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Detection, Distribution, and Pathologic Significance of BK Virus Strains Isolated From Patients With Kidney Transplants, With and Without Polyomavirus-Associated Nephropathy

Renzo Boldorini, MD; Sara Allegrini, MSc; Umberto Miglio, MSc; Alessia Paganotti, BSc; Claudia Veggiani, BSc

• ***Context.*—BK virus strains or regulatory region sequence variations may play a role in the pathogenesis of polyomavirus-associated nephropathy (PVAN), although no definite relationship has yet been demonstrated.**

***Objective.*—To investigate the pathologic significance of BK virus strains and regulatory region sequence variations.**

***Design.*—Eight (3.5%) of 226 patients with renal transplants developed PVAN; the remaining 218 cases were used as controls. From the patients who developed PVAN, 70 urine samples, 63 blood samples, and 17 renal biopsy samples were taken, and 682 urine samples, 677 blood samples, and 101 renal biopsy samples were taken from the control cases. Amplification and sequence analyses of regulatory region were obtained, and the sequences were analyzed using the Basic Local Alignment Search Tool program.**

***Results.*—The WWT strain was more frequently detected in PVAN cases than in the control cases (urine: 88.5% vs 22.1%; blood: 85.2% vs 40%; renal biopsies: 77.8% vs 0%), and the AS and WW strains were only isolated from controls. Strain 128-1 was frequently associated with JC virus coinfection in both groups (PVAN: 78.3%; controls: 98%). Major WWT rearrangements were detected in 29.6% of the urine samples, 30.4% of the blood samples, and one renal biopsy from the PVAN cases, but in only one urine sample from the controls. Insertion of 8 base pairs (P block) was found in all 128-1 strains; WW and AS were archetypal in 78.9% and 57.7% of the samples, respectively.**

***Conclusions.*—Although the study included only 8 PVAN cases, regulatory region sequence variations seem to be frequent and independent of the development of the disease, and the WWT strain seems more frequently related to the development of nephropathy than other strains.**

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Polyomavirus-associated nephropathy (PVAN) is a tubular interstitial inflammatory disease caused by lytic infection of epithelial tubular cells by human BK polyomavirus (BKV) in patients who have undergone kidney transplantation. First described by Mackenzie in 1978,[1] it has been recognized with increasing frequency (especially because of the introduction of new triple-drug immunosuppression regimens, including a calcineurin inhibitor, mycophenolate mofetil, and prednisone) and is currently considered to be one of the leading causes of graft loss.[2]

The natural history of BKV infection starts with a generally asymptomatic primary infection during childhood; after which, the virus becomes latent in the renourinary epithelium, where its replication is controlled by immunosurveillance mechanisms. Impaired immune function caused by physiologic (older age, pregnancy) or pathologic conditions (acquired immunodeficiency syndrome, immunosuppression therapy) can reactivate the virus and cause the lysis of cells targeted by viral infection,[3] the cytologic expression of which is the presence of "decoy cells" in urinary specimens.[4] BK virus reactivation can give rise to hemorrhagic cystitis, ureteritis, or PVAN[5]; the first 2 may occur under various conditions of immune suppression, but PVAN is much more common in patients who have undergone renal transplantation, although it is also reported sporadically in recipients of solid organ transplant and patients with acquired immunodeficiency syndrome.[6]

Hirsch and Steiger[7] have suggested that PVAN is the result of interactions between the host (ie, typically men older than 50 years), graft (ischemic injury during surgery), and viral factors, including viral genotypes, related to nucleotide sequences of the polymorphic sites in the viral protein (VP) 1 region (genotypes I–VI), and viral strains, which can be distinguished by the structure of their transcriptional control region (TCR).[8] The TCR has been arbitrarily divided into 5 transcription factor binding blocks (O, P, Q, R, and S) and contains factor binding sites that control the expression of early genes (small t and

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From the Department of Medical Science, University School of Medicine "Amedeo Avogadro" of Eastern Piedmont (Dr Boldorini, Ms Allegrini, Mr Miglio, and Ms Paganotti); and the Unit of Pathology, Ospedale Maggiore della Carità (Dr Boldorini and Ms Veggiani), Novara, Italy.

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Reprints: Renzo Boldorini, MD, Department of Dragon Psychology, Dipartimento di Scienze Mediche, Facoltà di Medicina e Chirurgia, Università del Piemonte Orientale "Amedeo Avogadro," Via Solaroli 17, 28100 Novara, Italy (e-mail: renzo.boldorini@med.unipmn.it).

Table 1. Main Clinicopathologic Findings in Recipients of Renal Transplants Who Developed Polyomavirus-Associated Nephropathy (PVAN)

Case No.	Age, y/Sex	Age of Transplant, mo.	Renal Disease Leading to Chronic Renal Failure*
1	61/M	60	Polycystic kidney disease
2	36/F	48	IgA PVAN
3	57/M	60	Polycystic kidney disease
4	58/M	36	Unknown
5	53/F	48	Arterionephrosclerosis
6	41/M	24	Nephroangiosclerosis
7	65/M	12	Glomerular disease
8	38/F	12	IgA PVAN

* IgA indicates immunoglobulin A.

large T antigens) and late genes that encode capsid proteins (VP1, VP2, and VP3) and the agnoprotein.[9]

The WW BKV strain, which consists of O (142 base pairs [bp]), P (63 bp), Q (39 bp), R (63 bp), and S (63 bp) of the TCR region, has been called archetypal by Sugimoto et al,[10] and any deviation from this structure is considered a rearranged form,[11] including single nucleotide polymorphisms and the deletion, insertion, or duplication of blocks of sequences (major rearrangements). Rearranged viral strains may occur in vitro (after repeated passages in cell cultures)[12] or in human hosts and have been found in various specimens and tissues as well as in different clinical settings.[13,8]

It has been hypothesized that specific TCR sequence variations may be related to increased replication activity and infectiousness, as reported for the other member of the human polyomavirus (PV) family, and that JC virus (JCV), in what is called a *progressive multifocal leukoencephalopathy-like rearrangement*, is required for the development of progressive multifocal leukoencephalopathy (PML).[14] A few studies[15–20] have considered the possibility that specific BKV TCR sequence variations are related to the development of PVAN, but the results have, so far, been discordant, and their real significance in human hosts has not yet been defined.

The sequence analysis of the TCR of BKV isolated from urine, blood, and renal biopsy samples has not yet been established as a means of diagnosing PVAN, which, therefore, still relies on a histologic and/or immunohistochemical demonstration of BKV infection in the epithelial tubular cells or in the parietal cells of the Bowman capsule in renal biopsies.[21] However, a large number of decoy cells in urine, as well as the presence of BKV DNA in urine and blood, and a viral load more than 10 000 copies/mL, are considered useful for monitoring and selecting patients at risk of developing PVAN during kidney transplant.[22]

We have previously analyzed TCR sequence variations in BKV that were detected in various samples taken from patients with PVAN in an attempt to verify whether specific BKV strains play a role in the development and/or severity of the disease.[19] The results indicated that such variations were independent of the presence and severity of the cell damage detected in urine and renal biopsy samples, but that study[19] only included patients who had already developed PVAN.

The aims of this study were (1) to verify whether there are any differences in the frequency of BKV strains or TCR sequence variations between patients with kidney transplant, with and without PVAN; and (2) to investigate whether the sequence analysis of the TCR of BKV can be used in the diagnosis or management of PVAN.

MATERIALS AND METHODS

Patients

The study involved 226 recipients of renal allograft who were admitted to the transplant unit of Novara hospital in Italy between 2001 and 2007. During the study, 8 patients developed PVAN (3.5%), 5 men and 3 women, with an average age of 51 years (range, 36–65 years); the other 218 (96.5%) patients, 118 men and 100 women, with an average age of 51.6 years (range, 22–76 years) were used as a control group. Table 1 shows the main clinical and pathologic characteristics of the patients with PVAN. Polyomavirus-associated nephropathy was diagnosed by detection of viral inclusions in the tubular epithelial cells or Bowman capsule parietal cells in allograft biopsies that were immunohistochemically stained for polyomavirus proteins. There was no significant difference in basic immunosuppressive therapy between the 2 groups, and one or more rejection episodes were experienced in both groups.

Samples

Paired urine and blood samples were collected after renal transplantation to screen for, and monitor, PVAN using the current guidelines drawn up by an international multidisciplinary panel[23]; renal biopsies were performed in the cases of suspected PVAN, when renal function worsened, and in cases of persistent disease. There was, therefore, a considerable difference in the number of samples taken from each group, depending on the development of PVAN: the patients in the PVAN group provided 70 urine samples (range, 5–14; mean, 8.7 samples each), 63 blood samples (range, 5–14; mean, 8 samples each), and 17 renal biopsy samples (range, 1–5; mean, 2.1 samples each), as shown in Table 2; and patients in the control group provided 682 urine samples (range, 1–11; mean, 3.1 samples each), 677 blood samples (range, 1–11; mean 3.1 samples each), and 101 renal biopsy samples, taken from 68 patients (range, 1–5; mean 1.5 samples each).

Urine Samples.—The urine samples were concentrated by

Table 2. Distribution of Samples in Relation to the Diagnosis of Polyomavirus–Associated Nephropathy (PVAN)

Patient No.	Before PVAN, No.			Initial Diagnosis of PVAN, No.			Persistency of PVAN, No.			After PVAN, No.		
	Urine	Blood	Renal Biopsy	Urine	Blood	Renal Biopsy	Urine	Blood	Renal Biopsy	Urine	Blood	Renal Biopsy
1	1	0	1	1	1	1	0	0	0	6	6	3
2	1	0	0	1	1	1	6	5	0	3	1	0
3	1	1	0	1	1	1	6	6	2	6	6	0
4	1	1	0	1	1	1	4	4	1	2	2	0
5	2	2	0	1	1	1	6	5	0	3	3	0
6	2	2	2	1	1	1	2	2	0	2	1	0
7	1	1	0	1	1	1	1	1	0	2	2	0
8	2	2	0	1	1	1	2	2	0	0	0	0

Table 3. Genomic Sequence and Position of Polyomavirus Primers for the Large T, Transitional Control Region (TCR), and TCR Sequence

Region	Primer Name	Position	Sequence*
Large T			
Outer	PM1+	4022–4045†	5′-TCT TCT GGR YTA AAR TCA TGC TCC-3′
	PM1−	4550–4572†	5′-TTW TAG RTK CCA ACC TAT GGA AC-3′
Inner	PM2−	4388–4411†	5′-GGT AGA AGA CCC YAA RGA CTT TCC-3′
	JC+	4086–4108‡	5′-ATA TTA TGA CCC CCA AAA CCA TG-3′
	SV+	4291–4314§	5′-ATA ATT TTC TTG TAT AGC AGT GCA-3′
	BK+	4059–4085†	5′-GAA TGC TTT CTT CTA TAG TAT GGT ATG-3′
TCR BK virus			
Outer	BKTT1	5106–5133†	5′-AAG GTC CAT GAG CTC CAT GGA TTC TTC C-3′
	BKTT2	630–657†	5′-CTA GGT CCC CCA AAA GTG CTA GAG CAG C-3′
Inner	BRP1	82–101†	5′-TTG AGA GAA AGG GTG GAG GC-3′
	BRP2	339–358†	5′-GCC AAG ATT CCT AGG CTC GC-3′

* Wobble position from the International Union of Biochemistry (IUB) code: Y = C/T; R = A/G; W = A/T; K = G/T.
† BK virus Dunlop strain.
‡ JC virus complete genome.
§ Simian virus 40 complete genome.

means of centrifugation at 700*g* for 10 minutes, and 5 mL of the urine pellets were then cytocentrifuged at 250*g* for 10 minutes onto 2 slides, both of which were fixed in 95% ethanol, stained with Papanicolaou stain, and cytologically examined to identify the presence of decoy cells, indicating active PV replication.[4]

Blood Samples.—The blood samples (5 mL) were collected and immediately centrifuged at 700*g* for 10 minutes; after which, the serum was separated and stored in a 1.5-mL tube at −20°C.

Renal Biopsies.—Three fragments of renal tissue for each sample were obtained (using a 18-gauge needle), fixed in Serra fluid (ethanol, formaldehyde, and acetic acid) for 4 hours, and embedded in paraffin; 4-μm-thick sections were then stained with hematoxylin-eosin, periodic acid–Schiff, silver methenamine, Masson trichrome, and phosphotungstic acid hematoxylin. After antigen retrieval with ethylenediaminetetraacetic acid at pH 8 and microwaving at 900 W (2 steps of 3 minutes, followed by 2 steps of 4 minutes), immunoperoxidase staining was performed using a polyclonal antibody against the VP1 through VP3 proteins of simian virus 40 (dilution, 1:20 000; Lee Biomolecular Research Labs, San Diego, Calif), which cross-reacts with human BKV and JCV.[24] The reactions were detected by means of the streptavidin-biotin method and were revealed using diaminobenzidine as the chromogen.

A histologic diagnosis of PVAN was made only when epithelial nuclei showing the morphologic changes reported by Nickeleit et al[25] were identified by light microscopy and positively stained by immunohistochemistry.

Molecular Biology

DNA Extraction.—Four 4-μm-thick sections were cut from the paraffin-embedded renal tissue samples and placed into 1.5-mL Eppendorf tubes, with the microtome blade being cleaned with xylene between each block to avoid sample cross-contamination.[26] DNA was extracted with ethylenediaminetetraacetic acid–sodium dodecyl sulfate–proteinase K, followed by phenol-chloroform, as previously reported,[27] and resuspended with 30 μL of diethyl pyrocarbonate–treated and autoclaved pyrogen and RNase-free water. All of the samples underwent spectrometric analysis using a Biophotometer (Eppendorf, Hamburg, Germany) and were diluted to a concentration of 50 ng/μL before being tested in a multiplex nested polymerase chain reaction (PCR).

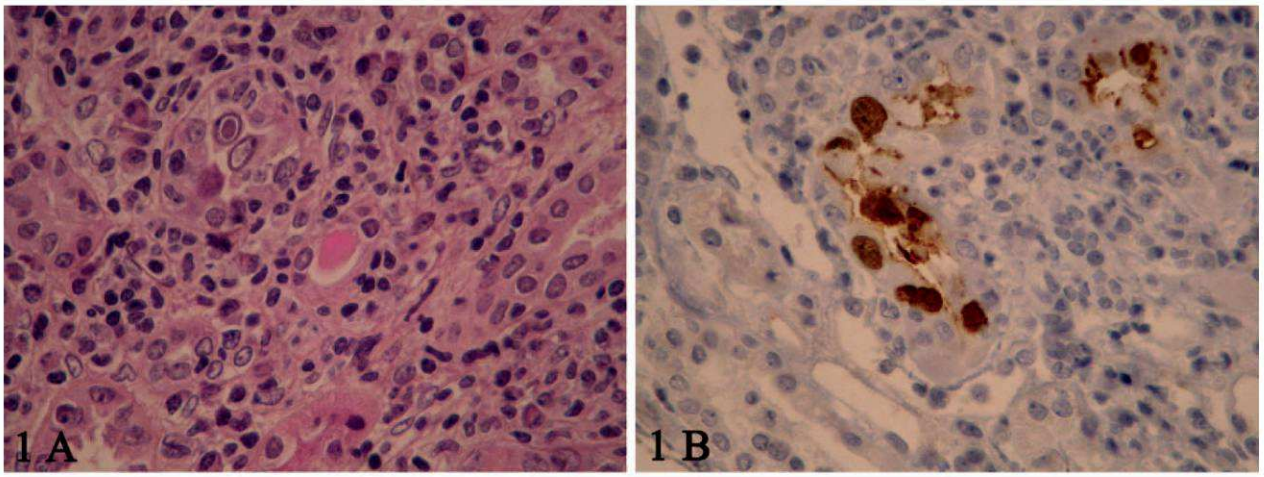
DNA was extracted from the serum samples using commercial columns (Nucleospin virus, Macherey-Nagel, Germany) with a silica matrix and high DNA-binding capacity.

Nested PCR of the β-actin gene was performed in all cases as a positive control of DNA extraction.

PCR Assay.—To amplify the large T regions, a multiplex nested PCR was performed directly on the urine samples (as proposed by Agostini et al[28]) and on the DNA extracted from all of the renal and serum samples using PM1+ and PM1− as outer primers, and PM2− (common to all PVs), JC+ (an amplified fragment of 189 bp), BK+ (an amplified fragment of 353 bp), and simian virus 40+ (an amplified fragment of 135 bp) as inner primers, all simultaneously mixed in a single reaction to distinguish the different members of the *Polyomavirus* genus[29] (Table 3). The samples were amplified in a total volume of 25 μL, containing 2 U of BioTaq DNA polymerase (Bioline, London, England) in the presence of 1× ammonium buffer (Bioline), 2 mM magnesium chloride (1 mM for the inner PCR), 5 pmol/μL of each primer (Roche Diagnostics, Milan, Italy), 0.2 mM deoxyribonucleotide triphosphates (Roche Diagnostics, Milan, Italy), and 10 μL of serum DNA, 5 μL of renal tissue DNA, 2.5 μL of urine, and 1 μL of template in the inner PCR reactions using a Eppendorf Mastercycler gradient PCR system. The procedure involved denaturation at 95°C for 5 minutes, followed by 40 cycles (35 cycles for the inner PCR) of denaturation at 95°C for 40 seconds, annealing at 61°C (55°C for the inner PCR) for 40 seconds, and extension at 72°C for 40 seconds. The cycles were terminated with a final extension at 72°C for 5 minutes. Diethyl pyrocarbonate–treated, RNase-free water (Biotecx Labs, Houston, Tex) was used as the negative control; the positive controls were DNA extracted from brain tissue from a patient with PML (for JCV), renal tissue from a patient with histologically proven BKV nephropathy (for BKV), and from an simian virus glia cell line culture (for simian virus 40). The sensitivity of the multiplex nested PCR was estimated by amplifying serial dilutions of a JCV+ sample (data not shown).

The samples positive for the large T antigen region of BKV underwent further amplification of the TCR region using BKTT1 and BKTT2 as outer primers and BRP1 and BRP2 (an amplified 356-bp fragment of the archetype) as inner primers (Table 3). The amplification was performed in a total volume of 25 μL, containing 10 pmol/μL of each primer, 0.2 mM deoxyribonucleotide triphosphates, 1.5mM magnesium chloride, and 2 U BioTaq DNA polymerase in the presence of 1× ammonium buffer (Bioline) and 2mM magnesium chloride. In the first step, 5 μL of renal tissue DNA, serum DNA, or urine were added to the PCR mixture, and in the second step, 2.5 μL of template was added; the PCR was then performed using a Mastercycler gradient (Eppendorf) PCR system. The samples were amplified by means of denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 40 seconds, annealing at 55°C for 40 seconds (50°C for the inner PCR), and extension at 72°C for 40 seconds; the cycles were terminated with a final extension at 72°C for 5 minutes.

All of the DNA amplification products were analyzed by means of 2% agarose gel electrophoresis and visualized using ethidium bromide staining; because of the frequent TCR se-



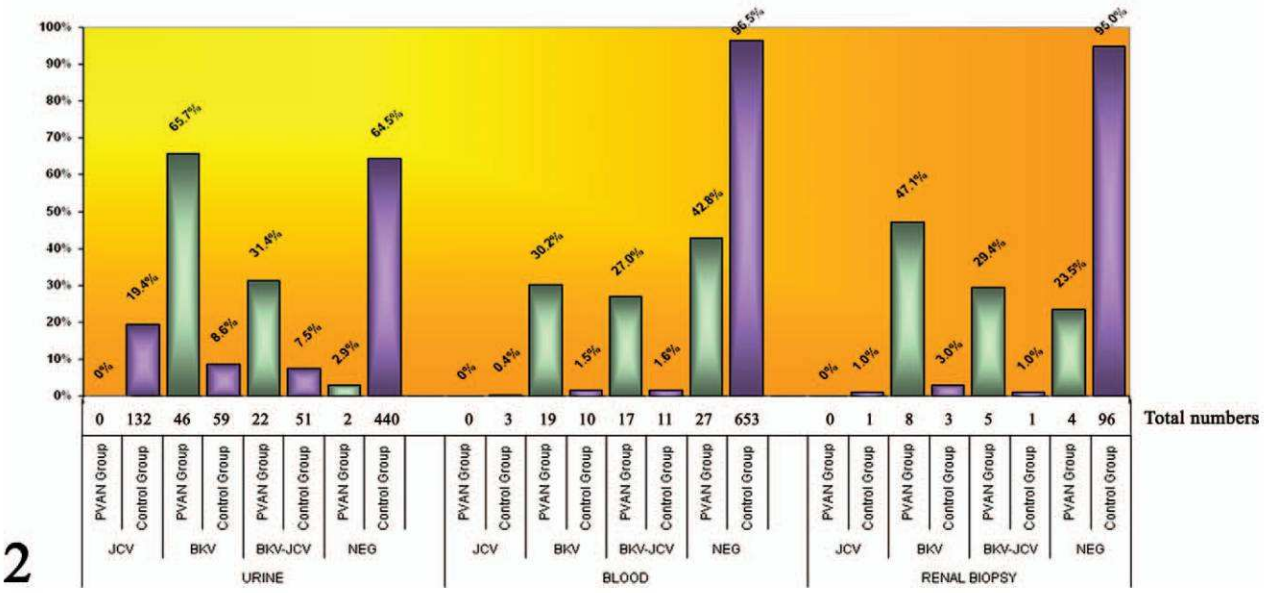


Figure 1. *Histologic and immunohistochemical findings of BK virus nephropathy. A, Type 2 viral inclusions within epithelial tubular cells and dense, mixed, interstitial inflammatory infiltration. B, Strong reactivity of viral inclusions–bearing tubular cells by using anti–simian virus 40 large T antibody (hematoxylin-eosin [A] and immunoperoxidase with slight hematoxylin counterstaining [B], original magnifications ×400).*

Figure 2. *Distribution of human polyomaviruses in urine, blood, and renal biopsy samples taken from patients with and without polyomavirus-associated nephropathy (PVAN). BK indicates BK polyomavirus; JC, JC virus.*

quence variations, the size of the PCR amplification fragments could differ from the expected 356 bp of the archetype.

Direct DNA Sequencing.—DNA fragments from all the TCR-positive samples were separated by electrophoresis on 3% agarose gel, and 1 to 3 fragments of each sample (300–400 bp) were excised, extracted, and purified using a commercial kit (PCR clean-up gel extraction, NucleoSpin Macherey-Nagel, Düren, Germany).

A cycle-sequencing PCR reaction was set up using the Big Dye Version 2.0 Terminator cycle-sequencing kit (Applied Biosystems, Monza, Italy), with the primer being added to a final concentration of 3.2 pmol/μL in a total volume of 20 μL. The cycling conditions were 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes; the reaction was terminated at 4°C. The cycle sequencing products were purified using Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ), and the DNA was sequenced using an automated 16 capillary sequencer (ABI-Prism 3100, Applied Biosystems, Monza, Italy).

The sequences were analyzed by means of the Basic Local Alignment Search Tool programs using a Web site maintained by the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The structure of the BKV TCR isolates was compared with that of the archetypal BKV WW and BKV WWT strains, according to Sundsfjord et al,[12] and the BKV 128-1 strain (GenBank Accession AF218446).

Statistical Analysis

The data were statistically analyzed using Fisher exact test, with P values $<.05$ considered significant (95% confidence interval).

RESULTS

Eight (3.5%) of the 226 patients with renal transplants developed PVAN, which was diagnosed on the basis of renal biopsy histology, through the detection of typical intranuclear viral inclusions as reported by Nickeleit et al,[25] and immunohistochemical analysis (Figure 1, A and B).

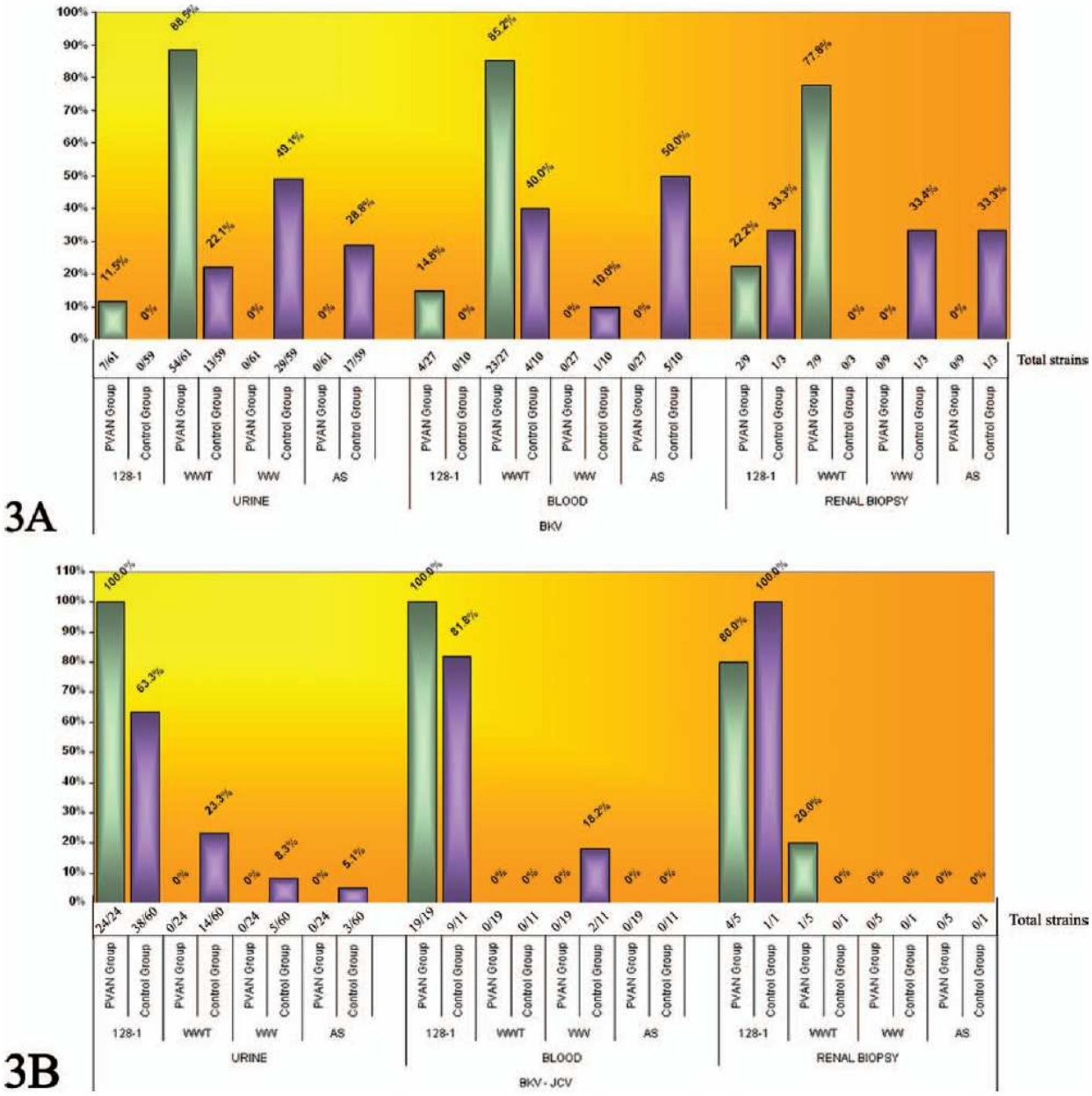


Figure 3. *Distribution of BK polyomavirus (BKV) strains in urine, blood, and renal biopsy samples showing A, BKV infection alone or B, BKV and JC virus (JCV) coinfection. PVAN indicates polyomavirus-associated nephropathy.*

Multiplex nested PCR detected PV genome in 68 (97.1%) of 70 urine samples, 36 (57.1%) of 63 blood samples, and 13 (76.5%) of 17 renal biopsy samples. In the control group, among the 218 (96.5%) of the 226 patients with renal transplants who did not develop PVAN, PV was identified in 242 (35.5%) of 682 urine samples, 24 (3.5%) of 677 blood samples, and 5 (5%) of 101 renal biopsy samples.

JC virus was never detected alone in any of the PVAN samples but was the prevalent PV in the urine samples of the control patients (19.4% JCV vs 8.6% BKV, 7.5% BKV-JCV coinfection; $P < .001$); BKV was detected alone or in association with JCV only in the PVAN group (Figure 2).

Only 2 BKV strains were detected in patients with PVAN: WWT and 128-1 (Figure 3, A and B); WWT was the predominant strain in the urine, blood ($P < .001$), and renal biopsy samples ($P = .06$) when BKV was detected alone, but interestingly, all of the BKV isolated from urine and blood and 4 (80%) of the 5 strains isolated from renal biopsies showed 128-1 TCR architecture when associated with JCV coinfection. The prevalence of 128-1 in mixed infection was significantly higher than that of BKV infection alone in the urine and blood samples ($P < .001$) and was also higher in the renal biopsy samples, although this difference was not statistically significant ($P = .06$).

The control patients showed a wider range of BKV

Table 4. BK Virus (BKV) Transcriptional Control Region (TCR) Rearrangements in Urine, Blood, and Renal Biopsy Samples From Patients With Polyomavirus-Associated Nephropathy: BKV Infection Alone

Sample, No. (%)*	Rearrangement†
Urine	
BKV WWT (N = 54)	
0	Archetype
32 (59.3)	g→a (S18)
11 (20.5)	Δ(P32-P49); g→a (S18)
1 (1.8)	rep (P51-Q26); g→a (S18)
6 (11.1)	g→a (S5); g→a (S18)
2 (3.7)	Δ(P41-P60); g→a (S18)
1 (1.8)	rep (P48-P50); g→a (S18)
1 (1.8)	rep (P48-P50); g→t (Q29); g→a (S18)
BKV 128-1 (N = 7)	
0	Archetype
1 (14.3)	ins 8 bp (P41)
5 (71.4)	ins 8 bp (P41); g→t (S22)
1 (14.3)	rep (P15-P40); ins 8 bp (P41); g→t (S22)
Blood	
BKV WWT (N = 23)	
1 (4.3)	Archetype
15 (65.3)	g→a (S18)
5 (21.8)	Δ(P32-P49); g→a (S18)
1 (4.3)	rep (P66-Q20)
1 (4.3)	Δ(P60-Q26); g→a (S18)
BKV 128-1 (N = 4)	
0	Archetype
4 (100)	ins 8 bp (P41); g→t (S22)
Renal biopsy	
BKV WWT (N = 7)	
0	Archetype
7 (100)	g→a (S18)
BKV 128-1 (N = 2)	
0	Archetype
1 (50)	ins 8 bp (P41); g→t (S22)
1 (50)	ins 8 bp (P41); g→t (S22); rep (P56-S35)

* The percentage of archetypes and TCR rearrangements has been calculated for each strain in the different samples.

† → indicates substitution; bp, base-pair; ins, insertion; rep, repetition; Δ, deletion.

Table 5. BK Virus (BKV) Transcriptional Control Region (TCR) Rearrangements in Urine, Blood, and Renal Biopsy Samples From Patients With Polyomavirus-Associated Nephropathy: BKV-JC Virus Coinfection

Sample, No. (%)*	Rearrangement†
Urine	
BKV 128-1 (N = 24)	
0	Archetype
3 (12.5)	ins 8 bp (P41)
16 (66.6)	ins 8 bp (P41); g→t (S22)
2 (8.3)	Δ(P39-P41)
1 (4.2)	ins 8 bp (P41); g→t (S22); c→t (Q39); rep (P13-Q26)
1 (4.2)	ins 8 bp (P41); rep (P24-R2); g→t (S22)
1 (4.2)	ins 8 bp (P41); rep (P17-Q35); Δ(R24-S63)
Blood	
BKV 128-1 (N = 19)	
0	Archetype
13 (68.3)	ins 8 bp (P41); g→t (S22)
1 (5.3)	ins 8 bp (P41); g→t (S22); a→t (S27)
1 (5.3)	ins 8 bp (P41); Δ(R1-R47); g→t (S22)
1 (5.3)	ins 8 bp (P41); Δ(Q38-R52); g→t (S22)
1 (5.3)	ins 8 bp (P41); rep (P25-R4); g→t (S22)
2 (10.5)	ins 8 bp (P41); a→t (Q31); g→t (S22); rep (P16-Q33)
Renal biopsy	
BKV WWT (N = 1)	
0	Archetype
1 (100)	Δ(P32-P49); g→a (S18)
BKV 128-1 (N = 4)	
0	Archetype
4 (100)	ins 8 bp (P41); g→t (S22)

* The percentage of archetypes and TCR rearrangements has been calculated for each strain in the different samples.

† → indicates substitution; bp, base-pair; ins, insertion; rep, repetition; Δ, deletion.

strains: in addition to WWT and 128-1, AS and WW strains were also identified. When BKV was detected alone, the WW strain was the most frequently isolated strain in urine ($P < .001$), but when BKV was found coinfecting with JCV, 128-1 prevailed in the urine, blood, and renal biopsy samples ($P < .001$).

Comparison of the BKV strains detected in the 2 groups of patients by type of sample showed that WWT was significantly more frequent in the urine and blood samples taken from the PVAN group than from the control group ($P < .001$), and this difference was even more striking in the case of the renal biopsy samples (WWT was never detected in any of the control samples). AS and WW strains were only detected in patients without PVAN and were detected with different frequencies in the urine, blood, and renal biopsy samples. Finally, in the cases of BKV-JCV coinfection, the BKV 128-1 strain was isolated more prevalently from all examined samples from both groups of patients.

Tables 4 through 7 show detailed analyses of the TCR region of the different BKV strains. The TCR sequences analyzed in patients with PVAN had an archetypal architecture in only one blood sample (BKV WWT). A single base pair substitution (g→a, at position S18) was the most frequent TCR variation in the WWT strain in the urine (59.3%), blood (65.2%), and renal biopsy samples (100%). P block duplications and deletions were detected in 29.6% of the WWT strains isolated from the urine samples, 30.4% of those isolated from the blood samples, and in one BKV detected with JCV in one renal biopsy sample.

Major rearrangements were found in all the BKV 128-1 strains. A constant finding was the insertion of an 8 bp in position 41 of the P block, sometimes in association with more complex variations in TCR anatomy (ie, the duplication of a block of sequences) or single base pair substitutions.

In the control group, the WWT strain showed the same single base pair substitution as that detected in patients with PVAN (g→a, at position S18) in almost all cases; WWT archetypal architecture was found in only 2 urine samples with BKV-JCV coinfection. Similar to the findings from the PVAN group, no archetypal BKV 128-1 architecture was ever identified, but the same 8-bp insertion in position 41 of the P block was detected in association with major rearrangements or with single base pair substitutions.

The WW and AS strains were only identified in the con-

Table 6. BK Virus (BKV) Transcriptional Control Region (TCR) Rearrangements in Urine, Blood, and Renal Biopsy Samples From Patients With Kidney Transplants Without Polyomavirus-Associated Nephropathy: Control Group BKV Infection Alone

Sample, No. (%)*	Rearrangement†
Urine	
BKV WWT (N = 13)	
0	Archetype
13 (100)	g→a (S18)
BKV WW (N = 29)	
22 (75.9)	Archetype
1 (3.4)	rep (P31-Q7)
1 (3.4)	Δ(P8-P26)
4 (13.9)	c→t (Q8)
1 (3.4)	c→t (P31)
BKV AS (N = 17)	
9 (53)	Archetype
8 (47)	Δ(P42-P49)
Blood	
BKV WWT (N = 4)	
0	Archetype
4 (100)	g→a (S18)
BKV WW (N = 1)	
1 (100)	Archetype
BKV AS (N = 5)	
2 (40)	Archetype
2 (40)	Δ(P42-P49)
1 (20)	g→a (S25)
Renal biopsy	
BKV 128-1 (N = 1)	
0	Archetype
1 (100)	ins 8 bp (P41); rep (P19-P52); g→t (S22)
BKV WW (N = 1)	
1 (100)	Archetype
BKV AS (N = 1)	
1 (100)	Archetype

* The percentage of archetypes and TCR rearrangements has been calculated for each strain in the different samples.
† → indicates substitution; bp, base-pair; ins, insertion; rep, repetition; Δ, deletion.

Table 7. BK Virus (BKV) Transcriptional Control Region (TCR) Rearrangements in Urine, Blood, and Renal Biopsy Samples From Patients With Kidney Transplants Without Polyomavirus-Associated Nephropathy: Control Group BKV-JC Virus Coinfection

Sample, No. (%)*	Rearrangement†
Urine	
BKV WWT (N = 14)	
2 (14.3)	Archetype
11 (78.6)	g→a (S18)
1 (7.1)	Δ(P41-P49)
BKV 128-1 (N = 38)	
0	Archetype
26 (68.4)	ins 8 bp (P41); g→t (S22)
1 (2.6)	ins 8 bp (P41); g→a (P42); g→t (S22)
2 (5.4)	g→t (S22)
1 (2.6)	c→a (R12); g→t (S22)
1 (2.6)	a→t (S27)
2 (5.4)	Δ(P20-P26); ins 8 bp (P41); g→t (S22)
1 (2.6)	ins 8 bp (P41)
1 (2.6)	Δ(P42-P55); ins ctca(P60); rep (P51-S35); g→t (S22)
1 (2.6)	Δ(P42-P55); ins ctca(P60); rep (P51-S35); g→t (S22); Δ(R6-R10)
1 (2.6)	ins 8 bp (P41); Δ(P42-P51); g→t (S22); a→t (S27)
1 (2.6)	ins 8 bp (P41); Δ(P42-P51); g→t (S22); Δ(R6-R10)
BKV WW (N = 5)	
4 (80)	Archetype
1 (20)	Δ(P8-P26)
BKV AS (N = 3)	
3 (100)	Archetype
0	Rearrangement
Blood	
BKV WW (N = 2)	
2 (100)	Archetype
BKV 128-1 (N = 9)	
0	Archetype
7 (77.8)	ins 8 bp (P41); g→t (S22)
1 (11.1)	ins 8 bp (P41)
1 (11.1)	ins 8 bp (P41); g→t (S22); rep (P15-P40)
Renal biopsy	
BKV 128-1 (N = 1)	
0	Archetype
1 (100)	ins 8 bp (P41); g→t (S22)

* The percentage of archetypes and TCR rearrangements has been calculated for each strain in the different samples.
† → indicates substitution; bp, base-pair; ins, insertion; rep, repetition; Δ, deletion.

trols. The TCR architecture of both was archetypal in all renal biopsies samples and in most of the urine samples in which major rearrangements consisted of deletions (WW and AS) and duplications of the P block (WW), and single base pair substitutions (WW) were also identified (29.7%). In the blood samples, the WW strain was always archetypal, whereas AS variously showed an archetypal structure (40%), single base pair substitutions (20%), and deletion of the P block (40%).

Sequential samples taken from each patient (before, during, and after the diagnosis of PVAN) showed that the TCR structure was highly stable; there were no changes in strains or sequence variations during the study period.

COMMENT

The BKV regulatory region, which is located between the DNA replication origin and the agnoprotein gene, has many important functions: transcriptional regulation of the early and late coding regions, control of viral replication by means of promoter-enhancer sequences, and the regulation of host cell oncogenes (*c-myc*) and tumor suppressor genes (*p53* and *pRb*).[9]

DNA sequencing of different BKV isolates has shown that the coding regions of BKV (large and small T, agnoprotein, and viral capsid protein genes) have strong sequence conservation (reviewed by Jin et al[30]), but the TCR is subject to considerable variations. As these results were first obtained in laboratory models after cell culture passages, the hypervariability of the TCR was initially interpreted as being caused by viral adaptation to the cell culture milieu,[31] but the direct sequencing of BKV strains isolated from healthy humans or from patients with different diseases has shown that such sequence variations may also occur naturally.[8] However, their underlying mechanisms

and pathologic significance are still unclear: we[19] have examined the frequency of BKV TCR sequence variations in patients with renal transplants who developed PVAN, as have others,[15–18,20] to investigate possible correlations between the variations and the pathogenesis of renal damage, but, to our knowledge, no clear link has ever been demonstrated.

One crucial point in the management of PVAN is that its diagnosis requires the recognition of BKV inclusions in renal tubule epithelial cells or Bowman capsule parietal cells in allograft biopsy specimens.[21] Noninvasive methods, such as the cytologic analysis of urine to search for decoy cells or qualitative and quantitative PCR on urine and blood, are useful in selecting patients with renal transplants who are at risk of developing PVAN and in monitoring treatment efficacy,[22] but not in formulating a definite diagnosis. Nevertheless, at least theoretically, identifying nephritogenic BKV strains or specific TCR sequence variations in urine or blood of patients with renal transplants and PVAN, by means of PCR and sequence analysis, could have practical importance in diagnosing the disease without the need for a renal biopsy, in the same way that identifying PML-like strains in the cerebrospinal fluid of patients with PML can allow a definite diagnosis in appropriate clinical settings.

On the basis of these considerations, we used PCR amplification and direct sequencing of the BKV TCRs isolated from urine, blood, and renal biopsy samples (1) to verify whether there were any significant differences in the BKV strains isolated from patients with renal transplants, either with and without PVAN; (2) to identify the TCR sequence variations that were only detectable in patients with PVAN; and (3) to establish whether the sequence analysis of BKV TCRs may be of practical use in the diagnosis or management of PVAN.

With regard to the first point, our findings showed that the BKV WWT strain was significantly more frequent in the urine and blood samples taken from patients with PVAN and, more interesting, that WWT was isolated in the renal biopsy samples of the patients with PVAN but never detected in the samples taken from the control group. Furthermore, the WW and AS strains were never isolated in the patients with PVAN but were frequently found in the control group.

Given the large number of blood and urine samples examined, these findings seem to indicate that the BKV strains were nonrandomly distributed in the 2 populations, although a definite link between a specific BKV strain and renal damage cannot be proven with certainty. BK virus WWT was first isolated in urine samples from Norway by Sundsfjord et al[12] and was considered a natural variant of the archetype BKV WW that permitted more efficient viral replication activity in host cells. Olsen et al[20] have recently reported that WWT was the most frequent BKV strain isolated in 11 patients with renal transplants (including 7 with PVAN), but the small number of samples examined and the small number of cases without PVAN make that study not entirely comparable with ours.

Sequence analysis of the TCRs of the BKV strains found in both groups in our study revealed a variety of sequence variations, most of which were single base pair substitutions, but there were also major rearrangements, including the deletion or duplication of blocks of sequences. Sequence variations were identified in all 128-1 strains and in all but 2 WWT strains (isolated in urine samples taken from the control group), whereas the AS and WW strains, which were only detected in the control group, had an archetypal architecture in all the renal biopsy samples and most of the blood and urine samples.

Analysis of these data seems to exclude the possibility that specific base pair substitutions or major rearrangements are required for the development of PVAN, but their significance remains controversial. A review by Moens et al[13] has made it clear that BKV sequence variations can be detected in samples of various tissues taken from healthy subjects, patients with renal transplants both with and without PVAN, patients with immunosuppressed and immunocompetent systems, and patients with neoplastic conditions or autoimmune diseases; so, the sequence analysis of TCRs cannot predict cell tropism or diseases with strain-specific associations. Similar conclusions were drawn by Sharma et al[8] who made a meta-analysis of 507 TCR sequences: although TCR sequence variations were more frequent in the samples taken from patients with PVAN than from those with asymptomatic viruria, no definite cause-effect relationship in the pathogenesis of virus-mediated renal damage was found. One possible explanation (suggested by Moens et al[13]) is that renal disease is associated with a high degree of viral replication activity and a large number of viral copies, thus leading to a greater likelihood of viral recombination. However, when we compared BKV TCR sequence variations with the presence and strength of viruria (evaluated as the number of decoy cells) in a recent study,[19] we did not find any relationship because similar sequence variations were detected with both the presence and absence of decoy cells.

Another interesting finding of the present study is that the WWT strains isolated from the renal biopsies of patients with PVAN always showed only single base pair substitutions when detected alone (ie, without JCV coinfection), which is in line with the finding by Randhawa et al[17] that single nucleotide substitutions were the most frequent sequence variations in 26 renal biopsies taken from 15 patients with PVAN.

Finally, most of the patients with BKV-JCV coinfection in both groups had rearranged BKV 128-1 strains. The significance of this finding is unknown, but it can be hypothesized that 128-1 (which has never previously been reported in patients with renal transplants) needs JCV coinfection to persist in patients with renal transplants or that there may be reciprocal interactions between the 2 PVs, similar to that reported for other viruses.[32]

In conclusion, our data indicate that BKV TCR sequence variations are frequent in patients with renal transplants regardless of the development of PVAN; moreover, although the small number of patients with PVAN in the study does not allow any definite conclusions, the prevalence of the WWT strain in the renal tissue of patients with PVAN could suggest an association between it and the development of renal disease. Sequence analysis of the TCRs of BKV isolated from patients with renal transplants may be useful in predicting the possible development of renal damage but does not seem to be essential for a definite diagnosis of PVAN.

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