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Mutations in the External Loops of Bk Virus Vp1 and Urine Viral Load in Renal Transplant Recipients

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Abstract

Polyomavirus Associated Nephropathy (PVAN) is a major complication that occurs after renal transplantation and is induced by reactivation of the human polyomavirus BK (BKV). The structure of the viral capsid protein 1 (VP1) is characterized by the presence of external loops, BC, DE, EF, GH, and HI, which are involved in receptor binding. The pathogenesis of PVAN is not well understood, but viral risk factors are thought to play a crucial role in the onset of this pathology. In an attempt to better understand PVAN pathogenesis, the BKV-VP1 coding region was amplified, cloned, and sequenced from the urine of kidney transplant recipients who did, and did not, develop the pathology. Urine viral loads were determined by using Real Time Quantitative PCR (Q-PCR). Amino acid substitutions were detected in 6/8 patients, and 6/7 controls. The BC and EF loop regions were most frequently affected by mutations, while no mutations were found within the GH and HI loops of both patients and controls. Some mutations, that were exclusively detected in the urine of PVAN patients, overlapped with previously reported mutations, although a correlation between changes in amino acids and the development of PVAN was not found. Urine viral loads were higher than that of the proposed cut-off loads for identification of patients that are at a high risk of developing PVAN (10^7 copies/mL), both in the PVAN and control groups, thus confirming that urine viral load is not a useful predictive marker for the development of PVAN.

Keywords

BK virus; Polyomavirus Associated Nephropathy; VP1 loop; genotype

Introduction

The human polyomavirus BK (BKV) is the causative agent of Polyomavirus Associated Nephropathy (PVAN) (Randhawa and Demetris, 2000), which has gradually emerged as a serious complication following renal transplantation. BKV is found worldwide, and

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approximately 80% of the adult population is seropositive for the virus (Knowles et al., 2003). Primary infection presumably occurs during childhood via a fecal-oral or a respiratory route and is usually asymptomatic (Stolt et al., 2003). The virus then establishes a life-long persistence in the renourinary tract as the principal site of latency, despite detection of BKV proteins and nucleic acid sequences in the brain (De Mattei et al., 1995), prostatic tissue (Zambrano et al., 2002), and leucocytes (Dorries et al., 1994). Reactivation of BKV in the primary sites of latency may occur, especially in immunocompromised individuals, and this may be associated with onset of pathological conditions. For instance, the use of anti-rejection immunosuppressive therapies in renal transplant recipients provides an environment for BKV replication within the allograft. BKV viruria and viremia may be detected in approximately 25-30% and 10-15% of patients, respectively, following renal transplantation and may progress into PVAN in approximately 5% of cases, resulting in renal damage and functional impairment (Hirsch et al., 2002).

The circular, double-stranded DNA genome (5153 bp) of BKV is divided into a non-coding control region (NCCR) with regulatory function and two coding regions: the early region, encoding the large and small T antigens, and the late region, encoding agnoprotein and the structural proteins VP1, VP2, and VP3, of which VP1 is the major capsid component. Based on its high homology with SV40 and mouse Polyomavirus VP1, whose crystal structures have already been determined (Griffith et al., 1992; Liddington et al., 1991), BKV-VP1 is predicted to be divided into five outer domains or loops, known as BC, DE, EF, GH, and HI, that connect the different β -strands and α -helix of the polypeptide. The BC loop of BKV-VP1 contains a short sequence, named BKV-subtyping region, which spans nucleotides 1744-1812. This region has been used to identify the four main viral genotypes (I, II, III, and IV) (Jin et al., 1993), which are differentially distributed within the human population (Takasaka et al., 2004; Zheng et al., 2007; Zhong et al., 2007). The subtyping region is also responsible for the existence of antigenic variants of BKV (Jin et al., 1993; Knowles et al., 1989). In addition, the external loops of polyomavirus VP1 have a crucial role in mediating host-cell receptor-binding and capsid-structure maintenance (Dugan et al., 2007; Gee et al., 2004; Stehle et al., 1994). Amino acid changes within the outer loops of polyomavirus VP1 were demonstrated to alter the biological properties of the virus in vivo in the ability to induce tumors in mice (Bauer et al., 1995; Freund et al., 1991a) and in vitro in virus hemagglutination properties, propagation in cell cultures, and capsid integrity (Dugan et al., 2007; Freund et al., 1991b). Given the importance of the outer loops of BKV-VP1, it has been proposed that amino acid changes within this protein may be associated with an increase in the pathogenic potential of this virus and therefore may contribute to the development of PVAN. To this purpose, Baksh et al. and Rhandawa et al. analyzed the VP1 subtyping region of allograft biopsies from PVAN patients, showing a strong genetic instability and suggesting a possible implication of VP1 amino acid changes for evasion to the host immunity (Baksh et al., 2001; Randhawa et al., 2002). A recent study reported frequent mutations within the BC and DE loops of BKV isolates from renal transplant patients but did not find any correlation between these amino acid substitutions and viruria (Krautkrämer et al., 2009).

In our study, VP1 sequences of BKV strains were amplified from the urine of kidney transplant recipients who did and did not develop PVAN. Following amplification, the VP1 sequences were analyzed in order to determine if amino acid changes within the five external loops of VP1 contribute to the development of PVAN. In addition, urine viral loads of allograft transplant patients enrolled in this study were determined. To our knowledge, this is the first study that aimed to identify specific amino acid substitutions within the complete VP1-loops sequences of BKV amplified from PVAN patients.

Materials and Methods

Urine samples and DNA purification

Fifteen BKV-positive patients were selected from a cohort of 226 renal allograft recipients who were admitted to the Transplant Unit of Ospedale Maggiore della Carità in Novara, between 2001 and 2007. During this period of time, all the cohort patients were screened and monitored for development of PVAN, as suggested by an international multidisciplinary panel (Hirsch et al., 2005). The diagnosis of PVAN was performed in the Laboratory of Pathology of Ospedale Maggiore della Carità and the progression of renal damage induced by BKV was classified as previously proposed (Nickeleit et al., 2000). At different time after transplantation, 8 patients, 5 males and 3 females, with a mean age at transplant of 51 years (range: 36-65), developed PVAN (Table 1). The remaining 7 patients enrolled in our study were selected among the group of renal transplant patients who did not develop PVAN, and they were included in the control group. The following criteria of selection were used: age (mean age at transplant: 55 years; range 39-72), sex (4 males and 3 females) and at least one renal biopsy performed during the study period.

None of the patients enrolled in the study experienced graft loss due to viral infection, although one PVAN patient showed disease progression leading to renal fibrosis. In this study, fifteen urine samples (one for each BKV-positive patient enrolled), collected when the allograft biopsy for PVAN diagnosis was performed, were investigated. An informed consent form was signed by each patient at the time of collection. DNA for molecular analysis was extracted from 200 µl of urine using the commercial kit Nucleospin RNA virus (Macherey Nagel, Germany).

Standard PCR for VP1 amplification

In order to amplify the genomic region encompassing the five external loops of VP1, two standard PCRs were performed with two different sets of primers: BK-1F (5'-AGTGCCAAACTACTAATAAAAG-3', nucleotides [nt] 1632-1654)/BK-1R (5'-CTGGGCTGTTGGGTTTTAG-3', nt 2121-2102) and BK-2F (5'-GAAAACCTATTCAAGGCAGTAA-3', nt 1988-2009)/BK-2R (5'-AAATTGGGTAAGGATTCTTTACA-3', nt 2470-2448). As shown in Figure 1, BK-1F/BK-1R and BK-2F/BK-2R amplified two partially overlapping fragments: fragment 1, delimited by BK-1F and BK-1R, was 489 bp in length, while fragment 2, delimited by BK-2F and BK-2R, was 482 bp in length (Fig. 1). The two amplifications were carried out in a total volume of 50 µl, containing 20 pmoles of forward and reverse primer, 0.6 mM dNTPs, 1.5 mM MgCl₂, and 2U of Euro Taq Polymerase (EuroClone, Italy) in the presence of 1× Reaction Buffer supplied by the manufacturer. 2, 5 or 7 µl of DNA extracted from urine were added to the PCR mixture. The two amplifications were performed running the same protocol in a GeneAmp PCR System 9700 (Applied Biosystems, USA): an initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C, 30 sec extension at 72°C, and a final extension step at 72°C for 7 min.

Precautions were taken to avoid contamination: three different rooms were used, one for DNA extraction, one for setting up the PCR reaction, and a third to analyze the PCR products. The products of amplification were analyzed by means of 1% agarose gel electrophoresis and visualization by ethidium bromide staining.

Molecular cloning and sequencing of PCR fragments

The products of amplification were cloned using the TA cloning kit (Invitrogen, USA) according to the manufacturer's instruction. After the transformation process, the plasmid DNA was extracted from the INVα'F strain of Escherichia Coli using the QIAGEN Plasmid

Minikit (QIAGEN, Germany). Purified plasmids were subjected to double digestion with HindIII and XbaI (Roche, USA) to select clones that contained the insert.

At least five representative recombinant clones for each PCR fragment were sent to an external facility for automated sequencing (Primm srl, Milan). Sequencing reactions were carried out using primers BK-1F for PCR fragment 1 and BK-2F for PCR fragment 2.

Translation of the nucleotide sequences into amino acid sequences was performed using ExPASy software (<http://www.expasy.org/ExpasyHunt/>; ExPASy & Health On the Net Foundation), whereas the alignment of multiple sequences was carried out using Clustal W (<http://www.ebi.ac.uk/Tools/clustalw/> (Chenna et al., 2003)).

Analysis of BKV VP1 sequences

The sequences of representative BKV isolates, belonging to genotypes I, II, III, IV and available on GenBank, were aligned in order to create a consensus sequence for each genotype (Table 2).

The BKV genotype of each patient was determined analyzing the polymorphisms within the nucleotide region 1744-1812, according to the classification method proposed by Jin et al. (1993).

In order to detect specific amino acid substitutions, the VP1-consensus amino acid sequence of each patient was compared to the consensus amino acid sequence of the corresponding BKV genotype.

Quantitative Real Time PCR for BKV

The urine viral load of PVAN patients and controls was determined by a Quantitative Real Time PCR assay (Q-PCR) that targeted a conserved region of the VP1 gene. Q-PCR was performed using a 7300 Real Time PCR System (Applied Biosystems, USA). Primers BKVPf (5'-AGTGGATGGGCAGCCTATGTA-3', nt 2511-2531), BKVPr (5'-TCATATCTGGGTCCCCTGGA-3', nt 2605-2586) and Taqman MGB probe BKVPp (5'-FAM-AGGTAGAAGAGGTTAGGGTGTGTTGATGGCACAG-3'MGB, nt 2578-2546) were used in this assay for amplification and detection of the target sequence. The reaction was performed in a final volume of 25 µl containing a 1× Taqman Universal PCR Master Mix (Applied Biosystems, USA), 0.4 µM primer BKVPf, 0.9 µM primer BKVPr, 0.2 µM BKVPp, and 5 µl of extracted nucleic acid. Thermal cycling was carried out according to the following steps: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, at the end of which the fluorescence was read.

Each sample was analyzed in triplicate, and each run contained a negative control containing the reaction mixture without a DNA template. A standard curve for quantification of BKV was constructed using serial dilutions of a plasmid containing the whole BKV genome (range: 10² to 10⁶ plasmid copies). The detection limit for this assay was determined to be 5 copies/reaction. Data were expressed as copies of viral DNA per milliliter of urine sample.

Statistical analysis

Statistical analysis of the data obtained by Q-PCR was performed with the Student's t-test.

Results

Two partially overlapping fragments, encoding the five external loops of BKV-VP1, were amplified from the urine of PVAN patients and controls. The amplified regions were cloned, and at least 5 positive clones for each PCR fragment were sequenced. Substitutions that

were detected in all or most of the clones were considered to be originally present in the urine samples, while those rarely detected were considered to be artifacts introduced by PCR or cloning (Eckert et al., 1990). However, it should be pointed out that if an heterogeneous population of BKV isolates is present in a given patient, rarely detected substitutions may also be linked to the amplification of less represented viral strains. These viral strains are therefore likely to be missed by using this approach.

Distribution of BKV genotypes

The BKV subtyping region of each patient and control was analyzed for the presence of specific polymorphisms in order to classify each BKV strain into the corresponding genotype. In the patient group, genotype I and IV were detected with the same frequency (4/8), while genotype II and III were not detected. In the control group, 3 out of 7 samples were assigned to genotype I, 2 out of 7 samples were assigned to genotype II and IV, and no sample was assigned to genotype III.

Identification of amino acid substitutions within the VP1 region—Amino acid changes within the VP1 region were detected in the urine samples collected from both the PVAN and control groups. Mutations were detected in 6 out of the 8 PVAN patients and in 6 out of the 7 controls. Amino acid changes were identified in the BC, DE, and EF loops. However, in the control group, mutations were also identified in the β -strains connecting the loops (β -C, β -D, β -E, β -F). The BC and the EF loops were the regions most frequently affected by mutations. No amino acid substitutions were detected in the GH and HI loops of the PVAN and control groups. Amino acid substitutions that resulted in a change of charge were observed in 3 patients and 3 controls (Table 3, Table 4).

A total of 8 and 18 mutations were identified in the PVAN patients and controls, respectively. The two groups shared four amino acid variations: D77E, E82D within the BC loop and D175E, V210I within the EF loop. The frequency of the mutations detected ranged from 1 to 4 for the PVAN group and from 1 to 2 for the control group. In the isolates from patients, the most frequent mutations were identified at position 175, where aspartic acid (D) was altered to glutamic acid (E), and position 210, where valine (V) was altered to isoleucine (I), whereas there was no specific amino acid prevalence within the control group (Table 5).

Quantification of viral load in the urine of patients and controls—BKV viral titers detected in the urine of patients and controls are shown in Tables 3 and 4. The urine median viral load of the PVAN group was $6.26\text{E}+08$ copies/mL (range: $1.25\text{E}+06$ - $7.93\text{E}+09$), whereas the urine median viral load of the control group was $5.87\text{E}+06$ copies/mL (range: $3.95\text{E}+04$ - $2.97\text{E}+10$) ($p=0.5$).

Discussion

PVAN is one of the major complications that occurs after renal transplantation and is induced by reactivation of BKV. Four genotypes of BKV have been identified on the basis of non-synonymous nucleotide polymorphisms clustered within the VP1 subtyping region, that corresponds to the BC loop of the protein. The pathogenesis of PVAN is not well understood, but different viral, host, and organ risk factors related to the transplant procedure are thought to play a role in the onset of this pathology. Among the viral factors, rearrangements within the highly variable NCCR (Azzi et al., 2006; Chen et al., 2001; Gosert et al., 2008; Olsen et al., 2006) and amino acid changes within the major capsid protein VP1 (Baksh et al., 2001; Krautkrämer et al., 2009; Randhawa et al., 2002) have been proposed by numerous authors, given their potential ability to generate viral strains with

altered pathogenic properties. In our study, the distribution of BKV genotypes, as well as the presence of amino acid changes within the outer loops of VP1, was investigated in urine collected from 8 biopsy-proven PVAN patients and 7 kidney-transplant patients who did not develop PVAN. In addition, the urine viral load was determined in these two groups.

Genotypes I and IV were detected in both the PVAN and control groups. Genotype II was detected only in two isolates from the control group, while no isolates of genotype III were identified in the PVAN patients or controls. The results from the PVAN patients are consistent with data from previous studies by Baksh et al. and Randhawa et al. that report a more frequent distribution of genotypes I and IV and a failure to detect sequences belonging to genotype III in a group of PVAN patients (Baksh et al., 2001; Randhawa et al., 2002). However, in regard to the control group, the results presented here are in contrast to what was previously reported by Di Taranto and colleagues, who analyzed the frequency of BKV genotypes in a group of healthy and HIV+ Italian children and found genotype I to be most frequently distributed, followed by genotype III and IV (Di Taranto et al., 1997). However, the differences in distribution pattern may be related to the small number of patients and controls enrolled. Amino acid changes in the VP1 sequence were detected in both groups and were mainly restricted to loops BC, DE, and EF, with the exception of a few sporadic mutations identified in the β -sheet regions of the BKV isolates from controls. On the other hand, the GH and HI loops amplified from all patients and controls were highly conserved, since no mutations were found in these regions. Mutations identified in the controls were more numerous than those identified in the PVAN group but also more sporadic, since the most frequent amino acid changes were detected in 2 out of 7 controls. In regard to the PVAN group, two mutations, D175E and V210I, were detected in 4 out of 8 patients. However, these amino acid substitutions had been previously described in some BKV strains isolated from healthy controls and from clinical settings different from PVAN (Chen et al., 2004). In addition, the same amino acid changes were also detected in 2 out of the 7 controls enrolled in our study. Thus, it may be speculated that positions 175 and 210 of VP1 are “hot spots” of mutations, that may be subjected to high interstrain diversity among different BKV isolates.

Interestingly, two of the amino acid changes exclusively found in the PVAN group, K69R and D75N, were previously reported following analyses of PVAN patients (Baksh et al., 2001; Randhawa et al., 2002). In vitro studies have shown that residue 69 of VP1 is important for virus viability, since an amino acid substitution at this position may induce a reduction of viral spread and receptor binding ability (Dugan et al., 2007). In addition, two amino acid substitutions, E73Q within the BC loop and H139N within the DE loop, were found in PVAN patients but not in controls, as previously reported (Krautkrämer et al., 2009). It should be pointed out that since a low number of patients and controls were involved in this study

The median urine viral load of the patients was higher than the median urine viral load of the controls, as demonstrated by Q-PCR; however, this difference was not statistically significant. Previous studies have proposed urine viral loads as a predictive marker for the development of PVAN in renal transplant patients with a cut-off value of 10^7 copies/mL (Randhawa et al., 2004). However, in our study we found a viral load higher than 10^7 copies/mL in both PVAN patients and controls, which supports recent findings that have failed to correlate urine viral load with the development of PVAN (Bressollette-Bodin et al., 2005). Thus, it has recently become clear that other markers, such as BKV viral load in plasma, should be considered in order to define the risk of PVAN development (Hirsch et al., 2002).

To our knowledge, this is the first study that has investigated the presence of mutations in PVAN patients and controls within the complete VP1-loops sequence. Interestingly, some mutations exclusively detected in the urine of PVAN patients overlapped with mutations that had been previously reported (Baksh et al., 2001; Randhawa et al., 2002; Krautkrämer et al., 2009), although a specific correlation between amino acid changes and PVAN development was not found. However, it should be pointed out that the small number of patients enrolled, due to the low percentage of renal allograft recipients that usually develop PVAN after transplantation, limits the conclusions that may be drawn from this work. Therefore, further investigations and an expansion of case studies are necessary to better understand the biological significance of VP1 amino acid substitutions in the pathogenesis of PVAN.

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Figure 1.

Target sites of primers BK-1F, BK-1R, BK-2F, and BK-2R within the VP1 region. The use of these sets of primers ensures amplification of all of the outer loops of VP1, together with their neighboring regions.

Main demographic and pathological features of PVAN patients whose urine was collected and analyzed in this study

TABLE 1

Patient	Gender	Age at transplant	Months ^a	Disease leading to chronic renal failure	PVAN stage
1	M	41	24	Nephroangiosclerosis	B
2	F	52	48	Arterionephrosclerosis	B
3	M	57	60	Polycystic kidney disease	B
4	M	65	12	Glomerular disease	A
5	F	38	12	IgA nephropathy	B
6	F	36	48	IgA nephropathy	B
7	M	61	60	Polycystic kidney disease	A
8	M	58	36	Unknown	C

^aMonths between renal transplantation and diagnosis of PVAN.

TABLE 2

BKV isolates used to obtain consensus sequences of the complete VP1 region for genotypes I, II, III, and IV.

Genotype	Isolate	Accession number	Reference
I	DUN	NC_001538	Seif et al., 1979
I	Gardner	Z19534	Jin et al., 1993
I	Dik	AB211369	Nishimoto et al., 2006
I	WW	AB211371	Nishimoto et al., 2006
I	JL	AB211370	Nishimoto et al., 2006
I	MT	AB211372	Nishimoto et al., 2006
I	MM	V01109	Yang and Wu, 1979
II	SB	Z19536	Jin et al., 1993
II	ETH-3	AB263916	Zheng et al., 2007
II	GBR-12	AB263920	Zheng et al., 2007
III	AS	M23122	Tavis et al., 1989
III	KOM-3	AB211386	Nishimoto et al., 2006
IV	RYU-3	AB211389	Nishimoto et al., 2006
IV	THK-8	AB211390	Nishimoto et al., 2006
IV	TW-3	AB211391	Nishimoto et al., 2006
IV	KOM-2	AB211387	Nishimoto et al., 2006
IV	FIN-2	AB260033	Igekaya et al., 2006

TABLE 3

Genotypes, viral load, and amino acid substitutions in the VP1 region of BKV isolates from urine of PVAN patients.

Patient	Genotype	Viral load ^b	Amino acid substitution ^a		
			BC loop	DE loop	EF loop
1	I	6,06E+07	K69R	-	D175E V210I
2	I	6,26E+08	E82D	H139N	D175E V210I
3	I	7,20E+08	D75N E82D	H139N	D175E V210I
4	IV	1,78E+06	D77E	-	-
5	IV	6,30E+08	-	-	-
6	IV	N/A ^c	D77E E73Q	-	-
7	I	7,93E+09	-	-	D175E V210I
8	IV	1,25E+06	-	-	-

^a amino acid numbers are those of Dunlop strain (Seif et al., 1979);

^b copies/ml;

^c N/A= not available;

amino acid substitutions that cause a change of charge are underscored.

TABLE 4

Genotypes, viral load, and amino acid substitutions in the VP1 region of BKV isolates from urine of controls.

Control	Genotype	Viral load ^b	Amino acid substitution ^a						
			BC loop	β-C	β-D	DE loop	β-E	EF loop	β-F
1	I	3.95E+05	-	-	-	-	-	D175E V210I	-
2	II	3.54E+08	-	-	-	E138Q	-	-	-
3	I	2.97E+10	-	-	-	-	-	-	-
4	IV	6.93E+05	N61D D62N D77G	-	-	-	V155I	E175D V178I I210V	Y225F
5	IV	3.24E+07	D77E	-	-	-	-	-	-
6	I	5.87E+06	E82D	-	-	-	-	D175E V210I	-
7	II	3.95E+04	-	L96P	K117Q	N139H V145I	-	Q175D I210V	Y225F

^a amino acid numbers are those of Dunlop strain (Seif et al., 1979);

^b copies/ml; amino acid substitutions that cause a change of charge are underscored; β-C, β-D, β-E, β-F; β-sheets.

TABLE 5

Frequency of amino acid substitutions detected in the VP1 region of BKV isolates from urine of PVAN patients and controls.

	Amino acid substitution ^a	Loop	Frequency
PATIENTS	K69R, E73Q, D75N	BC	1/8
	D77E, E82D	BC	2/8
	H139N	DE	2/8
	D175E, V210I	EF	4/8
CONTROLS	N61D, D62N, D77G D77E, E82D	BC	1/7
	L96P	β-C	1/7
	K117Q	β-D	1/7
	E138Q, N139H, V145I	DE	1/7
	V155I	β-E	1/7
	D175E, I210V, V210I	EF	2/7
	E175D, Q175D, V178I	EF	1/7
	Y225F	β-F	2/7

^a amino acid numbers are those of Dunlop strain (Seif et al., 1979).