RISULTATI

PUBBLICAZIONE 1

BK Virus è il principale agente eziologico della nefropatia associata a polyomavirus (PVAN); nel corso degli anni numerosi studi sono stati svolti per chiarire il suo ruolo nella patogenesi della malattia. La proteina capsidica VP1 è coinvolta nel riconoscimento di specifici antigeni cellulari di superficie e sembra avere un ruolo cruciale nell'internalizzazione del virus da parte della cellula ospite. Numerosi gruppi di ricerca si sono concentrati sullo studio della regione codificante tardiva del genoma di BKV, contenente il gene della VP1, identificando una regione compresa tra i nucleotidi 1744-1812, compresa nel loop BC, che codifica la porzione amminoacidica responsabile del riconoscimento antigenico. Questa regione è stata utilizzata per definire il genotipo e il sottotipo del virus e inoltre sono state ricercate mutazioni nucleotidiche che portano ad una sostituzione aminoacidica eventualmente correlata con una maggiore patogenicità del virus.

Il presente studio, realizzato in collaborazione con il Laboratorio di Patologia e di Ricerca Traslazionale del Ospedale San Giuseppe di Milano, è stato incentrato sull'analisi mutazionale dell'intera regione genomica virale codificante la protena capsidica VP1 (loops BC, DE, EF, GH e HI) in pazienti trapiantati renali che hanno sviluppato PVAN allo scopo di identificare specifiche sostituzioni aminoacidiche eventualmente correlate a ceppi virali con patogenicità maggiore. Per realizzare lo studio sono stati selezionati 15 pazienti BKV-positivi a partire da una coorte di 226 pazienti portatori di trapianto renale afferenti presso Centro Trapianti di Rene dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità" dal 2001 al 2007. I 15 pazienti BKV-positivi selezionati si dividono in due gruppi: otto hanno sviluppato dopo il trapianto PVAN, mentre gli altri sette sono stati selezionati tra il gruppo di pazienti portatori di trapianto renale che non ha sviluppato PVAN e sono stati utilizzati come gruppo di controllo. Per ogni paziente è stato analizzato con metodiche molecolari (PCR quantitativa e sequenziamento) il campione urinario raccolto contestualmente al momento dell'esecuzione della biopsia per sospetto diagnostico di PVAN.

I risultati del nostro studio mostrano come cambiamenti aminoacidi nella VP1 sono localizzati a livello dei loops BC, DE ed EF sia nei pazienti con PVAN che nei controlli. Alcune mutazioni sono state trovate solo nel gruppo dei pazienti con PVAN e, nonostante le differenze da noi rilevate tra i due gruppi non siano statisticamente significative probabilmente a causa dell'esiguo numero di casi in esame, le mutazioni a carico del loop BC coincidono con quelle riportate da altri studi antecedenti al nostro.

Journal of
Cellular
Physiology

Mutations in the External Loops of BK Virus VP1 and Urine Viral Load in Renal Transplant Recipients

SARA TREMOLADA,[1] SERENA DELBUE,[1] LORENZO CASTAGNOLI,[1] SARA ALLEGRINI,[2] UMBERTO MIGLIO,[2] RENZO BOLDORINI,[2] FRANCESCA ELIA,[3] JENNIFER GORDON,[4] AND PASQUALE FERRANTE[1,3]*

[1] *Department of Public Health-Microbiology-Virology, University of Milan, Milan, Italy*

[2] *Faculty of Medicine, Department of Medical Sciences, University Amedeo Avogadro of East Piedmont, Novara, Italy*

[3] *Center for Translational Research and Laboratory of Pathology, Saint Joseph Hospital, Milan, Italy*

[4] *Department of Neuroscience and Center for Neurovirology, Temple University School of Medicine, Philadelphia, Pennsylvania*

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.

J. Cell. Physiol. 222: 195–199, 2010. © 2009 Wiley-Liss, Inc.

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 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 the onset of pathologic 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 (5,153 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 viral capsid protein 1 (VP1), 2 (VP2), and 3 (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 (Liddington et al., 1991; Griffith et al., 1992), 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 1,744–1,812. 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 (Knowles et al., 1989; Jin et al., 1993). In addition, the external loops of polyomavirus VP1 have a crucial role in mediating host-cell receptor binding and capsid-structure maintenance (Stehle et al., 1994; Gee et al., 2004; Dugan et al., 2007). Amino acid changes within the outer loops of polyomavirus VP1 were demonstrated to alter the

Contract grant sponsor: NIH;
Contract grant number: R01-MH072528.

*Correspondence to: Pasquale Ferrante, Center for Translational Research and Laboratory of Pathology, Saint Joseph Hospital, Via San Vittore, 12, 20123 Milan, Italy.
E-mail: pasquale.ferrante@unimi.it

Received 17 June 2009; Accepted 14 August 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 24 September 2009.
DOI: 10.1002/jcp.21937

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biologic properties of the virus in vivo in the ability to induce tumors in mice (Freund et al., 1991a; Bauer et al., 1995) and in vitro in virus hemagglutination properties, propagation in cell cultures, and capsid integrity (Freund et al., 1991b; Dugan et al., 2007). 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 the 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, eight patients, five males and three females, with a mean age at transplant of 51 years (range: 36–65), developed PVAN (Table 1). The remaining seven 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 (four males and three 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, 15 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, Düren, 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′-AGTGCCAAAACT-ACTAATAAAAG-3′, nucleotides (nt) 1,632–1,654]/BK-1R (5′-CTGGGCTGTTGGGTTTTTAG-3′, nt 2,121–2,102) and BK-2F (5′-GAAAACCTATTCAAGGCAGTAA-3′, nt 1,988–2,009)/BK-2R (5′-AAATTGGGTAAGGATTCTTTACA-3′, nt 2,470–2,448). 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 pmol of forward and reverse primer, 0.6 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U of Euro Taq Polymerase (EuroClone, Pero, Italy) in the presence of 1× Reaction Buffer supplied by the manufacturer. A sample of 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): 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, Carlsbad, CA) 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, Hilden, Germany). Purified plasmids were subjected to double digestion with *Hind*III and *Xba*I (Roche, Nutley, NJ) 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).

TABLE 1. Main demographic and pathologic features of PVAN patients whose urine was collected and analyzed in this study

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	Polycistic 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	Polycistic kidney disease	A
8	M	58	36	Unknown	C

[a]Months between renal transplantation and diagnosis of PVAN.

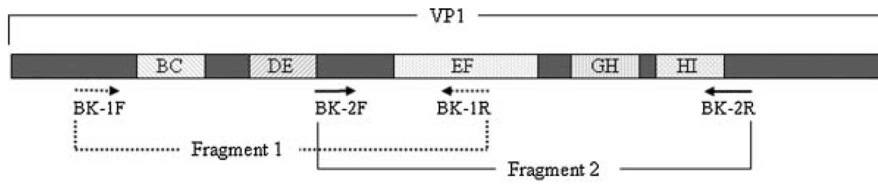

Fig. 1. Target sites of primers BK-1F, BK-1R, BK-2F, and BK-2R within the VP1 region. The use of these sets of primers ensures amplification of all of the outer loops of VP1, together with their neighboring regions.

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 1,744–1,812, 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, Foster City, CA). Primers BKVPf (5′-AGTGGATGGGCAGCCTATGTA-3′, nt 2,511–2,531), BKVPr (5′-TCATATCTGGGTCCCCTGGA-3′, nt 2,605–2,586) and Taqman MGB probe BKVPp (5′FAM-AGGTAGAAGAGGTTAGGGTGTTTGATGGCACAG-3′MGB, nt 2,578–2,546) 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), 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 sec 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: 10^2–10^6 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.

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	References
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	AB 263916	Zheng et al. (2007)
II	GBR-12	AB 263920	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	AB 260033	Ikegaya et al. (2006)

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 five 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 and Kunkel, 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, three out of seven samples were assigned to genotype I, two out of seven 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 six out of the eight PVAN patients and in six out of the seven 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, and 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 three patients and three controls (Tables 3 and 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

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[a]	Amino acid substitution[b]		
			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	D77E, E73Q	—	—
7	I	7.93E+09	—	—	D175E, V210I
8	IV	1.25E+06	—	—	—

Amino acid substitutions that cause a change of charge are underscored.
N/A, not available.
[a]Copies/ml.
[b]Amino acid numbers are those of Dunlop strain (Seif et al., 1979).

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 6.26E+08 copies/ml (range: 1.25E+06 to 7.93E+09), whereas the urine median viral load of the control group was 5.87E+06 copies/ml (range: 3.95E+04 to 2.97E+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 (Chen et al., 2001; Azzi et al., 2006; Olsen et al., 2006; Gosert et al., 2008) and amino acid changes within the major capsid protein VP1 (Baksh et al., 2001; Randhawa et al., 2002; Krautkrämer et al., 2009) 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 eight biopsy-proven PVAN patients and seven 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 et al., 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 two out of seven controls. In regard to the PVAN group, two mutations, D175E and V210I, were detected in four out of eight 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 two out of the seven 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).

TABLE 4. Genotypes, viral load, and amino acid substitutions in the VP1 region of BKV isolates from urine of controls

Control	Genotype	Viral load[a]	Amino acid substitution[b]						
			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

Amino acid substitutions that cause a change of charge are underscored; β-C, β-D, β-E, β-F: β-sheets.
[a]Copies/ml.
[b]Amino acid numbers are those of Dunlop strain (Seif et al., 1979).

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).

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 biologic significance of VP1 amino acid substitutions in the pathogenesis of PVAN.

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PUBBLICAZIONE 2

La trasmissione trans-placentare dei PVs umani è un argomento di aperto dibattito all'interno della comunità scentifica, gli studi condotti infatti sono pochi, eseguiti per la maggior parte con tecniche sierologiche ormai superate e hanno prodotto risultati altamemente contradditori.

Il presente studio si propone di investigare l'infezione da polyomavirus umani (BKV e JCV) e di SV-40 durante la fase terminale di gravidanza e di valutare la possibile trasmissione materno-fetale di tali virus. Lo studio è stato condotto su una popolazione non selezionata di 300 donne gravide, tutte con gravidanza fisiologica, arruolate presso la I divisione di Ginecologia e Ostetricia dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità"di Novara, e dei loro neonati. Sono stati raccolti e analizzati mediante metodiche di diagnostica molecolare (nested-PCR) per la ricerca di genoma virale: A) 300 campioni di urina e 300 campioni di sangue intero periferico appartenenti alle donne gravide prelevati durante il controllo di routine pre-parto alla trentasettesima settimana di gravidanza e B) i rispettivi 300 campioni di sangue intero di origine fetale pelevati dal cordone ombelicale durante il parto, come da routine per i normali esami diagnostici.

Gli isolati virali inoltre sono stati sottoposti ad analisi di sequenza della regione di controllo trascrizionale allo scopo di valutare eventuali riarrangiamenti genomici ed il loro significato in relazione alla trasmissione materno-fetale dell'infezione.

Questo studio rappresenta la prima investigazione sistematica della infezione da PVs eseguito durante la gravidanza; la limitazione di questo studio -di carattere osservazionale- è consistita nella possibilità di valutare solo la fase terminale della gravidanza, periodo nel quale le partorienti effettuano controlli di routine, mentre le fasi precedenti non sono state investigabili sistematicamente.

I risultati del nostro studio indicano la presenza di genoma virale di PVs del 26% dei campioni urinari e nel 5.5% dei campioni di sangue materno, mentre in nessun caso è stato identificato nei campioni di sangue fetale; inoltre decoy cells sono state identificate in un unico campione di urine. L'analisi di questi dati indica che nella popolazione da noi studiata non si è avuta evidenza di una trasmissione materno-fetale della infezione.

Inoltre la replicazione virale dei PVs umani durante la fase terminale della gravidanza è un evento molto raro ed infine i riarrangiamenti della regione di controllo virale sembrano non incrementare il rischio di trasmissione verticale dell'infezione.

Pathology (January 2008) 40(1), pp. 72–77

VIROLOGY

Latent human polyomavirus infection in pregnancy: investigation of possible transplacental transmission

Renzo Boldorini*, Claudia Veggiani*, Elena Amoruso†, Sara Allegrini*, Umberto Miglio*, Alessia Paganotti*, Raffaella Ribaldone† and Guido Monga*

*Scienze Mediche, Facolta di Medicina e Chirurgia, and †Clinica Ostetrica e Ginecologica, Ospedale Maggiore della Carità, Novara, Italy

Summary

Aims: The purpose of the study was to investigate the transplacental transmission of the human polyomaviruses JCV and BKV.

Methods: Urine and blood samples from 300 pregnant women underwent cytological analysis to search for 'decoy cells', nested PCR to identify presence and genotype of isolated polyomaviruses, and sequence analysis of the transcription control region. Nested PCR was also used to study the umbilical cord blood of all their newborns.

Results: Decoy cells were identified in only one urine sample (1/300; 0.33%); polyomavirus DNA was detected in 80 urine samples (26.6%) corresponding to BKV alone in 28 samples (9.3%), JCV alone in 49 samples (16.3%) and both JCV-BKV in three samples (1%). Blood samples were positive in 17 cases (5.6%), corresponding to BKV alone in 10 (3.3%), and JCV alone in 7 (2.3%). Rearrangements of the transcription control region were found in only one urinary JCV strain, consisting of the insertion of 13 bp at D block, whereas point mutations were identified in 11 BKV and 11 JCV strains detected from urine. Sequence analysis of the BKV strains detected in blood samples revealed a 20 bp insertion of P block (P42–61) in human chromosomes 20 (five cases) and 14 (three cases); two JCV strains had single bp point mutations. The search for polyomavirus DNA in umbilical cord blood samples was always negative.

Conclusions: Polyomavirus DNA was frequently detected in pregnancy, whereas genomic rearrangements were rare, and no evidence of transplacental transmission of polyomavirus was obtained.

Key words: Polyomavirus, pregnancy, polymerase chain reaction, sequence analysis.

Received 28 February, revised 30 April, accepted 7 May 2007

INTRODUCTION

The BK (BKV) and JC (JCV) human polyomaviruses (Family *Polyomaviridae*) are common in most human beings, as has been shown by serological studies that have identified BKV and JCV antibodies in 80% and 70% of healthy adults, respectively.[1] BKV and JCV are responsible for rare human diseases occurring in a setting of immune deficiency, such as BKV nephropathy in kidney transplant patients[2] or progressive multifocal leukoencephalopathy (PML) in AIDS patients,[3] and may be involved in the pathogenesis of human brain tumours such as glioblastomas and medulloblastomas.[4]

However, although widely studied, the natural route of transmission and mechanisms of polyomavirus infection are still not clear. On the basis of epidemiological findings and the detection of viral genome in the tonsillar tissue of healthy individuals, it has been hypothesised that JCV and BKV enter the body by the respiratory route, replicate in the lymphoid tissue of Waldayer's ring,[5,6] and then travel through the bloodstream to target organs (kidney and brain) where they persist indefinitely in a latent state.[7,8] Other routes of viral transmission have been postulated: ingestion of water contaminated by urine (due to urinary shedding of viral particles, latently infecting kidney tissue[9]), or stools,[10] sexual transmission,[11] transfusion of blood products,[12] or kidney transplantation.[13] Finally, some authors have suggested the mother–fetus pathway as an alternative or complementary route of viral transmission,[14,15] but this has been denied by others[16,17] and remains a subject of debate.

Polyomavirus genome can be functionally divided into two coding regions–the early large tumour (LT) region, and the late viral protein (VP) region–and the non-coding transcriptional control region (TCR). The TCR contains promoter/enhancer elements of early and late genes, and the origin of viral replication.[18] Sequence analysis of the BKV and JCV TCRs could reveal some unrearranged 'archetypal' strains, and strains with rearrangements that could modify viral replication and infectiousness, i.e., single or multiple base pair (bp) changes, insertions, deletions, or duplications.[19]

In order to investigate the hypothesis of the transplacental transmission of JCV and/or BKV, and the possible role of viral mutations of the TCR region in modifying their replicative ability and infectivity, we used nested polymerase chain reaction (nPCR) to study blood and urine samples from 300 unselected pregnant women, as well as umbilical cord blood samples of their newborns. The sequences of the viral isolates were analysed in order to evaluate the significance of TCR genomic rearrangements in relation to transplacental viral transmission.

Print ISSN 0031-3025/Online ISSN 1465-3931 © 2008 Royal College of Pathologists of Australasia
DOI: 10.1080/00313020701716458

MATERIAL AND METHODS

Sample collections

The study involved 300 pregnant women followed at the Obstetrics and Gynecology Unit of Novara hospital between February and September 2005; 282 women were European, 13 were Afro-Caribbean, and five were Asian, and the mean age was 32 years (median age 32 years, range 18–40). The pregnancy was uncomplicated in 280 of 300 women, whereas 12 (4%) developed gestational diabetes and 8 (2.7%) pre-eclampsia. All the pregnant women delivered a healthy newborn at term.

Peripheral blood (PB) and urine samples were collected from all 41 of the subjects during a visit for routine clinical and laboratory examinations in the 37th week of pregnancy.

Informed consent was obtained from each patient, and the study design was approved by the medical ethics committee of the Ospedale Maggiore della Carità, Novara, Italy.

Urine samples

Five millilitres of urine was cytocentrifuged at 250 g/min for 10 min on two slides, both fixed in 95% ethanol and stained with Papanicolau stain, and then cytologically examined in order to identify the presence of decoy cells indicative of active polyomavirus replication.[20]

Blood samples

The blood samples from the pregnant women, and the umbilical cord blood samples from their newborns, were collected and stored at -80°C.

DNA was extracted from 300 μL of whole blood treated with ethylenediaminetetracetic acid (EDTA) using the Wizard Genomic DNA purification kit (Promega, Italy) and re-suspended in a final volume of 100 μL following the manufacturer's instructions. All of the samples underwent spectrometric analysis using a DU530 (Beckman Coulter, USA), and were diluted to a concentration of 10 ng/μL before being tested in a multiplex nPCR.

PCR assay

In order to amplify the LT regions, a multiplex nPCR was performed directly on the urine samples (as proposed by Agostini *et al.*[21]) and on the DNA extracted from PB from mothers and umbilical cords, by using the following primers: (a) PM1+ and PM1− as outer primers; and (b) PM2− (common to all PVs), JC+ (amplified fragment of 189 bp), BK+ (amplified fragment of 353 bp) and SV40+ (amplified fragment of 135 bp) as inner primers, all simultaneously mixed in a unique reaction, in order to distinguish the different members of the *Polyomavirus* genus (Table 1). Each of the cloned polyomavirus DNAs was assayed between 0.1 and 1000 copies/tube. The limit of detection was 1.10 and 1 copy/tube for BKV, JCV, and SV40, respectively.[22] The samples were amplified in a total volume of 25 μL, containing 2 U BioTaq DNA polymerase (Bioline, UK) in the presence of 1× Bioline NH_4 buffer, 2 mM $MgCl_2$ (1 mM for the inner PCR), 5 pmol/μL of each primer (Roche Diagnostics, Italy), 0.2 mM dNTPs (Roche Diagnostics, Italy), two different concentration of DNA extracted from PB (10 μL and 7.5 μL), and 2.5 μL (1 μL for the inner PCR) of urine, using a Progene Techno PCR System (Duotech, Italy). The procedure involved denaturation at 95°C for 5 min, followed by 40 cycles (35 cycles for the inner PCR) of denaturation at 95°C for 40 s, annealing at 61°C (55°C for the inner PCR) for 40 s, and extension at 72°C for 40 s. The cycles were terminated with a final extension at 72°C for 5 min. DEPC-treated RNase-free water (Biotecx Labs, USA) was used as the negative control; the positive controls were DNA extracted from brain tissue with PML (for JCV) and renal tissue from a subject with histologically proven BKV nephropathy (for BKV). The sensitivity of nPCR multiplex was estimated by amplification of a serial dilutions of positive sample for JCV.

The samples that were positive for the LT region of BKV underwent further amplification of the TCR region using BKTT1 and BKTT2 as outer primers, and BRP1 and BRP2 (amplified fragment 356 bp of the archetype) as inner primers (Table 1). The amplification was performed in a total volume of 25 μL, containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U BioTaq DNA polymerase with an appropriate reaction buffer (Tris-HCl 100 mM, pH 8.3, KCl 500 mM). In the first step, 5 μL of DNA extracted from PB and urine was added to the PCR mixture and, in the second step, 2.5 μL of template; the PCR was then performed using a Progene Techno PCR System. The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, annealing for 40 s at 55°C in the first step and 50°C in the second step, and extension at 72°C for 40 s; the cycles were terminated with a final extension at 72°C for 5 min.

The samples that were positive for the LT region of JCV underwent further amplification of the TCR region using JRE1 and LP2 as outer primers, and RFOR and RREV (an amplified 358 bp fragment of the archetype) as inner primers (Table 1). The amplification was performed in a total volume of 25 μL, containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U BioTaq DNA polymerase with an appropriate reaction buffer (Tris-HCl 100 mM, pH 8.3, KCl 500 mM). In the first step, 5 μL of DNA extracted from PB, or 2.5 μL of urine, were added to the PCR mixture and, in the second step, 2.5 μL of template; the PCR was then

TABLE 1 Genomic sequence and position of PV primers for the LT, TCR regions, and TCR sequence

Region	Name	Position	Sequence
LT			
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 BKV			
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′
TCR JCV			
Outer	JRE1	†4989–5009	5′-CCT CCC TAT TCA GCA CTT TGT-3′
	LP2	†518–537	5′-TGC GGC ACC CAT GAA CCT GA-3′
Inner	RFOR	†5085–5104	5′-GCC TCC ACG CCC TTA CTA CT-3′
	RREV	†291–310	5′-CAG AAG CCT TAC GTG ACA GC-3′

Wobble position IUB code: Y, C/T; R, A/G; W, A/T; K, G/T.
*BKV Dunlop.
†JCV complete genome.
‡SV40 complete genome.

performed using a Progene Techno PCR System. The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, annealing for 40 s at 61°C in the first step and 58°C in the second step, and extension at 72°C for 40 s; the cycles were terminated with a final extension at 72°C for 5 min.

All of the DNA amplification products were analysed by means of 2% agarose gel electrophoresis, and visualised using ethidium bromide staining.

Direct DNA sequencing

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

A cycle sequencing PCR reaction was set up using the Big Dye Version 2.0 Terminator cycle sequencing kit (Applied Biosystems, 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 of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C; the reaction was terminated at 4°C. The cycle sequencing products were purified using Centri-Sep Spin Columns (Princeton Separations, USA), and the DNA was sequenced using an automated 16 capillary sequencer (ABI-Prism 3100, Applied Biosystems, Italy).

The sequences were analysed by means of the Basic Local Alignment Search Tool (BLAST) programs using a website maintained by the National Centre for Biotechnology Information of the United States (http://www.ncbi.nlm.nih.gov, accessed February 2007).

RESULTS

Cytology

Despite a careful search, decoy cells were identified with certainty in only one of the 300 urine samples (0.3%): they were rare and isolated, and characterised by the typical perfectly round nuclei with ground glass viral inclusions,[20] without inflammatory cells.

Molecular biology

LT region The polyomavirus LT region was amplified in 80 of the 300 urine samples (26.6%) and 17 of the 300 PB samples (5.6%) (Table 2): in detail, 49 JCV (16.3%), 28 BKV (9.3%), and three JCV/BKV co-infections (1%) were identified in the urine samples, whereas seven JCV (2.3%) and 10 BKV infections (3.3%) were identified in the PB samples. The urine sample containing decoy cells was positive for BKV DNA. None of the samples was SV40 DNA positive.

None of the umbilical cord blood samples contained polyomavirus DNA.

TABLE 2 LT region of JCV and BKV in urine and blood samples

	BKV	JCV	BKV and JCV	Total positive samples
Urine	28	49	3	80
Blood	10	7	0	17
Total positive samples	38	56	3	97
Cases with urine and blood postive samples	1	2	0	3

Sequence analysis The polyomavirus TCR region was amplified in all of the LT region positive samples of urine (80/80) and PB (17/17) and yielded to DNA fragments of 300–400 bp. All of the DNA isolated underwent sequence analysis using the following primers: for BKV, BRP1 (forward) and for JCV, RFOR (forward). Nucleotide changes detected using forward primers were confirmed using reverse primers: for BKV, BRP2, and for JCV, RREV.

Various polymorphisms and rearrangements were found in the TCR of each BKV and JCV strain in comparison with the consensus sequences (archetype strains).[23,24]

Urine samples

Four BKV strains were identified in urine (total number 28): BKV-WW (14 samples), BKV-WWT (nine samples), BKV-AS (three samples), and BKV-128 (two samples) (for reviews, see Negrini *et al.*[25]). As shown in Table 3, all the BKV-WWT and BKV-AS strains, and five of 14 BKV-WW strains were archetypes, whereas changes in the nucleotide sequence were identified in both BKV-128 strains [single nucleotide polymorphism (SNP) G → T at S block] and in nine BKV-WW (SNP C → T at P block).

All of the JCV isolated were CY strains (49/49): 37 archetype, 11 with single bp substitutions, deletions or insertions, and one rearrangement (insertion of 13 bp at D block) (Table 4).

Sequence analysis of the three co-infected cases revealed JCV-CY (three cases), BKV-WW (two cases) and BKV-WWT (one case). Only single bp changes were identified in the isolated fragments (Table 5).

Blood samples

Sequence analysis of TCR was possible in 14 of the 17 samples; the three failures (two BKV and one JCV) were probably due to low viraemia levels.[26]

As shown in Table 3, in all eight cases, sequence analysis of the BKV TCR revealed a 20 bp insertion of P block (P42–61) in human chromosomes: 20 (five cases) [BLAST: human DNA sequence from clone RP11–112L6 on chromosome 20 of *Homo sapiens*, inserted between CEBPB (20q13.1) and Kua-UEV (20q13.2) genes], and 14 (three cases) [BLAST human chromosome 14 DNA sequence BAC R-182E21 of library RPCI-11 from chromosome 14 of *Homo sapiens*, inserted into DPF3 (14q24.3–q31.1) gene between the exons 5 and 6] (Fig. 1).

All of the JCV were CY strains: four archetypes and two cases with single bp substitutions, deletions or insertions (Table 4).

DISCUSSION

Since the first reports by Gardner *et al.*[27] and Padgett *et al.*,[28] who identified BKV and JCV particles in urine and brain tissue of two patients by means of electron microscopy in 1971, the role of these two viruses in human diseases has been extensively investigated, and the pathogenesis of PML and BKV nephropathy adequately clarified.[29,30] However, despite the large number of studies, some aspects of the natural history of viral infection, the mechanisms of viral transmission, and the sites of viral

TABLE 3 BK virus strains and TCR rearrangements in urine and blood samples

No. of samples	Sample	Strain	Accession no.	Anatomy	Polymorphisms
3	Urine	BKV-AS	M23122	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	Not found
9	Urine	BKV-WWT	M34048	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	Not found
2	Urine	BKV-128	AF218446	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	S_{22}: G → T
5	Urine	BKV-WW	AF123397	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	Not found
9	Urine	BKV-WW		$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	P_{31}: C → T
5	Blood	Cr.20 + BKV		$P_{(42-61)}$	Not found
3	Blood	Cr.14 + BKV		$P_{(42-61)}$	Not found

TABLE 4 JC virus strains and TCR rearrangements in urine and blood samples

No. of samples	Sample	Strain	Accession no.	Anatomy	Polymorphisms
37	Urine	JCV-CY	AF123431	$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	Not found
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	F_{65}: ΔT
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	C_{46}: G → A
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	F_{41}: G → A
2	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	C_{49}: G → A
2	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	D_{19}: A → C F_{19}: A → G
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	C_{24}: C → G
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-52-52-64-64-66)}-E_{(1-18)}-F_{(1-69)}$	
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	D_{19}: A → C D_{44}: C → A F_{21}: A → G F_{35}: G → C
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	F_{21}: ΔG F_{22}: ΔG F_{24}: A → G
1	Urine	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	D_{51}: ΔA
4	Blood	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	Not found
1	Blood	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-67)}-E_{(1-18)}-F_{(1-69)}$	D3: ins C F57: ΔT
1	Blood	JCV-CY		$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-67)}-E_{(1-18)}-F_{(1-69)}$	B20: A → G

TABLE 5 Polymorphisms detected in JC-BK virus co-infections in urine samples

No. of samples	Strain	Accession No.	Anatomy	Polymorphisms
1	BKV-WW	AF123397	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	P_{31}: C → T
	JCV-CY	AF123431	$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	Not found
1	BKV-WW	AF123397	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	P_{31}: C → T
	JCV-CY	AF123431	$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	Not found
1	BKV-WWT	M34048	$P_{(1-68)}-Q_{(1-39)}-R_{(1-63)}-S_{(1-63)}$	Not found
	JCV-CY	AF123431	$A_{(1-25)}-B_{(1-23)}-C_{(1-55)}-D_{(1-66)}-E_{(1-18)}-F_{(1-69)}$	C_{49}: G → A

latency are still not fully understood. The transplacental transmission of polyomaviruses was initially anecdotally proposed[15,31] and has been more recently advanced after the nPCR detection of BKV DNA in placental and fetal tissues;[14] however, to the best of our knowledge, no studies have extensively investigated the possibility of this transplacental transmission in humans.

In this study, we evaluated the likelihood of the transplacental transmission of human JCV and BKV by applying nPCR to maternal peripheral blood and urine samples, and samples of the umbilical cord blood of all their newborns. Polyomavirus DNA was detected in 26.6% of the urine and 5.6% of the maternal peripheral blood samples, but in none of the umbilical cord blood samples, which seems to indicate that transplacental infection is not a significant route of either JCV or BKV transmission.

As there are no other published studies carried out using the same methods, our findings are not readily comparable with those of others. The results of previous studies of vertical polyomavirus transmission are conflicting. Rziha *et al*.[31] found anti-BKV IgM antibodies in the umbilical cord blood samples of 77/846 newborns using immunofluorescent assays, and therefore supported the hypothesis of transplacental transmission. However, the reliability of

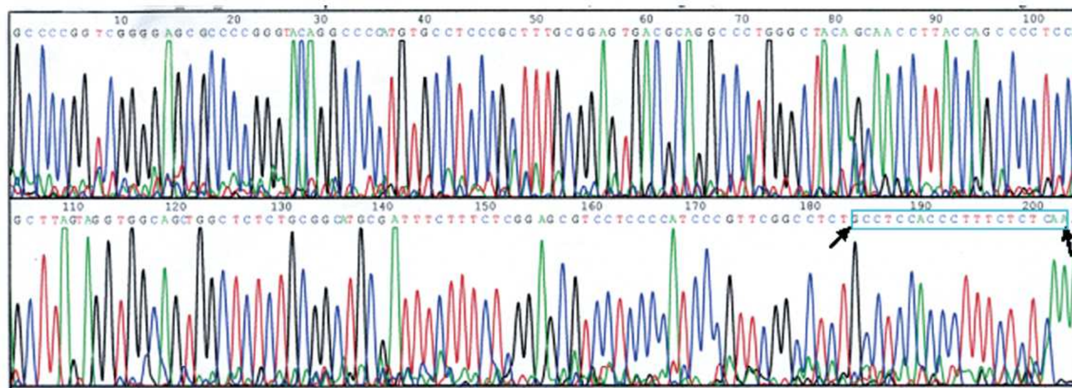

FIG. 1 Electropherogram showing a 20 bp insertion (arrows) of BKV transcription control region in human chromosome 20, isolated from blood sample. The *x* coordinate corresponds to the scan number throughout the time of data collection. The *y* axis is a relative height scale for peak intensity. Traces for A residues are shown in green, C in blue, G in black, and T in red.

this method was questioned by Shah *et al.*[16] on the ground that the results may be non-specific, and large-scale studies by Gibson *et al.*[32] and Coleman *et al.*[17] did not find anti-BKV IgM in the umbilical cord blood samples of newborns whose mothers' serum samples were anti-BKV positive.

In a study of 15 cases of aborted material, Pietropaolo *et al.*[14] detected BKV but not JCV DNA in 80% of the placental tissue, and 80% and 60% of the brain and kidney tissues of the aborted fetuses, and concluded that BKV could be transplacentally transmitted. As this study was carried out using highly sensitive nPCR, the results may have been due to contamination of placental and fetal tissues by maternal blood, although there were no data concerning the presence or absence of polyomavirus DNA in the peripheral blood of the pregnant mothers.

Interestingly, by means of real-time quantitative PCR using maternal and fetal tissue, Zhang *et al.*[33] have recently demonstrated the transplacental transmission of murine polyomavirus (MuPyV) in 86% of the litters born to mice intraperitoneally infected during late pregnancy. However, this could have been favoured by the different anatomical structure of murine placenta, the use of a *Polyomaviridae* that is structurally different from JCV and BKV or, more probably, the high blood MuPyV levels reached in this experimental study of infected mice, which were comparable with those found in acute viral infections/reactivations.

We did not perform quantitative PCR to evaluate blood or urine viral load, but the fact that decoy cells were found in only one of 300 cases, and that viral sequences of the TCR region were detected within human chromosomes in all of the BKV isolated in the maternal blood samples, suggests that the PVs were present in a latent state. Whether they might be vertically transmitted as in experimental animals under appropriate conditions remains to be clarified.

Sequence analysis of the TCR of JCV and BKV in the urine and blood samples showed that the viral isolates had mainly archetypal TCR sequences and that, when mutated, single bp changes were more frequent than rearrangements (which were only detected in two urine samples). The exact significance of these genomic changes is unknown;[19] however, our findings offer no evidence that either the presence or type of viral rearrangement may increase the risk of viral infectivity or transplacental transmission. It is interesting that the blood samples taken from the umbilical cord of the newborn whose mother showed these rearrangements were polyomavirus DNA negative.

In conclusion, the results of our study carried out using highly sensitive nPCR methods seem to exclude the possibility that the transplacental transmission of BKV and JCV is a frequent occurrence, and indicate that viral replication during late pregnancy is an exceedingly rare event and TCR rearrangements do not increase the risk of transplacental transmission. However, other ways of maternal–fetal transmission of polyomavirus infection, such as the ingestion or inhalation of maternal blood containing polyomavirus DNA by the newborn during the delivery, or by the infant during breast feeding, cannot be excluded by our study.

Address for correspondence: Professor R. Boldorini, Scienze Mediche, Facolta di Medicina e Chirurgia, via Solaroli 17, Novara 28100, Italy. E-mail: renzo.boldorini@med.unipmn.it

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