polyomavirus-associated chronic diseases [49, 50]. Previous studies have shown that BKPyV miRNAs limit viral replication through inhibition of large T antigen (LTA_g) expression [27, 28]. A study in a natural host cell model showed that high miRNA expression levels specifically controlled the replication of archetype BKPyV strains, but not the replication of rearranged variants with high LTA_g expression levels [27]. In our study, high viral loads of predominantly archetype BKPyV strains were detected along with high 5p miRNA expression

levels, suggesting that viral replication was not restricted. However, the correlations in individual patients suggest that viral replication was limited if only archetype BKPyV strains were present. This raises the question whether coexistence of rearranged variants, albeit in minority, may enable efficient virus replication with simultaneous high 5p miRNA expression. Coexistence of both rearranged and archetype strains in the plasma of PyVAN patients has been reported in a previous study, where the emergence of rearrangements was linked to increased viral replication [20]. For 3p miRNA the expression levels were considerably lower in most patients. Intriguingly, the patient with three modified BKPyV strains had extremely high 3p miRNA expression as compared to controls. By contrast, in patients harbouring solely archetype strains 3p miRNA expression levels were similar to controls. 3p miRNA has been shown to reduce the immune recognition of virus-infected cells, thus enabling viral persistence [30, 31]. Although low 3p miRNA expression levels do not support an essential role in the development of PyVAN, we show here that elevated and even substantially increased 3p levels may be detected in the presence of modified BKPyV strains. Previous studies have investigated BKPyV miRNA expression in plasma [28, 36] and in urinary exosomes [37] of PyVAN patients, and reported increased miRNA expression in PyVAN patients. These findings together with ours suggest that BKPyV miRNAs could have biomarker potential in monitoring renal transplant recipients, although their role in the development of PyVAN is still unclear and urges further studies.

Severe BKPyV disease is always associated with reactivation and enhanced replication of persistent virus. To our knowledge, this is the first study preliminary investigating both the expression of BKPyV-encoded miRNAs and the presence of modified BKPyV strains in the plasma of PyVAN patients. The predominance of archetype BKPyV strains in all our patients suggests that they are sufficient for PyVAN pathogenesis. Yet, our results also imply that even minor populations of rearranged viral strains may have an impact on the balance of miRNA expression and viral replication. In accordance with a previous study [36], significantly elevated BKPyV miRNA expression levels were detected in biopsy-confirmed PyVAN patients as compared to the controls

or presumptive PyVAN patients. Although host factors regulating polyomavirus miRNA expression and function have not been identified, we cannot exclude the possibility that the immunosuppressed status of our patients might have interfered with the regulation of viral replication by viral miRNAs. Despite our small study population, the frequent detection of viral miRNAs and their increased expression levels in PyVAN patients as well as in the presence of rearranged viral strains point to a role of viral miRNAs in the development of PyVAN. Although these results are preliminary and need further confirmation, they suggest that miRNA expression could provide an additional biomarker in kidney disease.

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Competing interests: None to declare

Ethical approval: The Helsinki University Hospital Ethics Committee approved the study (decision number 54/13/03/00/2015), and a written informed consent was obtained from all patients.

Table and figure captions

Table 1. Clinical patient characteristics.

Table 2. Results for miRNA detection and TCR characterization of nine PyVAN patients.

Table 3. Number of AP-1, NFAT, NF-1, Sp1 and p53 transcription factor binding sites in modified BKPyV strains.

Figure 1. TCR sequence comparison of archetype BKPyV strains. Archetype TCR regions from nine PyVAN patients (P1-9_arc, 'P' indicating patients 1 through 9 and 'arc' describing an archetype-like strain) were aligned with WW archetype BKPyV strain (BKPyV_WW). Dark and light blue colours indicate either purine (A, G) or pyrimidine (C, T) identity, and a dash indicates a gap. Black arrows indicate the starting position of sequence blocks. Black dots represent ten nucleotide intervals. Nucleotide numbering is according to archetype WW strain. A multiple sequence alignment tool (Kalign) and a multiple alignment viewer tool (MView) were used.

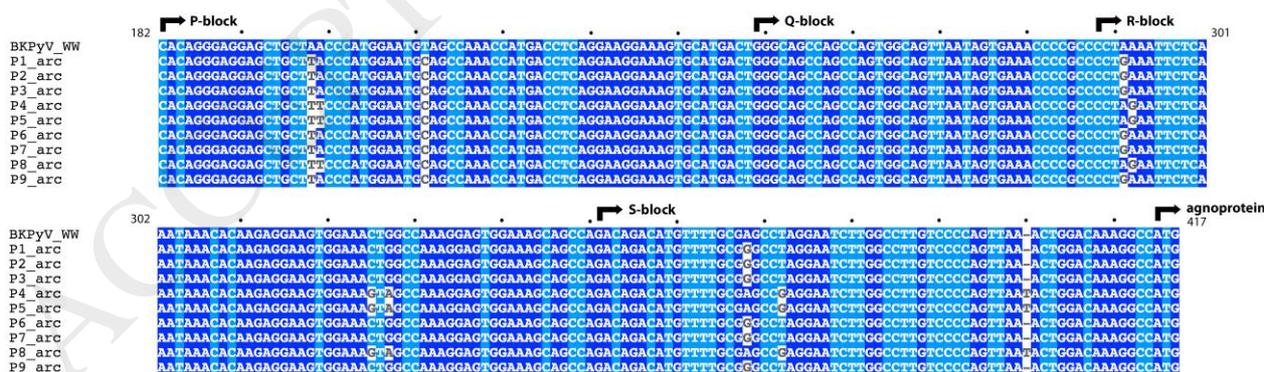
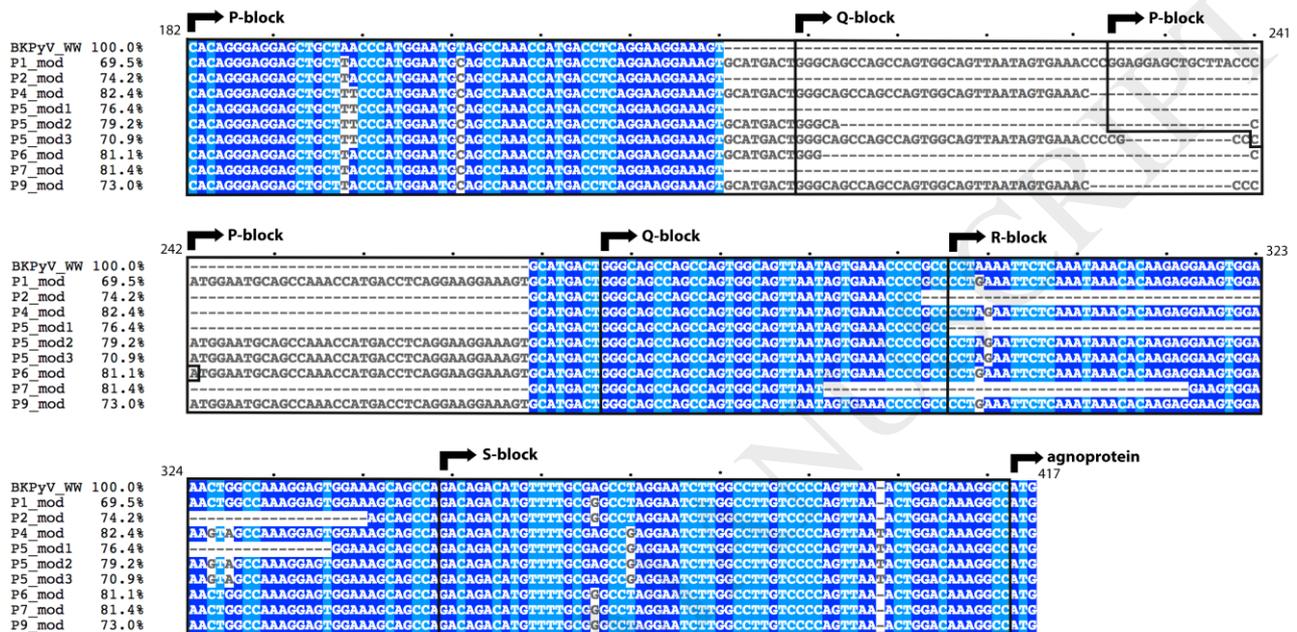


Figure 2. TCR sequence comparison of modified BKPyV strains. Modified TCR regions from seven (P1-9_mod, 'P' indicating the patient and 'mod' describing a modified strain) BKPyVAN patients were aligned with WW archetype BKPyV strain (BKPyV_WW). Dark and light blue

colours indicate either purine (A, G) or pyrimidine (C, T) identity, and a dash indicates a gap. Black arrows indicate the starting position of sequence blocks, which are defined with black lines. Black dots represent ten nucleotide intervals. Nucleotide numbering is according to the archetype WW strain. A multiple sequence alignment tool (Kalign) and a multiple alignment viewer tool (MView) were used.



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Table 1. Clinical patient characteristics.

Patient ID	IS ^a	Time of BKPyV viremia (months) ^b	Peak viral load in plasma (copies/mL)	Graft dysfunction at viremia diagnosis	Treatment of BKPyV viremia	Biopsy finding (grading) ^c	Follow-up (months)	Last eGFR (mL/min)
1	CsA	3	58600	No	MMF cessation, CsA dose reduction	PyVAN (A)	48	60
2	CsA	3	401000	No	MMF cessation, CsA dose reduction	PyVAN (A)	53	47
3	CsA	12	26700	No	No treatment	No PyVAN in biopsy	18	34
4	Tac	6	89100	No	MMF cessation	PyVAN (B)	91	49
5	CsA	6	256000	Yes	MMF cessation, CsA dose reduction	PyVAN (A)	60	93
6 ^d	CsA	3	11200	No	MMF cessation, CsA dose reduction	PyVAN (A)	45	11
7 ^e	CsA	5	29600	Yes	MMF cessation	PyVAN (B)	26	60
8	CsA	6	2700	No	MMF cessation	PyVAN (A)	72	68
9	Tac	12	102800	No	MMF dose reduction	No PyVAN in biopsy	14	47
10 ^f	Tac	-	-	-	-	-	50	26
11 ^f	CsA	-	-	-	-	-	26	92

^a Immunosuppression was a combination of cyclosporine or tacrolimus, mycophenolate, and steroids. Induction therapy was not used in any of these patients.

^b Time of BKPyV viremia diagnosis in months after kidney transplantation.

^c PyVAN stages and diagnosis of presumptive and definitive PyVAN were defined as described in [51].

^d After reduction of immunosuppression, patient 6 developed chronic antibody-mediated rejection and severe irreversible graft dysfunction and is approaching end-stage kidney failure 45 months after transplantation.

^e Patient 7 with a well-functioning kidney died at 26 months after transplantation due to causes unrelated to BKPyV (severe peripheral vascular disease and septic infection after limb amputation).

^f Controls, renal transplant recipients with stable graft function and no evidence of BKPyV viremia or viruria.

IS = immunosuppressive treatment; CsA = Cyclosporine-based immunosuppression; Tac = tacrolimus-based immunosuppression; MMF = mycophenolate; PyVAN = polyomavirus-associated nephropathy; eGFR = glomerular filtration rate, calculated with the CKD-EPI equation [52].

Table 2. Results for miRNA detection and TCR characterization of nine PyVAN patients.

Patient	Sample	Viral load (copies/mL)	3p ΔCt^a	5p ΔCt^a	3p fold change ^b	5p fold change ^b	Graft status	PyVAN status	TCR architecture (subtype/ subgroup)	TCR length (bp)	Sequence identity with archetype TCR (%)	Proportion of all sequences in sample (%)
1	a	–	24.7	22.5								
	b	58600	22.3	24.0					WW-like	233	98.6	96.5
	c	48700	21.6	21.4				(I/b-2)				
	d	29550	21.9	23.1								
	e*	45300	26.9	22.8	3.0	22.8	Stable	Biopsy- confirmed				
	f	19660	21.9	23.1								
	g	540	23.4	23.6				P-(Q)-(P)-Q-R-S	331	69.5	3.0	
	h	680	–	24.5								
	i	–	24.0	22.8								
2	a*	190100	19.9	20.9					WW-like	233	98.6	97.2
	b	401000	19.7	20.6	8.2	23.6	Stable	Biopsy- confirmed	(I/b-2)			
	c	225000	20.4	21.3								

	d	89200	20.4	23.0								
	e	37000	21.6	22.4								
	f	4000	23.7	25.5					P-(Q)-(R)-S	175	74.2	2.7
	g	1000	23.0	24.4								
	h	–	26.4	26.1								
3	a*	26700	23.0	25.8	0.7	4.8	Stable	Presumptive	WW-like (I/b-2)	233	98.6	99.9
	b	2600	27.7	24.9								
	a	3100	26.3	26.6					WW-like			
4	b*	89100	19.8	21.2					(IV/b-1)	234	97.1	96.5
	c	510	26.9	26.3	1.2	6.5	Stable	Biopsy- confirmed				
	d	490	25.6	25.5					P-(Q)-(P)-Q-R-S	275	82.4	2.5
	a*	217000	19.2	19.2					WW-like			
	b	223000	18.5	19.8					(I/b-2)	234	96.8	89.8
5	c	256000	18.4	23.2			Graft	Biopsy-				
	d	15000	19.2	19.8	31.2	10.9	dysfunction	confirmed				
	e	3400	20.1	25.8					P-Q-(R)-S	183	76.4	5.9
	f	1125	22.4	24.9								

	g	2300	–	26.6								
	h	1820	–	25.0					P-(Q)-(P)-Q-R-S	286	79.2	1.2
	i	960	–	26.0								
	j	1400	21.9	28.4								
	k	850	–	25.0					P-Q-(P)-Q-R-S	320	70.9	1.2
	l	760	–	26.3								
6	a	6020	–	24.9								
	b	11200	24.5	23.6					WW-like	233	98.6	83.9
	c*	8500	22.0	23.7					(I/b-2)			
	d	2800	27.7	23.4	1.0	11.5	Stable	Biopsy-confirmed				
	e	1000	25.3	25.2					P-(Q)-(P)-Q-R-S	283	81.1	15.9
	f	–	–	23.7								
7	a*	29600	23.3	25.2					WW-like	233	98.6	77.6
	b	3700	24.6	23.9	1.8	6.9	Graft dysfunction	Biopsy-confirmed	(I/b-2)			
	c	750	24.2	25.2					P-(Q)-(R)-S	192	81.4	22.4
8 ^c	a	2700	27.2	27.2								
	b	400	24.4	24.3	0.5	3.6	Stable	Biopsy-confirmed	WW-like	234	97.1	99.9
	c*	116000	ND	ND					(IV/b-1)			

	a	–	26.7	–									
	b	640	26.5	25.7					WW-like				
	c*	19300	21.4	22.0					(I/b-2)	233	98.6	97.6	
9	d	102800	23.2	23.6	1.0	6.2	Stable	Presumptive					
	e	13900	21.2	26.2									
	f	800	27.0	28.5					P-(Q)-(P)-Q-R-S	315	73.0	2.1	
	g	–	28.8	23.8									
10	Ctrl1	–	24.0	28.0									
11	Ctrl2	–	27.3	26.6									

* Sample from which the TCR architecture was characterized.

^a BKPyV miRNA Ct values were normalized by subtracting the mean Ct value of spiked cel-miR-39-3p miRNA from the mean Ct values of each BKPyV miRNA (Δ Ct).

^b Fold change of miRNA expression in sick versus healthy was calculated according to the $2^{-\Delta\Delta Ct}$ method [39] by comparing the results of each individual patient to the mean of both controls.

^c TCR sequence analysis of patient 8 was performed on a sample which was not analyzed for miRNA detection.

– = Viral load below detection range (<400 copies/mL), or miRNA not detected.

Ctrl = control sample; ND = not done; TCR = transcriptional control region

Table 3. Number of AP-1, NFAT, NF-1, Sp1 and p53 transcription factor binding sites in modified BKPyV strains.

Strain ^a	Accession number	AP-1	NFAT	NF-1	Sp1	p53	TCR architecture
Archetype WW	M15987.1	1	1	6	2	2	P-Q-R-S
P5_mod1	MG356964	1	1	5	2	2	P-Q-(R)-S
P2_mod	MG356960	1	1	4	1	2	P-(Q)-(R)-S
P7_mod	MG356970	1	1	6	1	2	P-(Q)-(R)-S
P5_mod3	MG356966	2	2	8	3	3	P-Q-(P)-Q-R-S
P1_mod	MG356958	2	2	8	3	3	P-(Q)-(P)-Q-R-S
P4_mod	MG356962	2	1	7	2	2	P-(Q)-(P)-Q-R-S
P5_mod2	MG356965	2	2	7	2	2	P-(Q)-(P)-Q-R-S
P6_mod	MG356968	2	2	7	2	2	P-(Q)-(P)-Q-R-S
P9_mod	MG356974	2	2	8	2	3	P-(Q)-(P)-Q-R-S

^a Name of the viral strain; 'P' indicating the patient and 'mod' describing a modified strain.

TCR = transcriptional control region