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TITOLO:

Ruolo dei riarrangiamenti genomici nella regione regolatoria virale e delle mutazioni nella proteina capsidica VP1 dei Polyomavirus umani in soggetti con trapianto di rene e di midollo osseo.

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<u>INDICE</u>

INTRODUZIONE

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I Polyomavirus sono agenti eziologici in grado di infettare diverse specie animali, quali topo, criceto, scimmia e l'uomo. Questi virus vennero inizialmente classificati nella famiglia delle *Papovaviridae* a causa della loro struttura con capside icosaedrico simile ai Papillomavirus (anch'essi membri della stessa famiglia), osservata al microscopio elettronico al momento della loro scoperta (Figura 1). Successivamente vennero poi classificati come famiglia delle *Polyomaviridae*[1]. Tra i Polyomavirus in grado di infettare l'uomo, i più studiati sono sicuramente i Polyomavirus hominis 1 e 2, meglio conosciuti con l'acronimo, rispettivamente, di BK Virus (BKV) e JC Virus (JCV), derivante dalle iniziali dei pazienti in cui sono stati isolati per la prima volta nel 1971. BKV fu isolato da un laboratorio inglese in un campione di urina di un paziente portatore di trapianto di rene con stenosi ureterale da causa ignota: le indagini ultrastrutturali rivelarono la presenza di particelle virali icosaedriche, prive di envelope, del diametro di circa 45 nm[2]. Nello stesso anno, in un altro laboratorio, furono studiati campioni cerebrali autoptici di soggetti deceduti per una malattia demielinizzante del sistema nervoso centrale, ad esordio acuto-subacuto, rapidamente progressiva, chiamata *leucoencefalopatia multifocale progressiva* (PML): anche in questo caso si osservò la presenza di un virus con caratteristiche simili a quello osservato in Inghilterra[3]. Di recente scoperta sono invece i Polyomavirus KI (Karolinsk Institute) e WU (Washington University), strettamente correlati tra loro ed isolati da escreati respiratori e il Merkel Cell Polyomavirus descritto come probabile agente eziologico del carcinoma a cellule di Merkel [4].
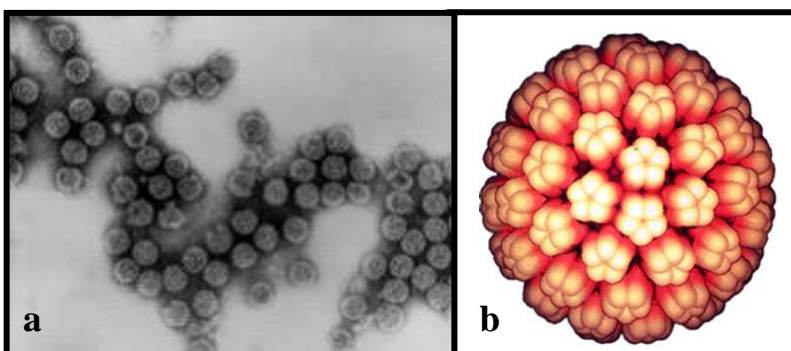

FIGURA 1
A) Fotografia al microscopio elettronico di SV40 (ingrandimento 10000).
B) Rappresentazione grafica 3D della struttura esterna dei Polyomavirus rappresentata dall'envelope formato nello strato esterno solo dalla VP.

Esiste inoltre un altro Polyomavirus il cui ospite naturale è la scimmia, ma che è stato osservato trovarsi anche nell'uomo; si tratta del virus vacuolizzante della scimmia, o SV40. SV40 è uno dei virus più studiati di cui si conosce sia l'intero genoma, sia il suo modo di interagire con la

cellula ospite e sembra essere stato introdotto accidentalmente nell'uomo tramite la somministrazione di vaccini antipoliomielite contaminati tra il 1955 e il 1963; il vaccino veniva allestito in colture di cellule renali di scimmia in cui il virus era presente come contaminante e si suppone che, una volta inoculato nell'uomo, sia stato poi in grado di infettare nuovi individui[5]. Tuttavia, alcune osservazioni epidemiologiche su popolazioni accidentalmente infettate con vaccini antipoliomielite non hanno evidenziato apparenti legami del virus con l'insorgere di specifiche patologie. Recenti osservazioni hanno ipotizzato che esso possa prendere parte al processo di cancerogenesi umana soprattutto per ciò che riguarda l'insorgenza di mesoteliomi maligni[6, 7].

Studi strutturali e genomici successivi alla loro scoperta hanno mostrato presenza di capside icosaedrico privo di envelope del diametro di 40-45 nm (variabile in relazione ai mezzi di fissazione e di inclusione utilizzati per l'indagine ultrastrutturale) e DNA a doppia elica della lunghezza di circa 5300 paia di basi (bp)[8]. Strutturalmente il DNA genomico è racchiuso da un capside virale composto da 360 copie della proteina capsidica maggiore VP (viral protein) 1, che si organizza in pentameri legando le proteine capsidiche minori, VP2 e VP3[7].

Il genoma virale è costituito da due regioni codificanti e una regione di controllo trascrizionale[9], in dettaglio (Figura 2):

- regione precoce (Early region), codificante per una proteina tumore-associata, Large-T (LT), e una proteina dalla funzione non ancora completamente nota, small-t (t).

- regione tardiva (Late region), codificante per le proteine capsidiche VP1, VP2, VP3, e per l'agnoproteina, che si suppone essere coinvolta nell'assemblaggio del capside virale.

- regione di controllo trascrizionale (TCR), non codificante, posizionata tra le due regioni codificanti. Presenta siti di legame per fattori di trascrizione e, pertanto, riveste un ruolo chiave nella storia naturale dell'infezione.
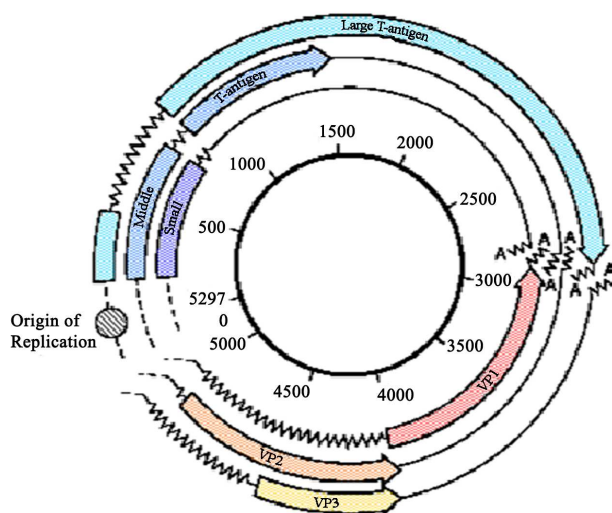

FIGURA 2

Rappresentazione schematica del genoma dei Polyomavirus umani. La regione TCR è posizionata tra la regione early (Large T, Small t), che codifica in un verso, e la regione late (VP1, VP2, VP3), che codifica in senso opposto.

La regione regolatoria di JCV è stata schematicamente suddivisa in 6 blocchi di sequenza genica da Ault e Stoner[10], convenzionalmente definiti A, B, C, D, E, F, ciascuno composto da un determinato numero di bp (A:25bp; B:23bp; C:55bp; D:66bp; E:18 bp; F:69bp). Analogamente Moens et al[11] hanno suddiviso la regione TCR di BKV in 5 blocchi di sequenza genica (O:124bp; P:68bp; Q:39bp; R:63bp; S:63bp).

TCR di entrambi i virus si è dimostrata essere conservata nella maggior parte dei casi, con occasionali mutazioni puntiformi (sostituzioni, delezioni o inserzioni di singole basi nucleotidiche); queste sequenze sono state definite da Yogo et al.[12] archetipi. L'archetipo rappresenta il genotipo virale prevalente nella popolazione umana derivante dalla co-evoluzione con l'ospite. L'ipotesi è che dalle sequenze archetipo presenti in un individuo si originano, per fenomeni di riarrangiamento genico, le sequenze ricombinanti a causa dell'ipervariabilità della regione, allo scopo di adattarsi alle condizioni presenti nell'ospite[13].

All'interno della regione TCR di BKV e JCV sono stati osservati siti di legame per fattori trascrizionali[14,15] (Figura 3), quali:

- **Sp1**: proteina implicata nella differenziazione cellulare; sembra avere un ruolo nel mantenere libere da metilazione le isole CpG, mantenendo attiva la trascrizione.
- **Nuclear Factor-1 (NF1)**: media le reazioni infiammatorie e immunologiche in risposta a diversi stimoli; mutazioni in questo sito hanno mostrato avere conseguenze nella trascrizione dei geni tardivi.
- **Large T**: agisce con un meccanismo di controllo negativo, inibendo l'attività del promotore precoce e bloccando la sua stessa espressione.
- **Purα**: proteina implicata nel controllo della replicazione e della trascrizione del DNA, agisce promuovendo l'espressione delle proteine precoci; inibita da Large T.
- Altri fattori di trascrizione che si legano alla regione TCR sono: **GM-CSF** (granulocyte/macrophage-colony stimulating factor), **PEA3**, **AP1**.

La regione TCR sembra essere determinante per la trascrizione cellulo-specifica del DNA virale, infatti è stato osservato che le regioni promotrici presenti nella TCR di JCV aumentano notevolmente la trascrizione nelle cellule gliali rispetto alle cellule non gliali in coltura, probabilmente anche grazie alla presenza in questi tipi di cellule di fattori di trascrizione più specifici per le sequenze regolatrici[14]. Risulta evidente quindi che significative mutazioni (come ad es. grosse delezioni, inserzioni o duplicazioni genomiche) possano, almeno

teoricamente, modificare la capacità replicativa virale, l'infettività, il tropismo cellulare od anche gli effetti patogeni di tali virus.
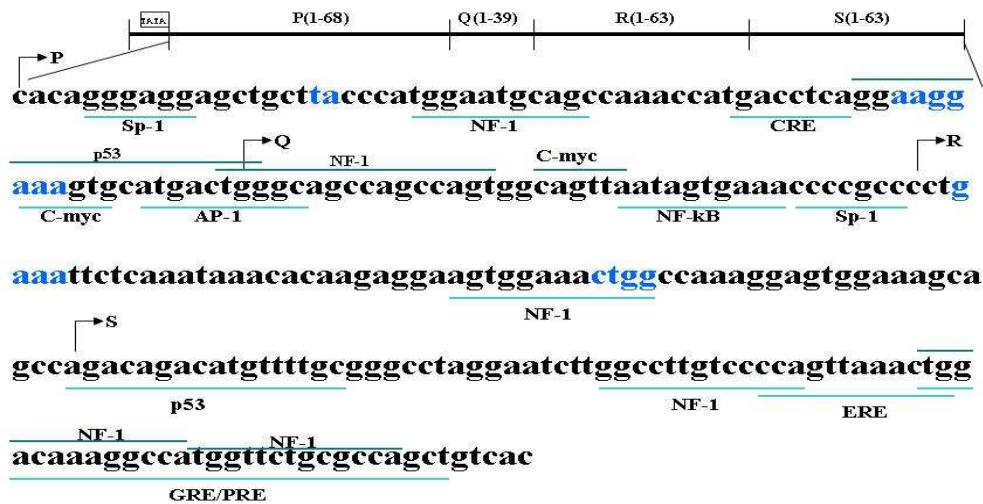

FIGURA 3

Rappresentazione schematica della regione regolatoria (TCR) BKV.
I numeri nelle parentesi indicano il numero di paia di basi presenti in ogni blocco. I nucleotidi polimorfici sono colorati in blu. I siti di legame con fattori di trascrizione sono segnati sopra la sequenza.

Analogamente alla regione TCR, si suppone che anche mutazioni aminoacidiche nella sequenza proteica di VP1 potrebbero influenzare la capacità del virus di infettare con diverso tropismo le cellule bersaglio[16]; per questo motivo nell'ultimo decennio un certo numero di studi si è proposto di analizzare e di caratterizzare in dettaglio la struttura della proteina VP1, le proteine bersaglio presenti sulla cellula e il sito di legame con tali proteine[17,18]. Le prime conoscenze riguardanti la funzione di VP1 sono relative a SV40, il quale è stato osservato essere in grado di riconoscere le proteine del complesso maggiore di istocompatibilità (MHC), presenti sulle cellule bersaglio. Successive analisi sui Polyomavirus umani hanno però evidenziato che VP1 di BKV e JCV non lega le molecole MHC, ma presenta un legame selettivo con oligosaccaridi leganti all'N-terminale una molecola di acido sialico[18,19].

La proteina VP1 è costituita da 362 aminoacidi, tradotti da una sequenza di circa 1086 nucleotidi presenti nella regione tardiva del genoma, e viene suddivisa in 5 loops: BC, DE, EF, GH e HI (Figura 4). La struttura terziaria di ogni monomero forma un "barile" β composto da filamenti β antiparalleli tra i quali si posizionano 3_{10}-eliche e α-eliche. Nella regione C-terminale il loop DE

si inserisce in profondità nel monomero VP1 adiacente consentendo il legame con gli altri monomeri, formando infine un pentamero. La struttura capsidica viene completata attraverso l'N-terminale di ogni monomero che si inserisce in un pentamero adiacente, stabilizzando così la struttura del capside virale (Figura 5 A e B)[20].
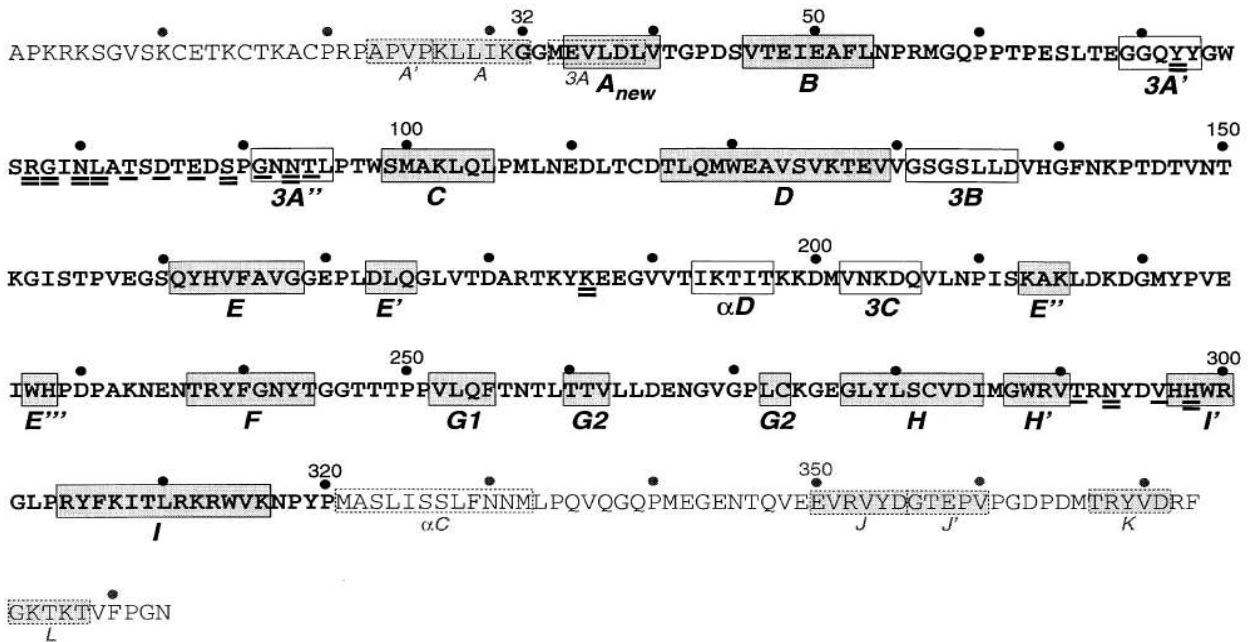
FIGURA 4

Sequenza regione VP1 con struttura secondaria determinata in relazione alla formazione dei legami idrogeno e le principali conformazioni di catena.
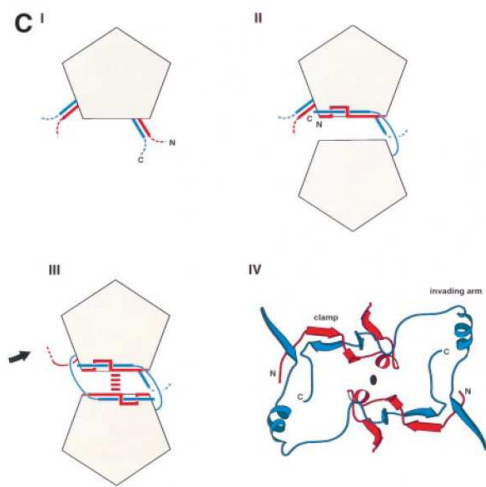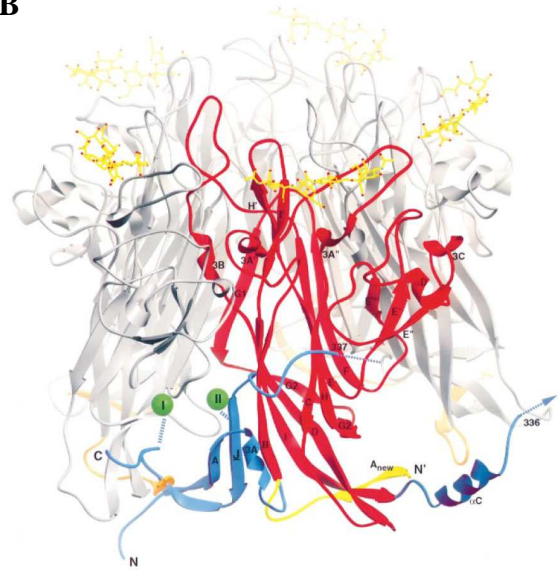
FIGURA 5

A- modello di legame tra i pentameri. I: pentamero libero; II: un braccio C-terminale (blu) invade il pentamero adiacente e si lega all'N-terminale (rosso); III: legame stabile tra due pentameri; IV: rappresentazione del legame stabile illustrato schematicamente al punto III.

B- Pentamero completamente assemblato. In blu sono illustrati le braccia che "invadono" la molecola adiacente. Due ioni calcio (verde) aiutano a stabilizzare la particella formatasi
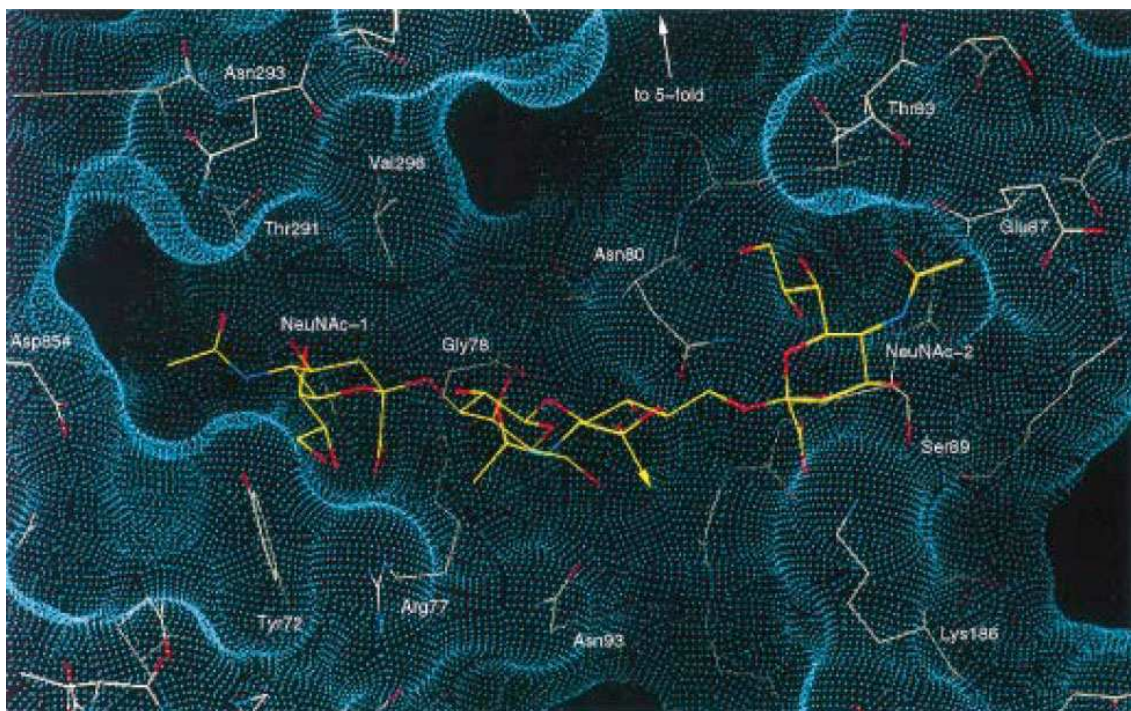
FIGURA 6

Superficie di legame della proteina VP1 in cui si lega la molecola di acido sialico (in giallo).

Studi in vitro utilizzando cellule *Vero* hanno dimostrato l'interazione tra VP1 e gangliosidi a cui è legata in posizione N-terminale una o più molecole di acido sialico. Low et al.[21] hanno recentemente condotto uno studio trattando le cellule con neuroamidasi, un inibitore dell'acido sialico, e incubandole con BKV: il virus non era in grado di infettare le cellule. Successive analisi hanno confermato il legame tra la proteina VP1 e acido sialico e hanno permesso di identificare due particolare gangliosidi interessati nel legame: GD1b e GT1b. Un terzo ganglioside preso in considerazione, GM1, ha portato infezione se incubato con SV40 ma non con BKV, indicando che l'acido sialico legato in posizione α2-8, mancante in GM1 ma presente in GD1b e GT1b, è essenziale per il legame con VP1. Il modello ad alta risoluzione proposto da Stehle et al.[20] ha mostrato che la superficie di legame di VP1 consiste in una tasca piana formata dai loop di foglietti β, nella quale si inserisce la molecola di acido sialico (Figura 6).

La sequenza aminoacidica di BKV interessata nel legare il ligando è compresa nella regione tra l'aminoacido 61 e l'aminoacido 83. Jin et al.[22] hanno analizzato la sequenza nucleotidica di 69 bp codificante per questa regione, individuando polimorfismi nucleotidici caratteristici, che hanno permesso di classificare il virus in quattro principali gruppi sierologici, successivamente caratterizzati in sottotipi da Baksh et al.[23]:

- GRUPPO I: Dunlop (DUN), MM, GS
- GRUPPO II: SB
- GRUPPO III: AS
- GRUPPO IV: IV, MG

BKV viene anche classificato in relazione alla sequenza della regione TCR, che mostra dei siti polimorfici caratteristici e conservati tra i vari genotipi[24]. I più rappresentati sono:

- WW
- WWT
- AS
- 128-I

I Polyomavirus sono largamente diffusi nella popolazione umana, dato rappresentato da uno studio di Shah[25] dove, analizzando la presenza di IgG anti-BKV e anti-JCV nel siero, si osserva che rispettivamente il 60% e 80% della popolazione mondiale risulta essere infettata da Polyomavirus.

Le modalità attraverso cui il Polyomavirus infetta e si trasmette non sono ancora interamente note: la via di trasmissione principale sembra essere quella respiratoria [26,27], anche se sono possibili altre modalità quale quella oro-fecale[28], urinaria[29] e trans-placentare[30,31], quest'ultima è stata presa in esame in un recente studio condotto dal nostro gruppo di ricerca[32]: non è stata

osservata trasmissione verticale madre-feto durante l'ultimo trimestre di gravidanza, tuttavia ulteriori studi sono in corso al fine di valutare la possibile trasmissione durante tutto il corso della gestazione. Indipendentemente dalle modalità di trasmissione, l'infezione da Polyomavirus risulta quasi sempre asintomatica od oligosintomatica ed è seguita da una fase viremica nella quale virioni o più probabilmente frammenti genomici virali, veicolati da linfociti circolanti attraverso il torrente circolatorio raggiungono gli organi bersaglio. Questi ultimi sono in parte noti, in parte non noti e differenti per JCV (encefalo, vie escretrici urinarie) e BKV (rene, vie escretrici urinarie). Raggiunta la cellula bersaglio il virus si localizza sulla membrana plasmatica in modo casuale, distribuendosi o a piccoli gruppi di copie virali oppure in gruppi di numerosi virioni; in questa sede riconosce i gangliosidi leganti acido sialico e viene veicolato all'interno della cellula mediante endocitosi[33]. Sono due le vie endocitotiche con cui BKV e JCV superano la membrana plasmatica[34]:

- JCV entra nella cellula attraverso vesciole ricoperte di molecole di clatrina;
- BKV utilizza caveole, vescicole delle dimensioni di 50-70 nm, a forma di fiasca, presenti sulla superficie di molti tipi di cellule.

Nel corso di una infiammazione matura e produttiva, le particelle virali si uniscono a formare aggregati tubulo-vescicolari all'interno della cellula, osservati mediante microscopia elettronica in continuità con il reticolo endoplasmatico rugoso e in prossimità dell'apparato di Golgi[33]. Il meccanismo mediante il quale i Polyomavirus penetrano nel nucleo ancora non è chiaro, alcuni studi hanno ipotizzato che i pori nucleari siano troppo piccoli per consentire il passaggio del virione, oppure che siano presenti degli inibitori che riducano il diametro dei pori; tuttavia, altri studi hanno proposto proprio il passaggio attraverso i pori nucleari come via di ingresso nel nucleo[35,36].

Durante la prima infezione asintomatica, successivamente all'ingresso nella cellula, i Polyomavirus diventano latenti negli organi bersaglio, in forma di DNA extracromosomico (episomiale) o di virus integrato e in questa fase sono rilevabili solo mediante metodiche di biologia molecolare[37,38]. Tuttavia, in condizioni di immunodeficienza di variabile grado (farmacologiche, indotte da infezione da HIV, gravidanza, deficit di immunità cellulo-mediata), il virus può andare incontro a riattivazione con conseguente infezione produttiva che porta alla produzione di nuova progenie virale, lisi cellulare e conseguente danno d'organo.

All'inizio della riattivazione il virus trasloca nel nucleo in cui ha inizio la trascrizione dei geni precoci: Large-T e small-t, derivanti da uno splicing alternativo di un singolo trascritto primario. Large-T si lega con, e inibisce attraverso degradazione, la proteina regolatrice del ciclo cellulare nota come retinoblastoma (pRB) e membri della stessa famiglia quali p107 e p130. Il legame permette la liberazione del promotore del ciclo E2F, che consente l'ingresso nella fase S,

dove la cellula produce proteine utili sia per la trascrizione sia per mantenere l'infezione virale. Large-T ha una funzione di controllo per le fasi iniziali della replicazione virale, legandosi a sequenze genomiche del virus formando complessi con diverse proteine: complesso polimerasi α-primasi; proteina A replicativa; proteina che lega DNA a singola elica. Inoltre possiede un'attività elicasica che permette la distensione del DNA e lo predispone per la sintesi. Nella fase tardiva, Large-T promuove la trascrizione dei geni tardivi e la repressione di quelli precoci. I meccanismi di inibizione non sono ancora pienamente conosciuti, ma quelli di attivazione si basano sul reclutamento di proteine cellulari. I geni tardivi VP1, VP2 e VP3, sono responsabili della formazione del capside, coadiuvati dalla agnoproteina. La capacità del large-T di trasformare le cellule è data da tre domini: dominio J, dominio legante pRB e dominio legante p53. Quest'ultimo dominio è responsabile del legame alla proteina, inattivandola e causando la crescita incontrollata della cellula. Durante l'infezione litica, l'interazione con queste proteine consente di prolungare la vita cellulare, per poter aumentare il numero di virioni prodotti[8,14].

L'effetto di degradazione delle proteine oncosoppressive p53 e pRB da parte di Large T sembra essere alla base del processo di trasformazione neoplastica proposto per alcune neoplasie umane[39,40].

Le patologie sicuramente causate da polyomavirus umani sono: leucoencefalopatia progressiva multifocale, cistiti ed uretriti emorragiche e la nefropatia da polyomavirus (PVAN)[41,42]. La PVAN consiste in una infezione litica di cellule epiteliali dei tubuli renali o delle cellule epiteliali della capsula di Bowman che riveste i glomeruli renali, con conseguente nefrite tubulo-interstiziale[8]. Tale patologia è causata in prevalenza da infezione-riattivazione di BKV[43], anche se di recente è stato riportato un caso di possibile coinvolgimento di JCV[44]. La PVAN, esclusiva dei soggetti immunodepressi in corso di trapianto renale, può essere causata da una riattivazione di virus latente nel soggetto ricevente l'organo trapiantato o, in alternativa, da una nuova infezione veicolata dall'organo trapiantato. Le cause principali della riattivazione sono le terapie farmacologiche anti-rigetto[45] (in particolare mophetil-mycophenolato, ciclosporina A e prednisone), ma ci possono essere altri fattori di rischio come l'età avanzata (le persone anziane rispondono di più all'immunosoppressione), il sesso (i maschi sono più a rischio), il siero-stato (l'80% dei pazienti con PVAN presenta BKV nel siero prima del trapianto)[8]. La diagnosi di PVAN si basa su elementi clinici, di laboratorio e su indagini cito-istologiche effettuate su urine e biopsia renale.

I sintomi della infezione sono aspecifici e in gran parte simili a quelli che si osservano in corso di rigetto acuto interstiziale mentre gli esami di laboratorio evidenziano generalmente un incremento aspecifico della creatinina sierica. Un importante elemento diagnostico è rappresentato dalla escrezione urinaria di cosiddette "decoy cells", identificabili con esame citologico urinario

sotto forma di cellule con nucleo ipercromico, con inclusioni intranucleari a vetro smerigliato, espressione morfologica di replicazione virale[46]. Tale reperto, è costantemente presente nei soggetti con PVAN, ma è scarsamente specifico, in quanto riscontrabile anche in soggetti in cui la riattivazione virale avviene nelle vie escretrici (ad es. nelle cistiti od ureteriti emorragiche) e deve essere associato ad una valutazione molecolare sulla presenza di viremia. Le attuali linee guida sulla diagnostica della PVAN prevedono poi che in caso di positività citologica alle decoy cells e molecolare su sangue debba essere eseguita una biopsia renale che attualmente rappresenta il *gold standard* diagnostico per questa patologia.

In caso di PVAN, la biopsia renale evidenzia un quadro di nefrite tubulo-intersiziale (Figura 7) (infiltrati mononucleari interstiziali, atrofia tubulare e fibrosi interstiziale) associata alla presenza delle tipiche inclusioni virali intranucleari, dimostrabili morfologicamente e con metodiche immunoistochimiche in cellule epiteliali.

Lo sviluppo della PVAN può essere diviso in stadi[8]:

➢ STADIO A: coinvolgimento focale midollare della cellule epiteliali del tubulo, inclusioni nucleari limitate

➢ STADIO B: estensivo coinvolgimento del rene con alterazioni citoplasmatiche diffuse o multifocali, necrosi e primi segni di fibrosi

➢ STADIO C: fibrosi interstiziale, i tubuli sono atrofici e appiattiti.

Dai dati della letteratura emerge che l'infezione-riattivazione di BKV nei soggetti trapiantati di rene rappresenta un evento molto frequente (nell'ordine del 70% dei pazienti) mentre la malattia conclamata si osserva in una netta minoranza (2-7%)[47]. Tale discrepanza può dipendere da vari fattori, in parte legati all'ospite (età, sesso, tipo e livello di immonodepressione, fattori geografici) e in parte legati al virus. Tra questi ultimi di particolare rilevanza sembra essere il genotipo virale, quale determinato dalle caratteristiche specifiche della regione regolatoria che, come precedentemente detto, presiede al controllo della replicazione ed infettività virale; più recentemente è emerso il possibile ruolo di JCV come co-fattore coinvolto nel danno renale da Polyomavirus.

Una seconda patologia associata ai Polyomavirus, in particolar modo BKV, è la cistite emorragica[42], caratterizzata da ematuria dovuta a infezione emorragica della mucosa vescicale, accompagnata da disuria, dolore sovrapubico e minzione frequente e impellente. Poiché, come già precedentemente detto, i Polyomavirus si riattivano in situazioni di immunodepressione, negli ultimi anni diversi studi si sono incentrati nel determinare il ruolo di BKV nello sviluppo di cistite emorragica in pazienti sottoposti a trapianto di midollo osseo o di cellule staminali della linea emopoietica[48,49,50]. Si è osservato che BKV nei campioni di urina in pazienti trapiantati di midollo

osseo è presente nel 50% dei casi[51] ed è stata associata a diverse manifestazioni cliniche come: ematuria asintomatica, cistite emorragica, stenosi ureterale, nefrite interstiziale[8,52]. Tra queste la complicazione più frequente in pazienti sottoposti a trapianto di midollo osseo è la cistite emorragica (10-25%) che, in rari casi, può condurre a morte[53]. I diversi gradi di ematuria hanno permesso di determinare una scala di gradazione crescente che aiuta a determinare la severità della cistite emorragica[54]:

- grado 1: ematuria microscopica
- grado 2: ematuria macroscopica
- grado 3: ematuria macroscopica con presenza di coaguli
- grado 4: grave ematuria con presenza di coaguli che causano ostruzione del tratto urinario

I potenziali fattori di rischio di sviluppo di cistite emorragica che portano a riattivazione di BKV sono: presenza di anticorpi IgG per BKV, regime di condizionamento (intenso o intensità ridotta), nei casi di trapianto allogenico si ha un aumentato rischio, insorgenza di Graft Versus Host Disease[49,55]. Per valutare la possibilità di insorgenza di cistite emorragica occorre valutare la carica virale nei campioni di urina dei pazienti, tuttavia questo metodo diagnostico non risulta decisivo poiché anche pazienti senza cistite emorragica presentano viremia[56].
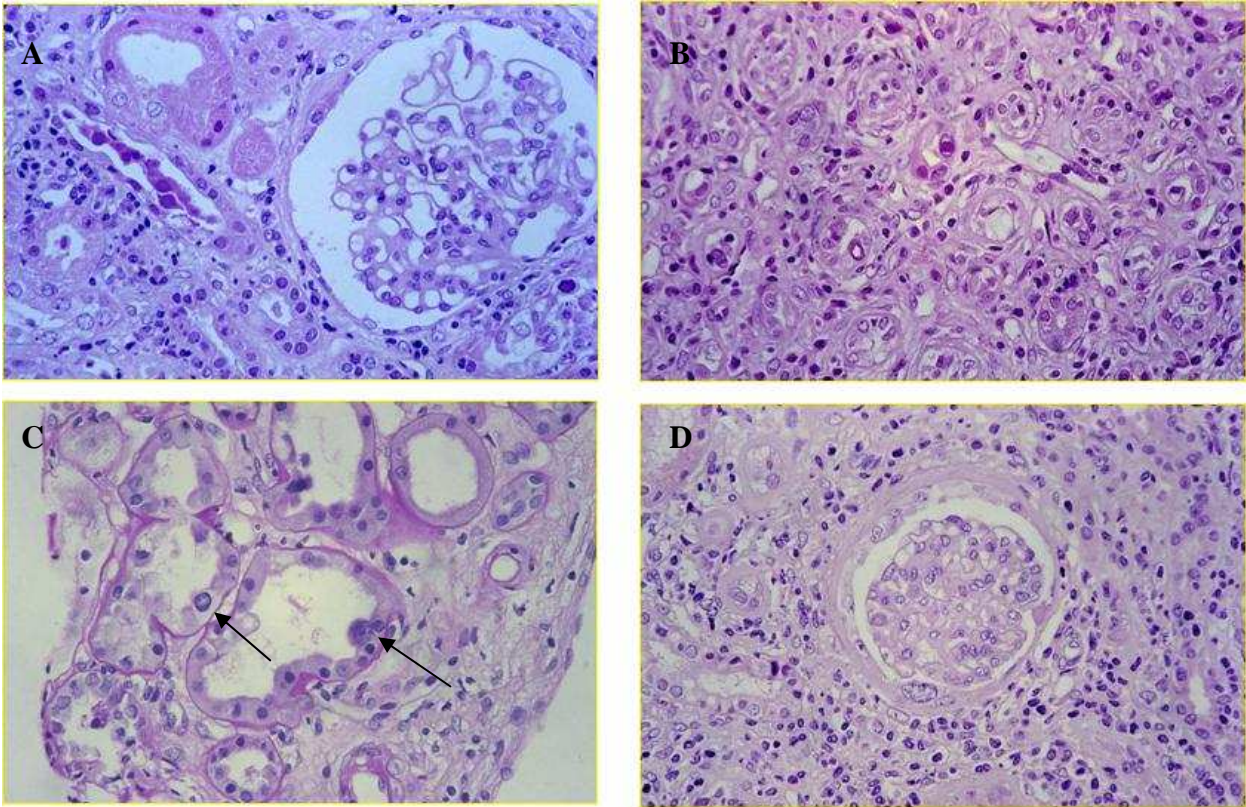
FIGURA 7

Biopsie renali di soggetti con nefropatia polyomavirus-associata: A,B,C) Varie tipologie di inclusi virali in cellule tubulari renali (frecce) e D) in cellula parietale di capsula di Bowman (freccia).
Ematossilina-Eosina, Ingrandimenti originali: 250x

SCOPO DELLA TESI

I Polyomavirus sono presenti in stato di latenza nel 90% della popolazione mondiale, ma in particolari condizioni di immunodepressione –quale quella farmaco-indotta in corso di trapianto di midollo osseo o di rene- essi si riattivano causando, in una bassa percentuale di pazienti (<10%), insorgenza di patologie quali la nefropatia Polyomavirus-associata e la cistite emorragica. La discrepanza tra frequenza di infezione e frequenza di patologia virus-indotta può essere correlata – almeno ipoteticamente- a vari fattori, in parte legati all'ospite, in parte alla tipologia di terapie immunosoppressive e in parte a caratteristiche strutturali del virus stesso. Lo scopo di questa tesi è di investigare se specifiche mutazioni nelle regioni genomiche virali TCR e VP di BKV e JCV possano essere correlate ad un incremento della patogenicità virale nel determinismo sia della PVAN in corso di trapianto di rene, sia della cistite emorragica in corso di trapianto di midollo osseo.

A tale scopo sono state caratterizzate le sequenze genomiche di TCR e VP di isolati virali da campioni (sangue, urine, eventuale biopsia renale) prelevati in pazienti provenienti da due casistiche distinte.

Nella prima lo studio è stato condotto su una casistica di pazienti portatori di trapianto renale, afferenti al Centro Trapianti di rene dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità", che hanno sviluppato PVAN, istologicamente dimostrata, e, per confronto, di pazienti con infezione da Polyomavirus senza PVAN (gruppo di controllo).

Nella seconda, in collaborazione con il Dipartimento di Sanità Pubblica dell'Università "La Sapienza", è stata analizzata una casistica di pazienti sottoposti a trapianto di midollo osseo con cistite emorragica da Polyomavirus.

Dopo estrazione ed amplificazione della regione meglio conservata di Polyomavirus (LT), sono state amplificate e sequenziate le regioni genomiche virali TCR e VP di BKV e JCV. La frequenza e la tipologia di mutazioni di TCR e VP, e le modificazioni aminoacidiche della struttura proteica della VP sono state analizzate e comparate tra il gruppo di pazienti con PVAN e il gruppo di controllo in termini statistici, così da evidenziare eventuali differenze significative tra i due gruppi, anche nell'ottica di utilizzare tali informazioni ai fini di una metodologia di approccio diagnostico non invasivo.

RISULTATI

Pubblicazione 1

BK virus è l'agente eziologico della nefropatia Polyomavirus-associata (PVAN); su di esso si sono incentrati numerosi studi nel corso degli ultimi decenni, principalmente al fine di stabilire il suo ruolo nella patogenesi della malattia. Alcuni gruppi di ricerca hanno analizzato la regione regolatoria virale (TCR) riscontrando delle mutazioni in campioni prelevati da soggetti affetti da PVAN, giungendo alla conclusione che determinate mutazioni possono essere causa di aumentata patogenicità virale e quindi maggiormente associate allo sviluppo di PVAN.

Alla luce dei risultati ottenuti in precedenti lavori del nostro gruppo di ricerca, il presente studio si propone di valutare su un'ampia casistica di pazienti portatori di trapianto renale, l'effettiva associazione tra riarrangiamenti nella regione TCR di BKV e l'insorgenza della PVAN. Dal 2001 al 2007 sono stati raccolti campioni di sangue e urine, prelevati durante i controlli di routine, e biopsie renali, di 226 pazienti portatori di trapianto renale afferenti presso il Centro Trapianti dell'Ospedale Maggiore di Novara. Nel corso dello studio 8 pazienti hanno sviluppato PVAN (5 maschi e 3 femmine, età media 51 anni), mentre i restanti 218 pazienti (118 maschi e 100 femmine, età media 51,6 anni) che non hanno sviluppato la patologia sono stati analizzati come gruppo controllo. Sui campioni di sangue, urina e biopsie renali è stata quindi eseguita amplificazione e successivo sequenziamento al fine di determinare la distribuzione dei genotipi di BKV e il significato patologico dei riarrangiamenti riscontrati nei diversi ceppi virali.

I risultati delle indagini hanno mostrato presenza di riarrangiamenti genomici sia in campioni provenienti da pazienti PVAN sia in campioni di pazienti controllo; la maggior parte dei riarrangiamenti era rappresentata da sostituzioni di singole basi nucleotidiche, ma sono state osservate anche grosse delezioni o duplicazioni in entrambe le popolazioni. Questi dati suggeriscono che i riarrangiamenti della regione TCR possano essere eventi casuali, non correlati con un incremento della virulenza o patogenicità virale. L'unico dato di un certo rilievo statistico è risultato la maggiore prevalenza di BKV WWT nei pazienti con PVAN rispetto al gruppo di controllo, suggerendo una possibile correlazione tra questo genotipo e sviluppo della patologia. Tuttavia anche questo dato necessita di ulteriori conferme, in considerazione del basso numero di pazienti con PVAN inclusi in questo studio.

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

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● **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.

(*Arch Pathol Lab Med.* 2009;133:766–774)

P olyomavirus-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 immu-

nosurveillance 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

BKV Strains in Patients With Renal Transplant—Boldorini et al

18

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 700g for 10 minutes, and 5 mL of the urine pellets were then cytocentrifuged at 250g 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 700g 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-
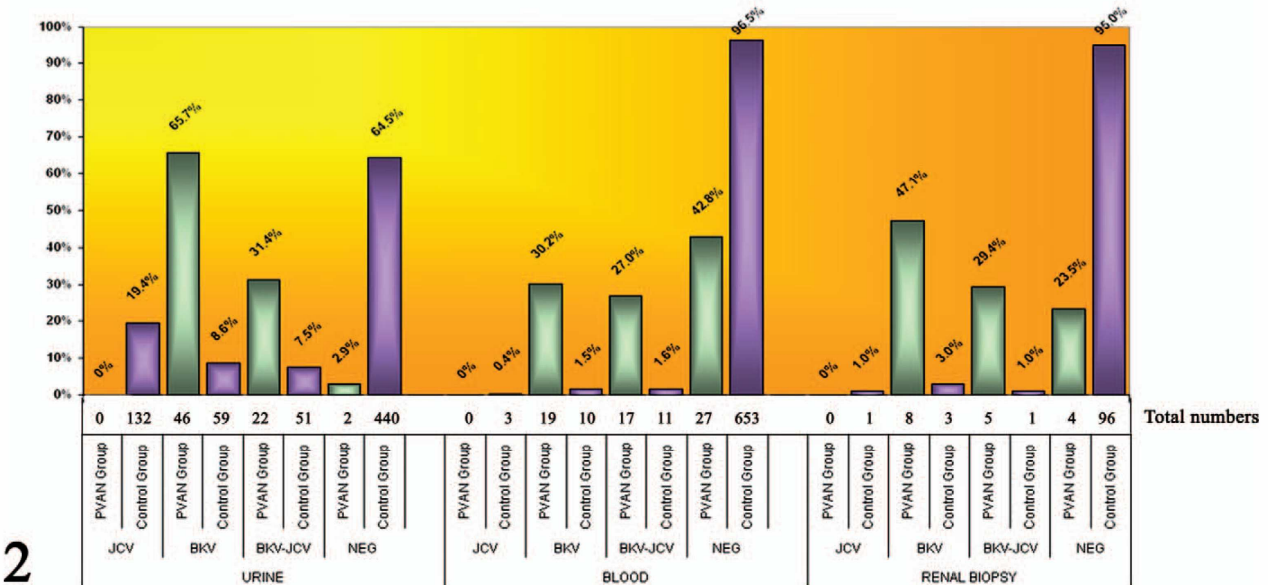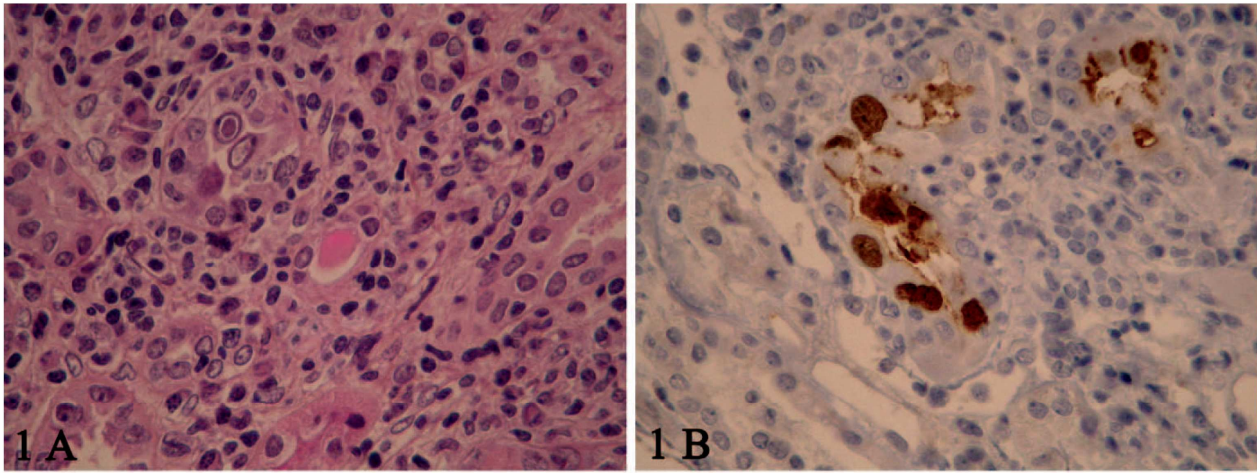
20

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).

21
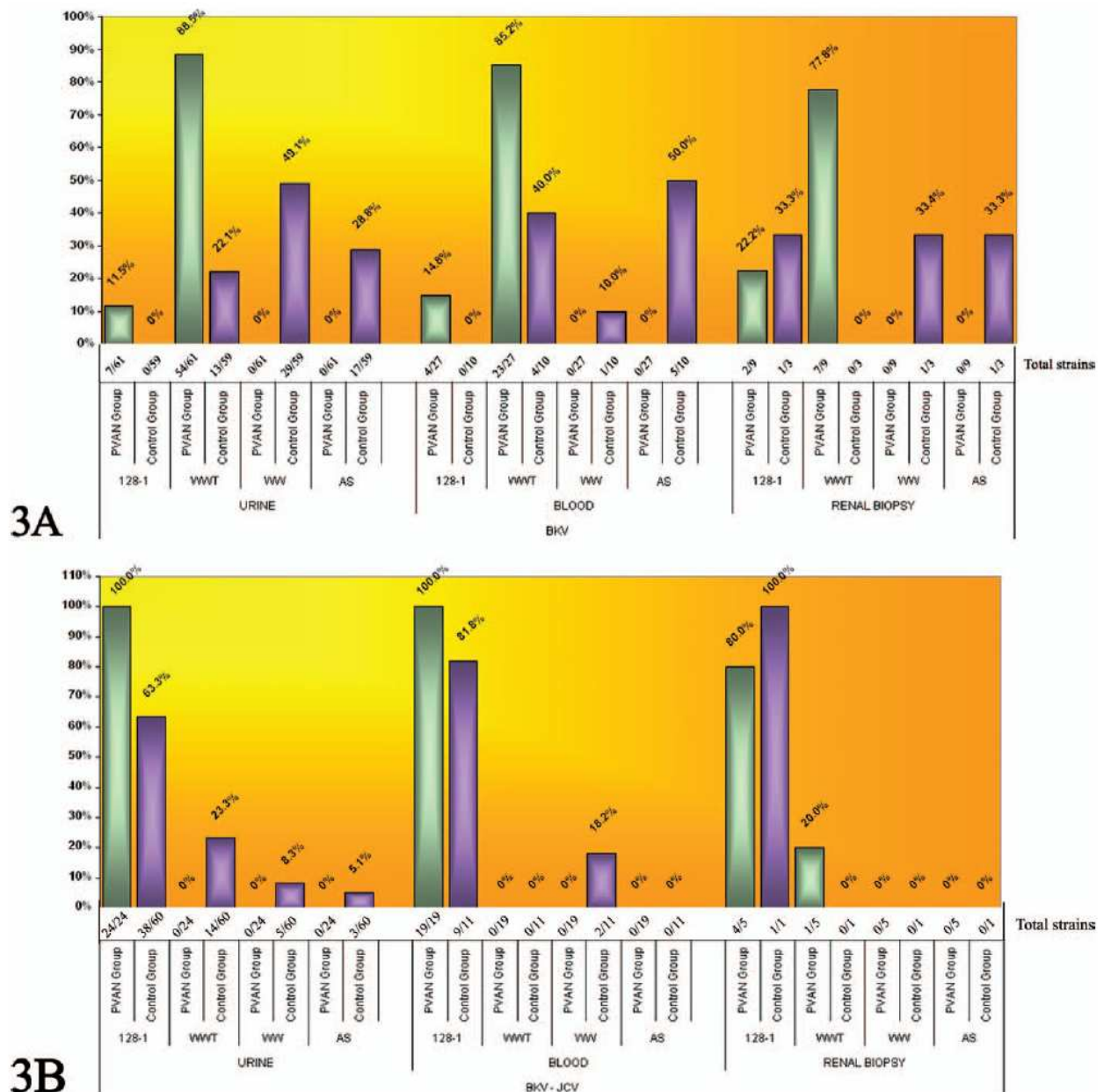
Figure 3. *Distribution of BK polyomavirus (BKV) strains in urine, blood, and renal biopsy samples showing A, BKV infection alone or B, BKV and JC virus (JCV) coinfection. PVAN indicates polyomavirus-associated nephropathy.*

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

22

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-

23

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

24

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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26

Pubblicazione 2

La regione tardiva del genoma virale dei Polyomavirus codifica per le proteine capsidiche VP1, VP2 e VP3; di queste solo VP1 è coinvolta nel riconoscimento dell'antigene. La sequenza genomica della regione compresa tra il nucleotide 1744-1812 di BKV, codificante per la porzione di VP1 coinvolta nel riconoscimento antigenico, viene utilizzata per definire il genotipo (I, II, III, IV) e il sottotipo (DUN, PT, MM, GS, SB, AS, IV, MG) del virus. I genotipi sono geograficamente distribuiti, ma possono essere osservate differenze anche tra pazienti trapiantati di rene e pazienti sottoposti a trapianto di midollo osseo.

Lo scopo del presente lavoro è di valutare la distribuzione dei genotipi e sottotipi di BKV, classificati in relazione alla proteina VP1, in pazienti portatori di trapianto renale che hanno e non hanno sviluppato PVAN, e di analizzare eventuali mutazioni genomiche che portano a un cambiamento amminoacidico, eventualmente correlato a una maggiore patogenicità virale o a una maggiore capacità del virus di riconoscere la cellula bersaglio presentante l'antigene. È stata quindi analizzata la sequenza compresa tra i nucleotidi 1744 e 1812, che codifica per la sequenza aminoacidica dall'aminoacido 61 all'83 coinvolta nel legame virus-antigene.

La casistica dei pazienti del presente lavoro è la medesima della precedente pubblicazione: 226 pazienti portatori di trapianto renale di cui 8 hanno sviluppato nefropatia Polyomavirus-associata (PVAN) nel corso dello studio, mentre i restanti 218 che non hanno presentato insorgenza di malattia sono stati analizzati come pazienti controllo. Sono stati raccolti campioni di urina, sangue periferico e biopsie renali, durante i controlli di routine.

L'analisi di distribuzione dei genotipi ha mostrato una prevalenza del sottotipo MM (genotipo I) nel gruppo controllo, mentre nei pazienti PVAN non sono stati osservati i sottotipi SB e AS (genotipo II e III rispettivamente). I dati ottenuti inoltre sembrano mostrare una maggiore frequenza di mutazioni in singole basi nucleotidiche nei campioni dei pazienti con PVAN, ciò nonostante cambiamenti aminoacidici abbiano presentato una distribuzione casuale in entrambi i gruppi.

Come dato osservazionale va infine sottolineato che un paziente ha mostrato una mutazione al residuo 75 in tutti i campioni di biopsia renale che mostravano progressione di malattia; ciò potrebbe suggerire una possibile correlazione tra una specifica mutazione aminoacidica e la progressione di PVAN.

Genomic Mutations of Viral Protein 1 and BK Virus Nephropathy in Kidney Transplant Recipients

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Genomic variability in the viral protein 1 region of BK polyomavirus (BKV) may change the ability of the virus to replicate. The significance of such changes was studied in clinical samples taken from kidney transplant patients with and without BKV nephropathy. A 94 base-pair fragment of viral protein 1 was amplified from 68 urine, 28 blood, and 12 renal biopsy samples from eight patients with BKV nephropathy, and from 100 urine samples, 17 blood and three renal biopsy samples from 41 of 218 controls. The DNA was sequenced and the amino acid changes were predicted by the Expert Protein Analysis System program (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland). Single base-pair mutations were detected more frequently in the samples from the BKV nephropathy patients than in the controls, and this was the only statistically significant finding of the study ($P < 0.05$), thus suggesting a greater genetic instability in BKV nephropathy associated strains. The amino acid changes were distributed at random in both BKV nephropathy patients and controls. However, one aspartic acid-to-asparagine substitution at residue 75 was detected in all samples of the one patient with BKV-associated nephropathy, who developed disease progression confirmed by histology, and not in any of the other patient or control samples. Whether this specific amino acid change plays a role in disease deserves further study. *J. Med. Virol. 81:1385–1393, 2009.* © 2009 Wiley-Liss, Inc.

KEY WORDS: polyomavirus; BK virus nephropathy; viral capsid protein; transcriptional control region; polymerase chain reaction; sequence analysis

INTRODUCTION

The human BK polyomavirus (BKV) may cause epithelial tubular cell damage and interstitial nephritis in kidney transplant patients [Nickeleit et al., 2000]. This polyomavirus or more appropriately BKV associated with nephropathy is thought to be due to latent BKV strains reactivated by anti-rejection immunosuppressive treatment [Nickeleit et al., 2000]. However, although the frequency of BKV reactivation is very high (estimated to be up to 68%) [Hirsch et al., 2002], it has been reported that BKV-induced renal damage occurs in only 2–10% of kidney transplant patients [Vasudev et al., 2005].

As pointed out in a review [Hirsch and Steiger, 2003], many host, environmental, and viral factors may be involved in the development of BKV-associated nephropathy. A previous study [Boldorini et al., 2009] investigated the pathogenic significance of genetic variation in the non-coding transcriptional control region (TCR) of BKV strains detected in blood, urine, and renal samples from patients with and without BKV nephropathy. It was found that BKV WWT, a strain first isolated in urine samples and considered a natural variant of the archetypal BKV WW with more efficient viral replication activity [Sundsfjord et al., 1990], was more frequent in the patients with BKV nephropathy than in the controls ($P < 0.05$), but neither the frequency nor specific types of TCR rearrangements seemed to be significantly different between the two groups. In addition to the TCR, the BKV genome contains two coding regions: one codes early small t and large T (LT) antigen proteins (oncoproteins), and the other late viral capsid proteins (VP1, VP2, and VP3). VP1 has many important functions: it is responsible for DNA packaging and virus assembly, and maintaining the

structural integrity of the virus, and it is also essential as a receptor site for the infection of host cells [Bolen et al., 1981; Jin and Gibson, 1996].

Although the genomic structure of the early region is stable within the same and different viral strains, the structure of the late VP1 region is characterized by nucleotide polymorphisms, and a 69 base-pair region (nucleotide positions 1744−1812 of the prototype Dunlop strain) has been used to identify four main viral genotypes: group I (including the DUN, MM, and GS strains), group II (the SB strain), group III (the AS strain), and group IV (the IV and MG strains) [Jin et al., 1993]. As the VP1 region is a coding region, single base-pair mutations (substitutions, deletions, or insertions) can lead to amino acid changes affecting the ability of the virus to recognize specific receptors on the surface of target cells: for example, it has been reported that a substitution of glutamic acid with glycine at residue 92 of VP1 affects mouse polyomavirus replication and spread in laboratory animals [Freund et al., 1991].

This study investigated whether specific BKV genotypes, VP1 genomic mutations, and/or amino acid changes in the VP1 protein may cause renal disease by comparing the sequences of the 69 base-pair region in the VP1 gene detected in urine, blood, and renal biopsy samples taken from kidney transplant patients who did and did not develop nephropathy associated with BKV.

MATERIALS AND METHODS

Patients and Samples

The study included 226 renal allograft recipients who had attended the Transplant Unit of Ospedale Maggiore della Carità between 2001 and 2007, eight of whom developed BKV nephropathy (3.5%); the 218 patients (96.5%) who did not develop the disease were considered controls. The diagnosis of BKV nephropathy was made on the basis of the morphological and immunohisto-

chemical detection of viral inclusions in tubular epithelial cells and parietal cells of Bowman's capsule in allograft biopsies [Nickeleit et al., 2000]. The epidemiological data of both groups have been published elsewhere [Boldorini et al., 2009] but, in brief, the eight patients who developed BKV nephropathy were five men and three women (mean age 51 years; range 36−65), and the 218 controls were 118 men and 100 women (mean age 51.6 years; range 22−76). As shown in Table I, the average time between organ transplantation and the development of BKV nephropathy was 37.5 months (range 12−60); the renal disease leading to chronic renal failure was: polycystic kidney disease (two cases), IgA nephropathy (two cases), arterionephrosclerosis (two cases), glomerular disease (one case), and unknown (one case). All of the patients developing BKV nephropathy had received a cadaveric organ and, during the study, none experienced graft loss due to viral infection, although one (#3) showed disease progression leading to renal fibrosis. There was no significant difference in immunosuppressive therapy between the patients with BKV nephropathy and the controls.

Post-transplant paired urine and blood samples were collected for the screening and monitoring of BKV nephropathy, as suggested by an international multidisciplinary panel [Hirsch et al., 2005]. Briefly, the samples were collected: (1) every 3 months during the first 2 years post-transplant; (2) when allograft dysfunction was noted; and (3) when allograft biopsy was performed.

A renal biopsy was performed when BKV nephropathy or transplant rejection were the suspected causes of a worsening in renal function and, in the case of persistent BKV nephropathy. The BKV nephropathy group provided 70 urine samples (a mean of 8.7 per patient; range 5−14), 63 blood samples (mean 8 per patient; range 5−14), and 17 renal biopsy samples (mean 2.1 per patient; range 1−5), and the control group provided 682 urine samples (mean 3.1 per patient;

TABLE I. Main Clinicopathologic Findings in Patients With BKV Nephropathy

Case no./ sex/age (y)	Age of transplant (mo)	Renal disease leading to chronic renal failure	Case no.	Morphological diagnosis
1/M/55	60	Polycystic kidney disease	First biopsy	Acute tubular necrosis
			Second biopsy	BKV nephropathy stage A
			Third biopsy	Cellular rejection
			Fourth biopsy	Drugs toxicity
			Fifth biopsy	Cellular rejection
2/F/32	48	IgA nephropathy	First biopsy	BKV nephropathy stage B
3/M/51	60	Polycystic kidney disease	First biopsy	BKV nephropathy stage B
			Second biopsy	BKV nephropathy stage C
			Third biopsy	BKV nephropathy stage C
4/M/55	36	Unknown	First biopsy	BKV nephropathy stage B
			Second biopsy	BKV nephropathy stage B
5/F/51	48	Arterionephrosclerosis	First biopsy	BKV nephropathy stage B
6/M/41	24	Nephroangiosclerosis	First biopsy	Polycystic kidney
			Second biopsy	BKV nephropathy stage B
7/M/65	12	Glomerular disease	First biopsy	BKV nephropathy stage A
			Second biopsy	Cellular rejection
8/F/38	12	IgA nephropathy	First biopsy	BKV nephropathy stage B

y, year; mo, month; M, male; F, female; BKV nephropathy stage according to Nickeleit et al. [2000].

range 1–11), 677 blood samples (mean 3.1 per patient; range 1–11), and 101 renal biopsy samples (taken from 68 patients: mean 1.5 per patient; range 1–5).

Urine Samples

The urine samples were concentrated by centrifugation at 700*g*/min for 10 min, and 5 ml of the urine pellets were then cytocentrifuged at 250*g*/min for 10 min onto two slides, both of which were fixed in 95% ethanol, stained with Papanicolau stain, and examined cytologically in order to identify the presence of decoy cells indicating active polyomavirus replication [Koss, 1979].

Blood Samples

The blood samples (5 ml) were collected and centrifuged immediately at 700*g*/min for 10 min, 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 hr, and embedded in paraffin; 4 µm thick sections were then stained with hematoxylin and eosin, periodic acid-Schiff, silver methenamine, Masson's trichrome, and phosphotungstic acid hematoxylin. All of the renal biopsies samples contained a minimum of four glomeruli and renal medullary, and were therefore adequate to establish a reliable diagnosis.

For immunohistochemical examination, ethylendiaminetetracetic acid (EDTA) at pH 8 and microwaving at 900 W (two 3-min steps followed by two 4-min steps) were used for antigen retrieval. Immunoperoxidase staining was performed using a polyclonal antibody against VP1-3 proteins of simian virus 40 (SV40) (Lee Biomolecular Research Labs, San Diego, CA, dilution 1:20,000), which cross-reacts with human BKV and JCV [Shinohara et al., 1993]. The reactions were detected by the streptavidin-biotin method, and revealed using diaminobenzidine as chromogen.

A histological diagnosis of BKV nephropathy was made when the epithelial nuclei showed typical morphological changes [Nickeleit et al., 2000] identified by light microscopy, positively stained by immunohistochemistry with anti-SV40 antibody. The progression of renal damage induced by BKV was classified as previously proposed [Nickeleit et al., 2000]: stage A, early, with the focal involvement of tubule epithelial cells positively stained by immunohistochemistry; stage B, extensive renal involvement by cytopathic viral alterations with necrosis, diffuse chronic inflammation, and initial fibrosis; and stage C, late, with interstitial fibrosis, scarring, tubular atrophy, and with few cells with cytopathic viral alterations.

Molecular Biology

DNA extraction and PCR assay. Four 4-µm thick sections were cut from the paraffin-embedded renal tissue samples after histological work-up using a microtome blade that was cleaned with xylene between each block in order to avoid sample cross-contamination [Wright and Manos, 1990], and were then placed in 1.5 ml Eppendorf tubes. DNA was extracted using EDTA–SDS/proteinase K followed by phenol–chloroform as previously described [Ferrante et al., 1995], and resuspended with 30 µl of DEPC-treated and auto-cleaved pyrogen and RNase-free water.

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

Polymerase chain reaction (PCR) of the β-actin gene was performed in all cases as a positive control of DNA extraction.

The BKV LT region was amplified in 68/70 urine samples (97.1%), 36/63 blood samples (57.1%), and 13/17 renal biopsy samples (76.5%) from all eight BKV nephropathy patients, and in 110/682 urine samples (16.1%), 20/677 blood samples (2.9%), and 3/101 renal biopsy samples (3%) from 41 patients (18.8%) in the control group.

The samples that were positive for the LT region of BKV underwent amplification of the VP1 region using VP1-7 (5′-ATC AAA GAA CTG CTC CTC AAT-3′) and VP1-2R (5′-GCA CTC CCT GCA TTT CCA AGG G-3′) as outer primers, and 327-1 (5′-CAA GTG CCA AAA CTA CTA AT-3′) and 327-2 (5′-TGC ATG AAG GTT AAG CAT GC-3′) as inner primers [Jin and Gibson, 1996]. A 327 base-pair fragment containing a region responsible for some BKV antigenic variation was obtained in all cases. Amplification was performed in a total volume of 25 µl containing 15 pmol of each primer (10 pmol for the inner PCR), 0.2 mM dNTPs, 1.5 mM MgCl$_2$, and 2 U BioTaq DNA polymerase in the presence of 1× Bioline NH$_4$ buffer. In the first step, 5 µl of urine or renal tissue DNA or serum DNA were added to the PCR mixture and, in the second step, 2.5 µl of template; the PCR was then performed using an Eppendorf Mastercycler gradient PCR System. The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 sec (30 cycles for the inner PCR), annealing at 55°C for 40 sec, and extension at 72°C for 40 sec; the cycles were terminated with a final extension at 72°C for 5 min.

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

The sensitivity of the multiplex nested PCR was estimated by amplifying serial dilutions of a BKV-positive sample (data not shown).

Direct DNA sequencing. DNA fragments from all of the VP1-positive samples were separated by electrophoresis on 3% agarose gel, and one fragment of each sample (327 base-pair) was excised, extracted, and purified using a commercial kit (PCR clean-up gel extraction, NucleoSpin, Macherey-Nagel).

A cycle sequencing PCR reaction was performed as previously described [Boldorini et al., in press]; in order to avoid Taq polymerase errors [Jin et al., 1993],

the sequence in each sample was analyzed twice. The sequences were read manually, compared with the prototype BKV Dunlop strain, and classified into the four known genotypes [Jin et al., 1993], which are distinguished on the basis of specific polymorphisms in the portion of the VP1 region spanning nucleotides 1744–1812 (shown in Table II); the presence of any single base-pair mutations of the known sequences was also recorded. When the electropherogram showed two peaks at the same height in the same nucleotide position, these were considered to be two distinct sequences.

The changes in one or more amino acid sequences encoded by the variant nucleotide(s) were predicted by the Expert Protein Analysis System program (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland—http://www.expasy.org), and the predicted amino acid variations were compared with the prototype amino acid sequence in order to identify those that might change the chemical properties of the VP1 protein in the investigated fragment.

Statistical Analysis

The data were analyzed statistically using Fisher's exact test, with P values of <0.05 being considered significant (95% confidence interval).

RESULTS

The BKV VP1 region was amplifiable in all 68 urine samples (100%), 28/36 blood samples (77.7%), and 12/13 renal biopsy samples (92.3%) from all of the BKV nephropathy patients who were positive for the LT region. One of the 13 renal biopsies with histologically proven BKV nephropathy was negative for VP1 amplification, probably because of the small quantity of renal tissue available for molecular analysis after histological and immunohistochemical processing. The VP1 region was amplifiable in 100/110 urine samples (91%), 17/20 blood samples (85%), and three renal biopsy samples (100%) from the 41 control patients.

Table III shows the distribution of BKV genotypes and subtypes in the two groups. The subtypes MM, GS, IV, and MG were distributed at random in the BKV nephropathy group (25% each), whereas SB and AS were never detected. Conversely, all of the BKV subtypes were represented in the control group, with MM, being the prevailing subtype (51.2%; $P < 0.05$) within this group and in comparison with the BKV nephropathy group, although this latter was not statistically significant (25%; $P = 0.25$).

Sequence analyses of the polymorphic VP1 gene sites showed single base-pair mutations in 75% of the BKV nephropathy patients and only 17% of the controls ($P < 0.05$); furthermore, they were only identified in the urine samples of the control group, but in samples of all types in BKV nephropathy group. Figure 1 shows the frequency of the genomic mutations in the VP1 region of

TABLE II. Nucleotide Sequences of VP1 Positions 1744–1812 Used to Assign BKV Genotypes and Subtypes [Jin et al., 1993] (A), and Related Amino Acids (B)

A

Subtype	1744–1746	1747–1749	1750–1752	1753–1755	1756–1758	1759–1761	1762–1764	1765–1767	1768–1770	1771–1773	1774–1776	1777–1779	1780–1782	1783–1785	1786–1788	1789–1791	1792–1794	1795–1797	1798–1800	1801–1803	1804–1806	1807–1809	1810–1812
I (DUN)	GAA	AAC	CTT	AGG	GGC	TTT	AGT	CTA	AAG	CTA	AGT	GCT	GAA	AAT	GAC	TTT	AGC	AGT	GAT	AGC	CCA	GAG	AGA
I (PT)																				AGT		GAA	
I (MM)																							
I (GS)																							
II (SB)	GAT					TAT			CAC		ACT				GCC		GAC					GAC	
III (AS)	GAT					TAT		CAG	AGA					ACT	GCC		GAG					GAC	AAA
IV (IV)	AAT	GAC				TAT			AGA		ACT			ACT	GCC		GAG					GAC	
IV (MG)	AAT	GAC				TAT					ACT			ACT	GCC		GAC					GAC	

B

Subtype	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
I (DUN-PT-MM)	Glu	Asn	Leu	Arg	Gly	Phe	Ser	Leu	Lys	Leu	Ser	Ala	Glu	Asn	Asp	Phe	Ser	Ser	Asp	Ser	Pro	Glu	Arg
I (GS)																							
II (SB)	Asp					Tyr			His		Thr				Ala		Asp					Asp	
III (AS)	Asp					Tyr		Gln	Arg					Thr	Ala		Glu					Asp	Lys
IV (IV)	Asn	Asp				Tyr			Arg		Thr			Thr	Ala		Glu					Asp	
IV (MG)	Asn	Asp				Tyr					Thr			Thr	Ala		Asp					Asp	

TABLE III. BKV Genotype and Subtype Distributions in
Patients From BKV Nephropathy and Control Group

		No. of patients	
Genotype[a]	Subtype[a]	BKV nephropathy group	Control group
I	MM	2/8 (25%)	21/41 (51.2%)
	GS	2/8 (25%)	4/41 (9.8%)
II	SB	0/8	4/41 (9.8%)
III	AS	0/8	1/41 (2.4%)
IV	IV	2/8 (25%)	4/41 (9.8%)
	MG	2/8 (25%)	7/41 (17%)
	Total number	8	41

[a]BKV genotypes and subtypes according to Jin et al. [1993] and
Randhawa et al. [2002].

all of the amplified fragments obtained from the samples of both the BKV nephropathy and control groups. As can be seen, genomic rearrangements of blocks of sequences in the VP1 region were never detected in the amplified fragments, but single base-pair substitutions were frequent in the urine samples of the BKV nephropathy patients (52% vs. 16.4%; $P < 0.05$), and were also detected in 21.2% of the blood and 29.4% of the renal biopsy samples.

Table IV shows the distribution and type of amino acid changes generated by the single base-pair mutations in the amplified fragments. The changes were distributed at random in both groups as only two patients in each group showed the same change: aspartic acid to asparagine at residue 62 (#2 and #4) and glutamic acid to alanine at residue 73 (#6 and #8) in the BKV nephropathy group, and arginine to lysine at residue 69 (#6 and #7) and glutamic acid to glutamine at residue 73 (#6 and #7) in the controls. It is worth noting that patient #3, who developed renal fibrosis due to BKV infection (BKV nephropathy stage C), showed an aspartic acid to asparagine substitution at residue 75 in all of the amplified fragments that was not identified in any of the samples from either group. Finally, it is also worth noting that the amino acid changes were stable in all of the patients from both groups.
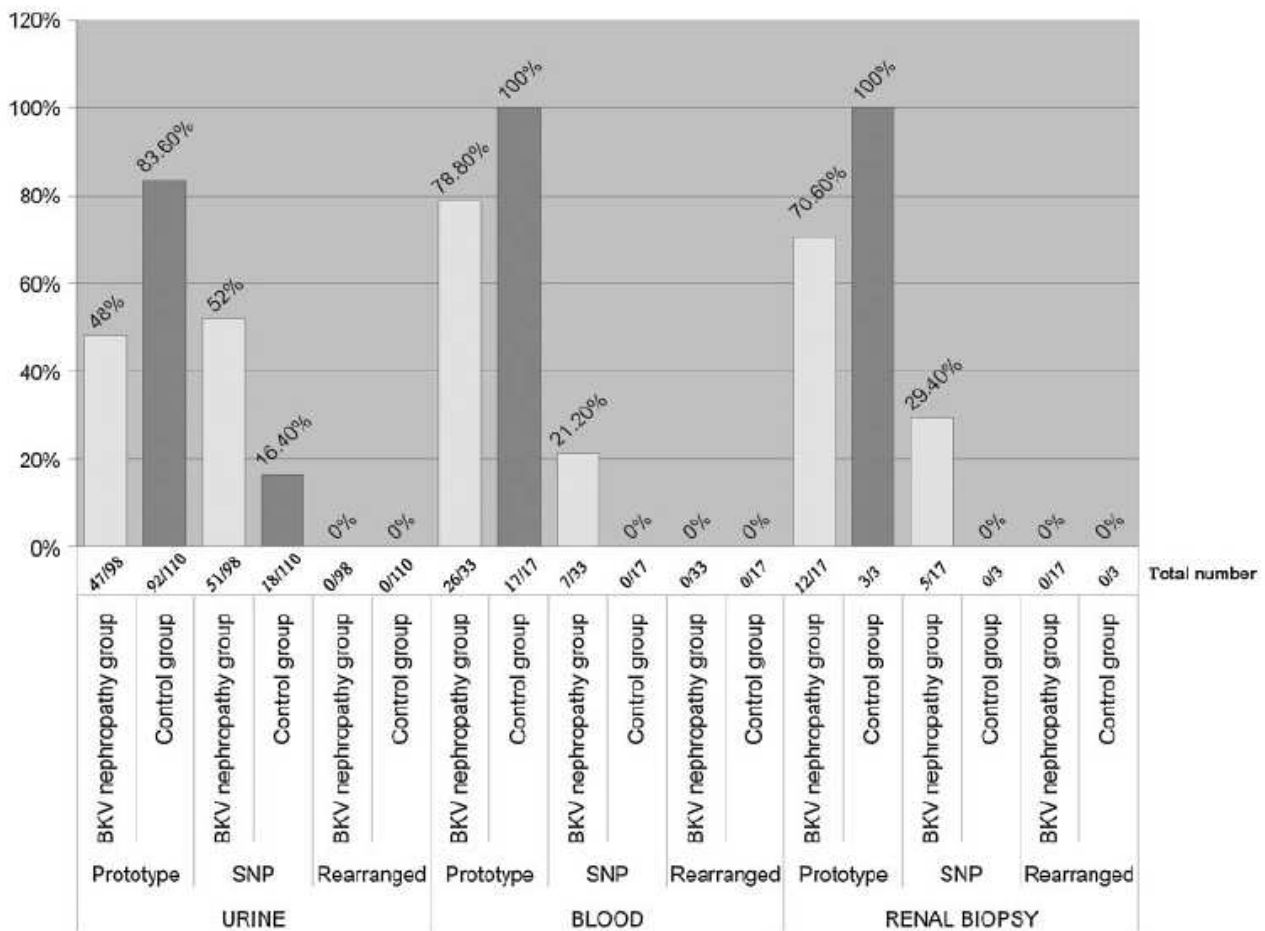

Fig. 1. Diagrammatic representation of BKV genomic mutations in Viral Protein 1 region detected in urine, blood, and renal biopsy samples from BK Virus nephropathy and control group. First line SNP, single nucleotide polymorphism.

32

TABLE IV. Viral Protein 1 Amino Acid Changes in Each Amplified Fragment From Patients of the BK Nephropathy Group (A) and Controls (B)

A

Patient	Sample	Amino acid positions														
		Asn→Lys 62	Asp→Asn 61	Leu→Pro 63	Lys→Arg 69	Glu→Thr 73	Glu→Ala 73	Glu→Gln 73	Glu→Lys 73	Thr→Ser 74	Asp→Asn 75	Glu→Gln 77	Ser→Arg 77	Ser→Lys 77	Asp→His 77	Asp→His 82
BKV nephropathy group																
#2	Urine		7					7				11				
	Blood		1					1				1				
	Renal biopsy							1				1				
#3	Urine					3					17					
	Blood										3					
	Renal biopsy										3					
#4	Urine		7	1												
#5	Urine	6							3	1			9	1	1	4
	Blood	1											1			1
#6	Urine				4		3									
	Blood				1		1									
	Renal biopsy				1											
#8	Blood						1									

B

Patient	Sample	Amino acid positions																
		Asn→Lys 61	Asp→Asn 61	Asp→Asn 62	Leu→Val 68	Arg→Lys 69	Glu→Lys 73	Glu→Gln 73	Asn→Lys 74	Δ Ala 75	Phe→Leu 76	Asp→Glu 77	Asp→Asn 77	Ser→Ile 78	Ser→Arg 80	Asp→Glu 82	Glu→Gln 82	Asp→Asn 82
Controls																		
#1	Urine		1									1						
#2	Urine										1							
#3	Urine						3									2		
#4	Urine				1									2	1		1	
#5	Urine								1									
#6	Urine					3		3					4					1
#7	Urine	2		3		3		3		3								

■, urine; ■, blood; ■, renal biopsy; Δ, deletion.

Table V shows the BKV genotypes and nucleotide changes in sequential samples taken before and after the diagnosis of BKV nephropathy from each patient. The BKV genotypes, subtypes, and single base-pair mutations were stable in all cases, regardless of the sample type or time of sample collection.

The BKV genotypes and subtypes in the control group were also stable in the urine samples taken at different times (data not shown).

DISCUSSION

The VP1 region of BKV encodes the major viral capsid protein, which plays a structural role in viral architecture and bears important antigen epitopes that are crucial for viral attachment to target cells [Jin and Gibson, 1996].

Nucleotide polymorphisms in the VP1 genomic sequence 1744–1812 are used to identify BKV genotypes

TABLE V. BKV Genotypes and Nucleotide Changes in Sequential Samples Before and After the Diagnosis of Nephropathy in BKV Nephropathy Patients

Case no.	Genotype	BKV nephropathy stage	Number of samples and nucleotide position of SNPs		
			Urine	Blood	Renal biopsy
#1	Genotype I (MM)	Before BKV nephropathy	1 prototype	N/A	1 negative
		BKV nephropathy	1 prototype	1 negative	1 prototype
		After BKV nephropathy	2 negative	6 negative	2 negative
			4 prototype		
#2	Genotype IV (IV)	Before BKV nephropathy	1 SNP 1792	1 prototype	
		BKV nephropathy	1 prototype	1 prototype	1 prototype
			1 SNP 1747; 1780; 1792	1 SNP 1747; 1780; 1792	1 SNP 1780; 1792
		After BKV nephropathy	7 prototype	2 prototype	N/A
			3 SNP 1792	1 negative	
			6 SNP 1747; 1780; 1792		
#3	Genotype I (GS)	Before BKV nephropathy	1 SNP 1786	N/A	N/A
		BKV nephropathy	1 SNP 1786	1 SNP 1786	1 prototype
					1 SNP 1786
		After BKV nephropathy	1 prototype	2 prototype	2 prototype
			12 SNP 1786	2 SNP 1786	2 SNP 1786
			3 SNP 1780; 1781; 1786	7 negative	
#4	Genotype IV (MG)	Before BKV nephropathy	1 prototype	1 prototype	N/A
			1 SNP 1747; 1751		
		BKV nephropathy	1 prototype	1 prototype	1 prototype
			1 SNP 1747; 1780		
		After BKV nephropathy	6 prototype	4 prototype	1 prototype
			1 SNP 1747; 1780; 1792	2 negative	
			1 SNP 1747		
			2 SNP 1747; 1780		
			1 SNP 1747; 1784		
#5	Genotype I (GS)	Before BKV nephropathy	2 prototype	2 prototype	N/A
		BKV nephropathy	1 prototype	N/A	1 prototype
			1 SNP 1749; 1794; 1807		
		After BKV nephropathy	4 prototype	2 prototype	N/A
			3 SNP 1749; 1794; 1807	1 SNP 1794; 1807	
			2 SNP 1749	6 negative	
			5 SNP 1794		
			1 SNP 1793; 1794		
#6	Genotype I (MM)	Before BKV nephropathy	2 prototype	2 prototype	1 prototype
			1 SNP 1769	1 SNP 1769; 1781	1 negative
		BKV nephropathy	1 prototype	1 negative	1 prototype
			1 SNP 1769; 1781		1 SNP 1769
		After BKV nephropathy	4 prototype	2 prototype	N/A
			2 SNP 1769; 1781	1 negative	
#7	Genotype IV (IV)	Before BKV nephropathy	1 prototype	1 prototype	N/A
		BKV nephropathy	1 prototype	N/A	1 prototype
		After BKV nephropathy	3 prototype	2 negative	N/A
#8	Genotype IV (MG)	Before BKV nephropathy	2 prototype	2 prototype	N/A
				1 SNP 1781	
		BKV nephropathy	1 prototype	1 prototype	1 prototype
		After BKV nephropathy	2 prototype	2 prototype	N/A

SNP, BKV single nucleotide polymorphism according to Jin et al. [1993].
N/A, not available; negative, BKV genome not found.

34

(I–IV) and subtypes (DUN, PT, MM, GS, SB, AS, IV, and MG) [Jin et al., 1993]. Genotype I is the most frequent in the human population, followed by genotypes II, IV, and III. There are significant geographical differences, as well as differences between bone marrow and kidney transplant patients, although not enough evidence to relate the different BKV genotypes and/or subtypes to reactivation, particular diseases or particular groups of immunosuppressed patients [Jin et al., 1993].

In this study the frequency of BKV genotypes and subtypes in eight patients with BKV nephropathy was examined and compared with those observed in 41 kidney transplant patients without BKV nephropathy. A different distribution was found between the two groups of patients, with the absence of subtypes SB and AS (genotypes II and III) in the BKV nephropathy group and the prevalence of subtype MM, (genotype I) in the controls. However, as both genotypes and subtypes were stable in sequential samples from the same patient, and there were only a few BKV nephropathy patients, these differences may simply reflect individual or geographical variability in the BKVs circulating in the population as reported in epidemiological studies [Knowels, 2001].

VP1 gene sequence analyses showed that single base-pair mutations were significantly more frequent in the patients with BKV nephropathy than in the controls and this was the only statistically significant finding of the study. Furthermore, they were identified in amplified fragments from urine, blood, and renal biopsy samples taken from the BKV nephropathy patients, but only in the urine samples of the controls. The biological and clinical significance of this genetic variability is unknown but, in line with a previous study [Randhawa et al., 2002], it can be explained tentatively by hypothesizing that more genomically unstable viral strains may have a particular tendency to cause clinical disease. Alternatively, it is possible that the heterogeneity of BKV strains circulating in the human population is greater than previously thought.

The amino acid changes generated by base-pair mutations were distributed at random in the two groups; interestingly, a mutation converting aspartic acid to asparagine at residue 75 was detected in all of the samples taken at different times from one patient with BKV nephropathy, but never in any of the other samples from either group. Three sequential renal biopsies of this patient showed disease progression leading to renal fibrosis, thus suggesting that this specific amino acidic change may have played a role in the worsening of the disease. As there are no other published studies comparing BKV VP1 sequences in urine, blood, and renal biopsy samples taken at different time from kidney transplant patients with and without BKV nephropathy, the results of the present study are not readily comparable with those of other authors. One study of the significance of VP1 mutations in JC polyomavirus in patients with progressive multifocal leukoencephalopathy found that mutations were more frequent in those who survived longer, thus suggesting that they may be associated with disease progression [Zheng et al., 2005].

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Previous studies [Dubensky et al., 1991; Freund et al., 1991] have found that amino acid changes occurring in specific positions of the VP1 gene of mouse polyomaviruses generated from PTA, RA, and A2 strains lead to in vitro plaque size and viral hemoagglutination behaviors that affect their ability to replicate and spread in mice. However, these results were obtained in experimental animals using a polyomavirus species that is different from BKV and may not be comparable with the results obtained in the present study.

Finally, a study that sequenced the DNA of the VP1 region of BKVs isolated from 49 sequential renal biopsies taken from 24 BKV nephropathy patients at different times during the course of disease (no data concerning urine or blood samples were reported) found frequent base-pair mutations and amino acid changes [Randhawa et al., 2002]; furthermore, unlike the results of the present study in which the genomic structure of the isolated BKVs were stable in sequential samples, examination of the data at different times indicated that the genetic structure of the virus may have undergone continuous evolution related to the progression of the disease. The authors hypothesized that VP1 nucleotide and amino acid variation may have implications for escaping host immunity and the development of antiviral drug resistance but, as their study was retrospective, and the interval between successive samples was up to 6 years, it is possible that the variability in genomic structure was related to the long intervals between the renal biopsies.

In conclusion, clinical sample data collected during this study seem to indicate greater genomic instability in the structure of the BKV VP1 region in patients who developed renal disease than in the controls but, as the single base-pair mutations and amino acid changes were distributed at random, their clinical significance is unknown. Finally, the mutation converting aspartic acid to asparagine at residue 75, which was detected in all of samples of the only BKV nephropathy patient in whom the disease progressed is of potential interest, but further studies of a larger number of BKV nephropathy patients are needed to understand the real significance of this change.

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Pubblicazione 3

È stato stimato che il 50% dei pazienti sottoposti a trapianto di midollo osseo presentano infezione da BKV, e diversi studi hanno dimostrato che la presenza di tale virus può essere associata ad insorgenza di cistite emorragica.

Il seguente lavoro, condotto in collaborazione con il laboratorio di Microbiologia del Diaprtimento di Scienze di Sanità Pubblica dell'Università "La Sapienza" di Roma, si pone lo scopo di valutare, in sette pazienti sottoposti a trapianto di midollo osseo e affetti da cistite emorragica, la presenza dei Polyomavirus BKV e JCV, determinando il titolo virale e i genotipi presenti. Inoltre sono stati ricercati, tramite sequenziamento diretto, eventuali riarrangiamenti genomici nella regione regolatoria virale e nella regione codificante la proteina VP1, ed è stata valutata la possibile associazione di tali riarrangiamenti con una aumentata patogenicità virale.

Dei sette pazienti analizzati, quattro hanno mostrato infezione da BKV, due presenza di coinfezione BKV-JCV e un solo paziente è risultato positivo per il solo JCV. Dati quantitativi hanno mostrato che in campioni consecutivi di pazienti coinfetti, l'aumento della carica virale di un virus corrispondeva alla diminuzione dell'altro, questo sembrerebbe indicare un ruolo di entrambi i virus nello sviluppo della cistite emorragica. L'analisi dei genotipi ha confermato quanto già presente in letteratura: BKV genotipo I è il più frequente nei campioni di urina di soggetti sottoposti a trapianto di midollo osseo e affetti da cistite emorragica, mentre JCV genotipo I, osservato sia da solo sia in associazione con infezione da BKV in tutti i pazienti della nostra casistica, è il più comunemente distribuito in Italia.

La ricerca di mutazioni nelle sequenze genomiche TCR e VP1 non ha mostrato presenza di riarrangiamenti o di sostituzioni di basi nucleotidiche, pertanto sembra non esserci nessuna associazione tra mutazione e insorgenza di cistite emorragica.

Infine, la identificazione di infezione da JCV in un paziente con cistite emorragica suggerirebbe un coinvolgimento anche di questo virus nell'insorgenza di questa patologia.

Viral Infection In Bone Marrow Transplants: Is JC Virus Involved?

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Hemorrhagic cystitis is characterized by hematuria due to inflammation of the bladder. In bone marrow transplants, this disease is linked to the infection by human polyomavirus BK, whereas the role of the human polyomavirus JC is unclear. The transcriptional control regions of both viruses contain important cellular transcription factor binding sites that undergo rearrangement process generating suitable variants that could be more active for viral replication and for the onset of hemorrhagic cystitis. In this study urine obtained from seven patients with bone marrow transplant were examined. Polyomavirus genomes were quantified by PCR and viral loads were compared. The transcriptional regions of both viruses were amplified and sequenced to determine the presence of variants. Subtypes of polyomaviruses were determined by amplification and sequencing of the viral protein 1 region. The results showed that four of seven patients were positive for BK DNA, two of seven patients had BK and JC DNA and one of seven had JC DNA. Positive samples were amplified and sequenced successively for transcriptional regions. The viral archetype was always found in both viruses. Finally, typing showed that BK virus subtype I infected patients with BK, whereas JC virus genotype IA and genotype 1B were found in patients infected with JC. The data suggest that new and different approaches are required to improve the morbidity and mortality caused by polyoma-associated hemorrhagic cystitis, since it known that BK virus is involved in the onset of hemorrhagic cystitis, whereas the role of JC virus should be investigated further. *J. Med. Virol. 82:138–145, 2010.* © 2009 Wiley-Liss, Inc.

KEY WORDS: JC virus infection; bone marrow transplants; hemorrhagic cystitis; quantitative PCR; sequencing analysis

INTRODUCTION

Hemorrhagic cystitis is characterized by painful hematuria due to hemorrhagic inflammation of the bladder mucosa. It is a distinct clinical disease that has been associated with various predisposing factors [DeVries and Freiha, 1990] and it is an important cause of morbidity and occasional mortality in patients undergoing bone marrow transplantation [Sencer et al., 1993]. The clinical features of hemorrhagic cystitis vary from microscopic hematuria to severe hemorrhage in the bladder leading to clot retention and renal failure. Cystitis can be classified into two types: early onset hemorrhagic cystitis, which occurs within 48–72 hr of a conditioning regimen, and a later onset hemorrhagic cystitis, which occurs beyond 72 hr from the preparative regimen. The incidence of hemorrhagic cystitis varies from 7 to 68% [Santos et al., 1983; Brugieres et al., 1989; Sencer et al., 1993; Russel et al., 1994; Yang et al., 1994; Bedi et al., 1995; Leung et al., 2001; Xu et al., 2007].

Numerous causes, such as chemical immunosuppression and irradiation, cytopenia and viral infections, can contribute to the onset of hemorrhagic cystitis. Infection or reactivation of the human polyomavirus BK is common and represents a consistent risk factor for the development of this disease. It has been associated with a later onset hemorrhagic cystitis in patients undergoing allogeneic hematopoietic stem cell transplantation [Reploeg et al., 2001; Leung et al., 2005; Giraud et al., 2006; Giraud et al., 2008]. On the other hand, the role of the human polyomavirus JC is still poorly understood.

The two human polyomaviruses BK and JC belongs to the *Polyomaviridae*, a family of small non-enveloped

icosahedral DNA viruses, which infect human population worldwide [Imperiale, 2001; Stoner and Hubner, 2001].

The BK viral genome can be divided into an early region (regulatory proteins), a late region (viral capsid proteins and the agnoprotein) and a transcriptional control region, which contains the origin of replication and sequences for transcriptional cell factors involved in early and late viral transcription [Moens and Van Ghelue, 2005]. These binding sites undergo deletion and enhancement process that could generate variants that could offer advantages to the virus in its host [Sharma et al., 2007].

Four antigenic subtypes of BK virus (I–IV) have been characterized by genomic subtyping and serological reactivity [Jin et al., 1995]. The association of a particular BK virus subtype with clinical features is controversial, although some investigators demonstrated that BK virus subtype I was the predominant in urine samples taken from bone marrow transplants affected by hemorrhagic cystitis [Bogdanovic et al., 1998; Fioriti et al., 2005].

Like BK virus, the JC viral genome is divided into three regions: the early, the late and the transcriptional control region which includes the origin of replication and contains binding sites for transcriptional cell factors involved in viral early and late transcription [Frisque et al., 1984; Kim et al., 2001]. Like BK virus, deletion and enhancement process could give rise to more active variants with altered tissue tropism and pathogenic capability [Vaz et al., 2000; Pietropaolo et al., 2003; Mischitelli et al., 2005]. The analysis of the viral protein 1 gene allowed to distinguish eight major genotypes and numerous subtypes of JC virus [Kmieciak et al., 2008]. Types I and IV predominate in Europe and in the USA [Jobes et al., 1998; Stoner et al., 2000; Agostini et al., 2001b; Pagani et al., 2003].

Primary polyomavirus infection is usually asymptomatic and is acquired during childhood probably via the upper respiratory tract and other ways [Brown et al., 1984; Sundsfjord et al., 1994; Pietropaolo et al., 1998; Zambrano et al., 2002; Ahsan and Shah, 2006; Knowles, 2006; Boldorini et al., 2008]. Following primary infection, these viruses disseminate and establish lifelong persistence especially in the kidney [Imperiale, 2000; Rabenau et al., 2002]. Reactivation may occur in conditions of immunosuppression such as following bone marrow and kidney transplantations [Drachenberg et al., 2007; Dropulic and Jones, 2008; Boldorini et al., 2009]. JC virus is the etiologic agent of a rare neurodegenerative disease named Progressive Multifocal Leukoencephalopathy, whereas BK virus causes nephropathy in kidney transplant patients and it is associated with hemorrhagic cystitis in allogeneic bone marrow transplants [Khalili et al., 2006; Mischitelli et al., 2007; Giraud et al., 2008].

About 50–100% patients with a bone marrow transplant develops BK viruria, nevertheless only 5–40% progress to hemorrhagic cystitis indicating that a specific subtype, genome mutations and graft versus host disease may contribute to its development [Bogdanovic et al., 1996; Azzi et al., 1999; Leung et al., 2001; Carr et al., 2006; Ikegaya et al., 2006; Giraud et al., 2008]. Nevertheless, very few studies have been carried out linking hemorrhagic cystitis with polyomavirus infection and some studies pointed to a significant correlation between the pathogenesis and shedding of polyomavirus in urine, whereas data from other studies showed no such relationship [Rabenau et al., 2002; Bogdanovic et al., 2004; Erard et al., 2005; Fioriti et al., 2005; Giraud et al., 2006].

In order to assess better the association of BK and/or JC viruses infection with the onset of hemorrhagic cystitis or progression, urine taken from patients with bone marrow transplants and immunocompetent individuals was analyzed. BK virus and JC virus genomes were quantified and viral loads were compared to determine whether high peak urine viral loads were linked to the onset of hemorrhagic cystitis and with variation of immunosuppressive therapy. In addition, BK virus and JC virus transcriptional control regions were amplified and sequenced to determine whether if mutations in transcription factor binding sites were associated with a more aggressive pathology. Finally, the BK and JC viruses were subtyped to determine whether a particular subtype was circulating most commonly in bone marrow transplants.

MATERIALS AND METHODS

Patients and Clinical Specimens

This study was based on seven Caucasian patients (two males and five females) who underwent allogeneic bone marrow transplantation and developed post-transplant hemorrhagic cystitis. The average age of the patients was 35 for females (30–45) and 32 for males (24–39). They were admitted to "Policlinico Gemelli" Hospital of Rome with a different diagnosis of hematopoietic malignancies, none had a history of urinary tract infection, coagulopathy, pelvic irradiation or evidence of microscopic hematuria prior to bone marrow transplantation. All patients received allogeneic bone marrow transplants. One of these patients received a bone marrow transplant from a relative and six from matched unrelated donors. The seven patients developed hemorrhagic cystitis at an average of 33 days after bone marrow transplantation. The clinical data of the patients are shown in Table I. Seven healthy individuals were recruited as controls.

Specimens were collected when hematuria was evident or when the symptoms were of hemorrhagic cystitis. Two patients were also infected with BK virus and JC virus and had recidivant hemorrhagic cystitis. Serial urine specimens were collected from these two patients from the onset of the symptoms to determine whether high peak urine viral loads were associated with hemorrhagic cystitis episodes and with variation of immunosuppressive therapy. Investigations were made on fresh uncentrifuged urine.

TABLE I. Clinical Data of BMT Patients

Pz	Clinical history	BMT	HC onset	Polyomavirus infection	Other viral infections[a]	Other complications	Outcome
F1	AML	MUD 03-18-05	60 days after BMT	BKV	AdV, CMV, EBV	LPD-EBV related	Dead
F2	NHL	MUD March 2008	10 days after BMT	BKV	None	Numerous bacterial infections	Dead
F3	AML	MUD 05-05-07	20 days after BMT	BKV	EBV	/	Dead
M4	ALL T cells	BMT from relative 05-09-07	60 days after BMT	BKV	CMV	Acute GVHD	Dead
M5	ALL	MUD 05-10-05	30 days after BMT	JCV	EBV	/	Dead
F6	ALL PH +	MUD 11-21-06	30 days after BMT	BKV, JCV	CMV, EBV	/	Remission: dasatinib administration; DLI
M7	M3-AML	MUD 02-16-07	20 days after BMT	BKV, JCV	CMV, EBV	/	M3-AML recidivant

ALL PH +, acute lymphoblastic leukemia chromosome philadelphia positive; M3-AML, M3 acute myelocytic leukemia; AML, acute myelocytic leukemia; NHL, non Hodgkin's lymphoma; MUD, match unrelated donors; LPD, linfoproliferative disorders; GVHD, graft versus host disease; DLI: donor leukocyte infusions.
[a]AdV, EBV and CMV infections were monitored according to BMT guidelines and they were recruited from patient's medical case sheets.

Informed consent was obtained from each patient and each volunteer and the study design was approved by the medical ethics committee of the "Policlinico Gemelli" Hospital of Rome.

Diagnosis and Treatment

Hemorrhagic cystitis was diagnosed by urine culture and PCR to distinguish bacterial disease from viral etiology. The treatment is shown in Table II.

DNA Extraction

One milliliter of urine was incubated in lysis buffer and proteinase K (200 mg/ml). DNA extraction was performed by the DNeasy® Tissue Kit (QIAGEN S.p.A., Milan, Italy), according to the manufacturer's instructions.

PCR Analysis

Urine was examined using quantitative assays for quantitation of BK virus and JC virus DNA. The specimens with BK and/or JC DNA were then analyzed using qualitative assays for the detection of viral transcriptional control regions and for virus typing.

BK Virus Quantitative PCR

The assay was performed using 7300 Real Time PCR System (AB Applied Biosystems, 850 Lincoln Centre DriveFoster City, CA 94404, USA). PCR amplifications were run in a reaction volume of 20 µl (optimized mix including forward and reverse primers and hydrolysis probes) containing 5 µl of DNA sample. 50 ng/µl of total purified DNA was used.

Thermal cycling was initiated with a first denaturation step of 10 min at 95°C, followed by 45 cycles of 95°C for 15 sec, 60°C for 1 min, 72°C for 1 min, at the end of which, fluorescence was read. Amplification data were analyzed with software provided by the manufacturer. Standard curves for the quantitation of the viral genome were constructed using serial dilutions of a plasmid containing the target sequences (Large T Antigen). Concentrations of the plasmid ranged from 10^2 to 10^5 copies of target plasmid. All samples were tested in triplicate and the number of viral copies in each sample was calculated from the standard curve. The results were expressed as copies of viral DNA per milliliter (c/ml) of sample. Standard precautions to prevent contamination were followed. In each run non-template control lanes

TABLE II. Patients Treatments

Pz	GVHD prophylaxis	Conditioning regimen	Antiviral therapy	Other therapies
F1	MTX, CYSP	Bu, Cy, ATG	Rib, Gancyclovir, Foscarnet, Rituximab	/
F2	MTX, CYSP	TBI, Cy, Adriamycin	Rib	Antibiotic therapy
F3	MTX, CYSP	Bu, Cy, ATG	Cid, Rib	/
M4	MTX, CYSP	Bu, Cy, AraC	Gancyclovir,	Corticosteroids, MMF, Thalidomide, Alemtuzumab Infliximab
M5	MTX, CYSP	Bu, Cy, AraC	Cid, Rib	/
F6	MTX, CYSP	TBI, Cy, ATG	Cid, Rib, Gancyclovir, Rituximab	Antibiotic therapy, Probenecid, Risperidone
M7	MTX, CYSP	Bu, Cy, ATG	Cid, Rib	Antibiotic therapy, Probenecid

MTX, methotrexate; CYSP, cyclosporin; TBI, total body irradiation; Cy, cyclophosphamide; Bu, busulfan; ATG, antihuman thymocyte globulin; AraC, cytarabin; Cid, cidofovir; Rib, ribavirin; MMF: mycophenolate mofetil.

were included. The assay can to detect about 10 molecules of target sequences in 5 µl of DNA.

JC Virus Quantitative PCR

The assay was performed using 7300 Real Time PCR System (AB Applied Biosystems, 850 Lincoln Centre DriveFoster City, CA 94404, USA). PCR amplifications were run in a reaction volume of 40 µl containing 10 µl of the DNA sample, reaction mix for quantitative amplification, forward and reverse primers and finally SYBR GREEN I probes. 50 ng/µl of total purified DNA was used.

Thermal cycling was initiated with a first denaturation step of 2 min at 94°C, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 77°C for 15 sec at the end of which, fluorescence was read. Dissociation stage was characterized by a temperature curve with an initial step at 65°C and a final step at 95°C. The temperature increment was 0.3°C every 10 sec. The amplification data were analyzed with a specific software provided by the manufacturer. Standard curves for viral genome quantification were constructed using serial dilutions of a plasmid containing target sequences (Viral Capsid Protein 2). The plasmid concentrations ranged from 10^2 to 10^5 plasmid copies of the target. All samples were tested in triplicate and the number of viral copies in each sample was calculated from the standard curve. PCR specificity was guaranteed by a dissociation stage characterized by curves with melting point of $79 \pm 1°C$, corresponding to a 132 bp fragment. These data were expressed as c/ml of sample. Standard precautions to prevent contamination were followed. In each run nontemplate control lanes were included. The assay allows to detect about 20 molecules of target sequences in 10 µl of DNA.

PCR for Transcriptional Control Region of BK Virus

Samples positive for BK virus genome underwent further amplification for detection of the transcriptional region using a nested PCR with BKTT1 and BKTT2 as outer primers and BRP1 and BRP2 (356 bp) as inner primers. The amplification was performed in a reaction volume of 25 µl, containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, and 2U 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 was added to the PCR mixture and, in the second step, 2.5 µl of template. PCR was run in GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA). 50 ng/µl of total purified DNA was used for the assay.

The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 sec, annealing for 40 sec at 55°C in the first step and 50°C in the second step, and extension at 72°C for 40 sec. The cycles were terminated with a final extension at 72°C for 5 min. All assays included positive (purified viral DNA) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results. PCR products were analyzed by 2% agarose gel

electrophoresis and visualized using ethidium bromide staining [Boldorini et al., 2009].

PCR for Transcriptional Control Region of JC Virus

Samples positive for JC virus genome underwent further amplification for detection of the transcriptional region using a nested PCR with JRE1 and LP2 as outer primers and RFOR and RREV (358 bp) as inner primers. The amplification was performed in a reaction volume of 45 µl, containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, and 2U BioTaq DNA polymerase with an appropriate reaction buffer (Tris-HCl 100 mM, pH 8.3, KCl 500 mM). 5 µl of DNA was added to the PCR mixture at the first step, whereas 2.5 µl of template was added in the second step of PCR. PCR was run in GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA). 50 ng/µl of total purified DNA was used. The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 sec, annealing for 40 sec at 61°C in the first step and 58°C in the second step, and extension at 72°C for 40 sec. The cycles were terminated with a final extension at 72°C for 5 min. All assays included positive (purified viral DNA) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results. PCR products were analyzed by means of 2% agarose gel electrophoresis and visualized using ethidium bromide staining [Boldorini et al., 2009].

Sequencing of Transcriptional Control Regions of BK and JC Viruses

PCR products corresponding to transcriptional control regions of BK and JC viruses were purified and sequenced. Briefly, amplicons were purified prior sequencing to remove the excess of primers with QIAquick PCR purification kit, according to QIAGEN protocol. DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, 850 Lincoln Centre DriveFoster City, CA 94404, USA, mod. 370 A), according to manufacturer's specifications (Amplicycle Kit, Applied Biosystem, 850 Lincoln Centre DriveFoster City, CA 94404, USA). Sequences were organized and analyzed using homology analysis, multiple alignments and the Genetic Computer Group sequence analysis software package. In particular the obtained sequences were compared with BK virus archetype (Genbank Accession Number V01108) and JC virus archetype (Genbank Accession Number J02226).

PCR for Viral Capsid Protein 1 of BK and JC Viruses

In order to amplify genome region corresponding to viral capside protein 1 of BK virus, a nested PCR was performed using VP1-7 and VP1-2R as outer primers, and 327–1 and 327–2 as inner primers (327 bp), reported by [Jin et al., 1993]. The amplification was performed in a reaction volume of 20 µl containing 10 pmol/µl of each

primer, 0.2 mM dNTPs, 1.5 mM MgCl2, 2U BioTaq DNA polymerase and 10x Bioline NH4 buffer. In the first step, 5 µl of DNA was added to the PCR mixture and, in the second step, 2.5 µl of template. The nested PCR was performed using an Eppendorf Mastercycler gradient PCR System. 50 ng/µl of DNA was used. The samples were amplified by means of denaturation at 95°C for 5 min, followed by 35 cycles (30 cycles for the inner PCR), at 95°C for 40 sec, annealing at 55°C for 40 sec and extension at 72°C for 40 sec. The cycles were terminated with a final extension at 72°C for 5 min.

Single step PCR was used to amplify the genome region corresponding to viral capside protein 1 of JC virus, using JLP15 and JLP16 primers (215 bp) reported by [Pagani et al., 2003]. The amplification was performed in a reaction volume of 20 µl containing 25 pmol/µl of each primer, 0.4 mM dNTPs, 2.5 mM MgCl2, 2U BioTaq DNA polymerase, 10x Bioline NH4 buffer and 5 µl of DNA. 50 ng/µl of DNA was used.

After initial denaturation at 95°C for 9 min, followed by 40 cycles at 95°C for 40 sec, annealing at 63°C for 40 sec and extension at 72°C for 40 sec; the amplification protocol was concluded with a final extension at 72°C for 7 min. PCR products were analyzed using 2% agarose gel electrophoresis and visualized using ethidium bromide staining.

Direct DNA Sequencing for Viral Genotyping

PCR products corresponding to the viral capsid protein 1 region of both viruses were separated by electrophoresis on 3% agarose gel. The fragment corresponding to each sample (327 bp for BK virus and 215 bp for JC virus) was excised, extracted and purified using a commercial kit (PCR clean-up gel extraction, Nucleo-Spin Macherey-Nagel, 52355 Düren, Germany).

A cycle sequencing PCR reaction was performed as previously described [Boldorini et al., 2009]. In order to avoid Taq polymerase mistakes [Jin et al., 1993], the sequence was analyzed twice for each sample. The sequences were read manually, compared with the prototypes (Dunlop strain for BK virus and Mad-1 strain for JC virus) and classified into known genotypes [Jin et al., 1993; Agostini et al., 2001a]. The known genotypes are distinguished on the basis of specific polymorphisms within the portion of the viral protein 1 region spanning nucleotides 1744–1812 (BK virus) and nucleotides 1735–1902 (JC virus). The presence of single mutations was also noted.

RESULTS

In this study, urine from seven Caucasian patients who developed post-transplant hemorrhagic cystitis was analyzed.

Quantitative results showed that four of seven patients had BK DNA, two of seven patients had BK and JC DNA, whereas only one patient had JC DNA. Patient no.1 developed hemorrhagic cystitis 60 days after transplantation when, BK virus quantitative assay showed 65×10^8 DNA c/ml, whereas JC virus quantitative PCR revealed that the specimen was suitable for analysis, but it did not have viral DNA (Table III). In patient no.2, 15×10^7 c/ml of BK virus DNA was found, whereas the urine did not have JC viral DNA (Table III). The BK virus quantitative assay performed on the urine of patient no.3 revealed that there were 33×10^7 c/ml of viral DNA, but in the some specimen JC viral DNA

TABLE III. Summary of Urine Samples' Results for BK and JC Viruses

	BK DNA (copies/ml)	BK transcriptional control region	JC DNA (copies/ml)	JC transcriptional control region	BK virus type	JC virus type
Patients infected by BK virus						
F1	65×10^8	Archetype	NEG	ND	Sub-type I	ND
F2	15×10^7	Archetype	NEG	ND	Sub-type I	ND
F3	33×10^7	Archetype	NEG	ND	Sub-type I	ND
M4	14×10^7	Archetype	NEG	ND	Sub-type I	ND
Patient infected by JC virus						
M5	NEG	ND	63×10^7	Archetype	ND	Genotype I B
Patients infected by BK and JC viruses						
F6	12×10^8	Archetype	90×10^4	Archetype	Sub-type I	Genotype I A
F6	91×10^7	Archetype	90×10^5	Archetype	Sub-type I	Genotype I A
F6	41×10^7	Archetype	20×10^5	Archetype	Sub-type I	Genotype I A
F6	20×10^7	Archetype	90×10^5	Archetype	Sub-type I	Genotype I A
F6	33×10^7	Archetype	40×10^5	Archetype	Sub-type I	Genotype I A
F6	75×10^7	Archetype	50×10^5	Archetype	Sub-type I	Genotype I A
F6	90×10^4	Archetype	10×10^2	Archetype	Sub-type I	Genotype I A
M7	50×10^2	Archetype	40×10^5	Archetype	Sub-type I	Genotype I A
M7	20×10^3	Archetype	60×10^5	Archetype	Sub-type I	Genotype I A
M7	20×10^4	Archetype	50×10^4	Archetype	Sub-type I	Genotype I A
M7	30×10^2	Archetype	90×10^5	Archetype	Sub-type I	Genotype I A
M7	70×10^3	Archetype	70×10^4	Archetype	Sub-type I	Genotype I A
M7	20×10^3	Archetype	30×10^4	ND	Sub-type I	Genotype I A
M7	10×10^2	Archetype	10×10^2	Archetype	Sub-type I	Genotype I A

NEG, negative; ND, not determined; F, female; M, male.

was not found (Table III). In urine obtained from patient no.4, the quantitative PCR revealed the presence of 14×10^7 c/ml of BK virus DNA whereas JC DNA was not found (Table III).

In patient no.5, JC DNA quantification showed a number of viral DNA copies of 63×10^7 c/ml, whereas BK DNA was not found (Table III). The quantitative assays for patients nos 6 and 7 showed that these subjects were co-infected with JC and BK viral DNA. For patient no.6, the BK virus DNA copies were 12×10^8 c/ml in the urine collected on 12-22-2006, whereas JC virus DNA copies were 9×10^5 c/ml. During the serial collection of specimens, corresponding to a recidivant hemorrhagic cystitis, the BK virus DNA average was 52×10^7 c/ml, whereas the JC virus DNA average was 6×10^6 c/ml. In the sample analyzed during the follow-up after remission (07-24-2007), 9×10^5 c/ml of BK virus DNA were detected, whereas the JC virus DNA copies were 10^4 c/ml. For patient no.7, in the urine collected on July 3, 2007, the assays quantified 5×10^3 c/ml of BK virus DNA and 4×10^6 c/ml of JC virus DNA. In the specimens collected during recidive, a BK virus DNA average of 6×10^4 c/ml was detected, whereas the JC virus DNA average was 3×10^6 c/ml. In the urine collected on 09-03-2007 (follow-up after remission), the number of copies of BK and JC viruses DNA decreased to 10^3 c/ml for BK virus and 10^4 c/ml for JC virus (Table III). No control subject was found positive (data not shown).

Samples with viral DNA were amplified and sequenced successively for viral transcriptional control regions. Homology analysis, multiple alignments and the Genetic Computer Group sequence analysis always revealed the presence of the BK and JC viral archetypes in all sequences obtained from the specimens of all patients.

The antigenic subtyping of BK and JC viruses revealed that BK virus type I, corresponding to the MM strain, was found in patients infected with BK including those co-infected. JC virus genotype IA was found in all samples obtained from co-infected patients, whereas in the patient infected only with JC virus, genotype IB was detected (Table III).

DISCUSSION

Hemorrhagic cystitis is a distinct clinical disorder of multiple etiologies. It is characterized by painful hematuria due to hemorrhagic inflammation of the bladder mucosa [Leung et al., 2005]. The disease that occurs before the graft is mostly transient and self-limiting, whereas after the graft, it is severe and sometimes life threatening [Leung et al., 2005].

About 50% of bone marrow transplants show BK viruria within 2 months of transplantation; it is similar in allogeneic (range 46–53%) and autologous (range 39–54%) transplants [Bedi et al., 1995; Azzi et al., 1999; Dropulic and Jones, 2008]. Although the association of BK virus infection with hemorrhagic cystitis in bone marrow recipients was first demonstrated two decades ago, the role of JC virus remains obscure. However, it is known that bone marrow recipients are susceptible to the infection. Boubenider et al. [1999] demonstrated that BK and JC viruses reactivate in 55 and 6.7% of bone marrow patients, respectively. BK virus infection or reactivation alone is not sufficient to cause the disease. Nevertheless, the transcriptional control region rearrangements could strengthen the virus tropism and generate more suitable variants that could be more active for viral replication and for the onset or development of hemorrhagic cystitis. A particular BK virus subtype in patients with a bone marrow transplant, could be involved in the onset of hemorrhagic cystitis.

JC viral infection occurs in <10% of patients with bone marrow transplant in whom progressive multifocal leukoencephalopathy is a rare complication [Przepiorka et al., 1997; Yasuda et al., 2008]. Like BK virus, JC transcriptional control region variants could alter viral tissue tropism and increase pathogenic capability, even if the genomic structures isolated from urine samples are highly conserved in healthy and immunocompromised individuals and they always appear very similar to that of the archetype [Vaz et al., 2000; Pietropaolo et al., 2003]. Finally it is suggested that different genotypes of JC virus would act differently in immunocompromised individuals as in the case of progressive multifocal leukoencephalopathy [Ferrante et al., 2003].

Given this, urine specimens obtained from bone marrow transplanted and immunocompetent individuals were analyzed. BK virus and JC virus genomes were quantified and viral loads were compared to understand if high urine viral loads were correlated to hemorrhagic cystitis episodes. In addition BK virus and JC virus transcriptional control regions were amplified and sequenced to investigate if mutations in transcription factor binding sites were associated with a more aggressive pathology. Finally, the BK and JC subtypes were determined to establish whether a particular subtype circulated more commonly in patients with bone marrow transplant.

Quantitative assays showed that four of seven patients were positive for BKV DNA, two of seven patients were BK and JC co-infected, whereas only one patient was positive for JC virus DNA. In particular, it was observed that a high viral load in urine was related to hemorrhagic cystitis episodes in all patients. Interestingly, in serial urine specimens collected from co-infected patients, it was found that when BK virus replication increased, the JC virus replication decreased. Conversely, when the number of copies of JC DNA increased, BK viral DNA decreased. This suggests that both Polyomaviruses may contribute to the pathogenesis or development of hemorrhagic cystitis. It may be that JC virus involvement in transplant diseases is underestimated since the detection of this virus is not required in the diagnostic approach.

The sequencing of the transcriptional control regions of both viruses, revealed that no particular mutations or structures could be associated with a more strong viral replication and disease onset or recidive. Probably, in the genitourinary tract, viral replication did not require

specific transcriptional cell factors, so the host proteins may be sufficient to allow virus replication. Therefore, as demonstrated by Gosert et colleagues, the study of functional proteins in permissive cells could help to understand the possible associations between virus replication and the specific subset of trans-acting factors [Gosert et al., 2008].

According to the literature, BK virus typing showed that subtype I is found most commonly in infected individuals [Zheng et al., 2007; Krumbholz et al., 2008]. This study confirmed previous results that demonstrated how subtype I was predominant in urine samples obtained from patients with bone marrow transplants and hemorrhagic cystitis [Fioriti et al., 2005]. The epidemiology of JC virus shows that genotype I is the most common JC virus circulating in Europe and the genome detected most frequently in the Italian population [Stoner et al., 2000; Agostini et al., 2001a; Pagani et al., 2003; Rossi et al., 2007]. In these seven patients, the JC genotype IA was detected in co-infected individuals, whereas in patient no. 5 the genotype IB was found. Although there are few patients, it may be that this diversity could be used as a tool to outline the origin of infection and the selection of a particular JC genotype in patients co-infected with BK virus.

However, since hemorrhagic cystitis pathology is complex, it is essential to consider many cofactors in its development, such as the conditioning regimen, gender, the donor source and the histocompatibility between recipient and donor patients. Regarding polyomavirus infection, it is important to evaluate the dynamics of the infection, the timing and the frequency of monitoring and how BK and JC viruses are implicated in renal disease in patients with bone marrow transplant. Additional work is required to understand whether the JC virus acts only as a cofactor or it plays a role in the onset or development of hemorrhagic cystitis.

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<u>CONCLUSIONI</u>

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Gli studi condotti dai nostri gruppi di ricerca non hanno sostanzialmente evidenziato un ruolo prioritario di specifiche mutazioni genomiche né della regione regolatoria virale né di quella capsidica di BKV nel determinare, in pazienti portatori di trapianto di rene, un sostanziale incremento della potogenicità virale. Rimangono quindi aperti gli interrogativi iniziali in ordine alle vere motivazioni che portano ad un danno renale virus-indotto in una frazione relativamente bassa di pazienti con infezione latente o replicativi da BKV. E' anche possibile che ciò sia in parte legato all'analisi di una casistica tutto sommato limitata di pazienti affetti da nefropatia BKV correlata, o che in alternativa vi siano altre cause –non strettamente virus-correlate- di maggiore suscettibilità individuale ad una infezione virale che causi danno d'organo.

Analogamente, l'investigazione circa il possibile ruolo di JCV nel determinismo del danno vescicale (cistite emorragica) in soggetti portatori di trapianto di midollo osseo non ha sostanzialmente condotto a risultati definitivi. Dei sette casi analizzati, quattro hanno evidenziato una esclusiva infezione da BKV, due una coinfezione JCV-BKV e uno solo una infezione da JCV. In nessun caso i dati di analisi di sequenza delle regioni TCR e VP, né i dati quantitativi sembrano correlarsi in alcun modo ad una maggiore virulenza di BKV o JCV. Tuttavia la segnalazione, solo occasionalmente riportata in letteratura, di un caso di cistite emorragica da JCV suggerisce anche per questo argomento un ampliamento della casistica a conferma di un risultato per ora provvisorio.

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ALTRE PUBBLICAZIONI

V I R O L O G Y

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

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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.

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.

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 NH4 buffer, 2 mM MgCl2 (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 MgCl2, 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 MgCl2, 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 positive 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ C F_{19}: A $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ C D_{44}: C $\rightarrow$ A F_{21}: A $\rightarrow$ G F_{35}: G $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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
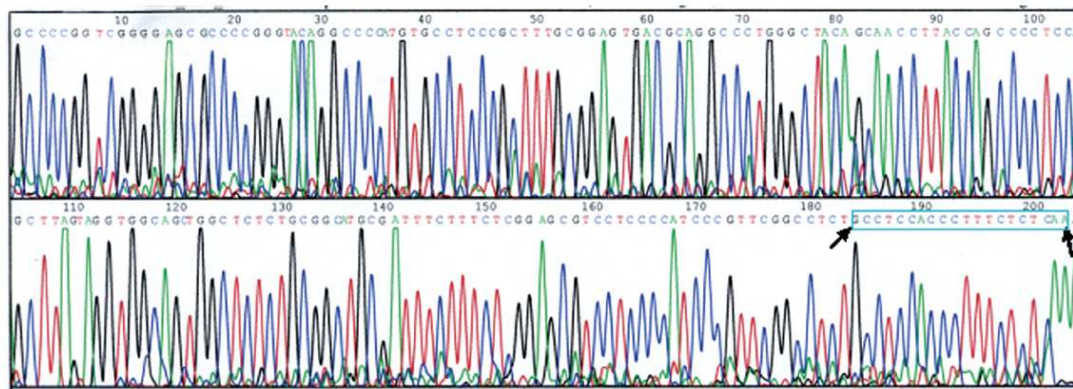
FIG. 1   Electropherogram showing a 20 bp insertion (arrows) of BKV transcription control region in human chromosome 20, isolated from blood sample. The *x* coordinate corresponds to the scan number throughout the time of data collection. The *y* axis is a relative height scale for peak intensity. Traces 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.

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Virology Journal

Complications post renal transplantation: literature focus on BK virus nephropathy and diagnostic tools actually available

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Abstract

Clinical diagnosis of kidney transplants related illnesses is not a simple task. Several studies were conducted to define diseases and complications after renal transplantation, but there are no comprehensive guidelines about diagnostic tools for their prevention and detection.

The Authors of this review looked for the medical literature and pertinent publications in particular to understand the role of Human Polyomavirus BK (BKV) in renal failure and to recognize analytical techniques for BK virus associated nephropathy (BKVAN) detection.

Introduction

Clinical diagnosis of kidney transplants related illnesses is not a simple task. Several studies were conducted to define diseases and complications after renal transplantation, but there are no comprehensive guidelines about diagnostic tools for their prevention and detection.

The Authors of this review looked for the medical literature and pertinent publications in particular to understand the role of Human Polyomavirus BK (BKV) in renal failure and to recognize analytical techniques for BK virus associated nephropathy (BKVAN) detection. For reviewing we used Medline and recent pertinent bibliographies.

Kidney pathologies in renal transplants are associated with graft function, immunosuppressive drugs and infec-

tions [1]. Moreover cardiovascular, bone and bone marrow diseases, metabolism dysfunctions and cancers could affect these patients [2,3]. Graft function is the most important parameter in evaluation of the allograft status; acute rejection, obstruction, renal artery stenosis could influence renal function resulting in graft dysfunctions and ultimately in chronic renal allograft failure [1,4,5]. Persistent urinary protein excretion and hyperlipidemia are associated with acute rejection, in particular heavy proteinuria has important consequences for extracellular fluid volume regulation and demonstrate the rapid deterioration of renal function associated with pathologic glomerular lesions [6,7]. Serum creatinine levels and urine protein/creatinine ratio (total protein excretion) should be used to screen for changes in renal function. Acute allograft rejection could be also due to interstitial

infiltrates and mild tubulitis that unfortunately are clinically silent and could be detected only by immunohistochemistry (IHC) [1].

Immunosuppression therapy

The morbidity and mortality rates associated with renal transplantation and the use of immunosuppressive medications are high. Conventional immunosuppression is based on azathioprine, nevertheless, other immunosuppressive drugs, such as cyclosporine A (CsA), tacrolimus, sirolimus, mycophenolate-mofetil (MMF) and corticosteroids are used [1,8]. To reduce adverse effects of immunosuppressive therapies, it is strongly recommended to monitor routinely blood level of CsA, tacrolimus and sirolimus. The nephrotoxicity associated with azathioprine and MMF is monitored by assessing hemoglobin levels, hematocrit value and white blood cell counts at least weekly for months 1 to 2, every 2 week for months 3 to 4, monthly for months 4 to 12, and then every 3 to 6 months [1,8-12]. Finally toxicity related to corticosteroids is monitored periodically by controlling blood pressure, lipoprotein levels and blood glucose levels [8,11]. Compared with conventional immunosuppression with azathioprine, CsA reduced the incidence of acute rejection and prolonged graft survival but caused chronic tubulointerstitial atrophy and fibrosis that are difficult to distinguish from chronic allograft nephropathy attributable to other causes [1,13]. Instead the role of acute and chronic tacrolimus nephrotoxicity in graft failure is unclear. However the incidence of renal toxicity is roughly proportional to tacrolimus doses and its blood levels [14]. In the other hand sirolimus seems to be efficacious in preventing acute rejection when used in place of, or in combination with, CsA. However very few studies have been conducted to determine the relationship between blood levels of sirolimus and either acute rejection or toxicity [10]. Regarding azathioprine and MMF, hematologic and gastrointestinal toxicities are usually dose-related and respond to dose reductions [12]. Moreover MMF causes leukopenia in renal transplants. Finally clinical signs of corticosteroid toxicity, which are observed relatively soon after the initiation of prednisone treatment, include skin changes, hypertension, peptic ulcer disease and myopathy [8].

Human Poliomavirus BK and BKVAN

Viral infections cause several complications in renal transplants that are closely related with the immunosuppressive therapy. On the basis of literature data, viruses implicated in graft failure we could number Varicella zoster, Cytomegalovirus, Influenza A and B, Hepatitis B and C and human Poliomavirus BK and JC [15-18]. In particular BK virus, described for the first time in a transplant recipient, has a remarkable tropism for the genitourinary tract, in fact BKVAN are recognized as an important cause of late allograft failure [19].

BKV is ubiquitous in human populations worldwide. BKV infects young children and the seroprevalence is 70%–80% in adults [20,21]. Serologic surveys of populations, using hemagglutination inhibition assay for the detection of antibodies, indicate that seroconversion takes place early in life, at 5–7 years of age [20,21]. Primary infection is usually inapparent and only occasionally may be accompanied by mild respiratory illness or urinary tract disease. During primary infection viremia occurs and the virus spreads to several organs of the infected individual where it remains in a latent state. After the initial infection, the virus disseminates and establishes a persistent infection in the urinary tract and maybe in lymphocytes [20,22,23].

The complete genome of BKV contains 5,153 bp and it is functionally divided into three regions: the early, the late, and the transcriptional control region (TCR). The first region codes for the small and large T-antigens (t-Ag and T-Ag), the second region codes for the viral capsid proteins VP1-VP2-VP3 and agno-protein, and the last region (TCR) contains the transcriptional control elements for both "early" and "late" gene expression [24] Primary transcripts are required for viral replication, in particular T-Ag promotes unwinding of the double helix and recruitment of cellular proteins required for DNA synthesis whereas in non permissive cells it is involved in neoplastic transformation [24,25] (Fig. 1). Late transcripts encode for viral capside proteins and agnoprotein, that has a critical role in the regulation of viral gene expression and replication, and in the modulation of certain important host cell functions including cell cycle progression and DNA repair [26]. TCR contains the origin of replication and it is arbitrarily divided into four box alphabetically designated P, Q, R and S. These sequence blocks serve as regulatory regions, or enhancer elements believed to contain several transcription factor binding sites involved in the modulation of viral transcription [24,27,28]. It is not known that genetic alterations are essential for the pathogenesis associated with BKV after kidney transplantation, nevertheless BK-strains with rearranged TCR have been particularly described in subjects under immunosuppressive therapies [24,29,30]. In renal transplants BKV infection may be transmitted via the donor organ, may be acquired in the community or latent BKV could reactivate [31,32]. The incidence of allograft failure has ranged from 15 to 50% in affected individuals [33], but few data are available about BKVAN; it probably due to recent emerging of this disease as an important cause of allograft failure following renal transplantation. BKV urinary shedding of infected urothelial cells occurs in 10 to 60% of renal transplant recipients [34] and literature data suggest that prospective
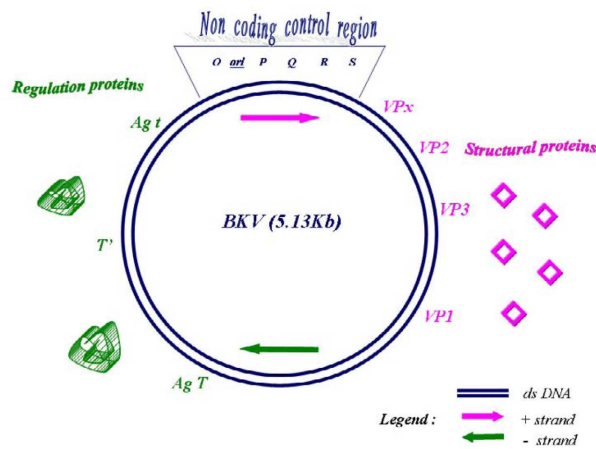
Figure 1
Schematic representation of the gene organization in the BK virus (BKV) genome. The double circle represents the double stranded DNA genomes. The genome is divided into three regions. The early region encodes three regulatory proteins (Agt, AgT, T'). The late region specifies four structural proteins and agnoprotein (VP1, VP2, VP3, VPx). The non-coding control region contains the elements for the control of viral DNA replication (ori) and viral gene expression. The arrows indicate the positive and negative strands according to the direction of viral transcription (24).

monitoring of patients at risk for BKVAN may identify those with active infection before renal function deteriorates [35-37]. Recent studies demonstrated that BKVAN develop in as many as 8% of renal allograft recipients, with as many as 50% of patients experiencing graft loss over the next 2 to 3 years of follow-up [34,38,39]. A current study performed by Giraldi et colleagues show that, in a cohort of the 117 patients followed up every three months during a two year period after transplantation, 4 had BKVAN (3.4%) confirmed by quantitative assays on plasma and urine and assessed by allograft biopsy [40].

BKVAN diagnosis
BKVAN diagnosis is very difficult since this disease is often misdiagnosed as acute rejection or drug toxicity. Diagnostic tools available include histopathology by means of renal allograft biopsy, detection of BKV DNA on plasma and urine by polymerase-chain-reaction (PCR) and quantitative PCR (QPCR) and presence of "decoy cells" in the urine sediment. Diagnostic confirmation may be obtained using IHC, in situ hybridization (ISH), and/or electron microscopy (EM) in renal biopsy specimens [34,41-45].

Early identification provides the opportunity for intervention with reduction of the immunosuppression in an effort to control BKV replication and prevent BKVAN. The

risk factors predisposing to BKVAN appear to be multiple, with immunosuppressive regimens containing tacrolimus and MMF representing recognized associations [41,46]. Several investigators have begun to define risk factors for BKV disease among renal transplant recipients. The serologic status of the donor and the recipient appears to be a predictor of BKV infection, but it is not currently clear whether it influences the development of BKV nephritis. Tubular injury could be a factor promoting viral replication in an immunocompromised state induced by tacrolimus or MMF. The load of dormant BKV in the grafted organ is likely to be another important risk factor: no dormant virus, no re-activation and most likely, no BKVAN [47]. On these basis, since no specific anti-viral therapy is available, reduction in immunosuppression remains the mainstay of treatment with an increased risk of subsequent rejection. Therefore an accurate diagnosis is important, as it allows for early intervention and possible recovery of renal function.

Urine cytology is based on decoy cells recovery. Decoy cells are epithelial cells with enlarged nuclei and large basophilic ground-glass intranuclear viral inclusions, screening for their presence provides a simple and an inexpensive tool for the diagnosis of BKV nephropathy, nevertheless, Papanicolaou-stained urine sediment is not to be considered a specific morphological marker of BKV disease [48,49].

Electron microscopy is very sensitive for detection of BK virions, but the finding of viral particles is not diagnostic of BKVAN, since the ultrastructural appearance of BK virus is poorly typical. Virions are arranged in paracrystalline arrays of naked, round, electron-dense structures that measure 45 nm in diameter. It is important to emphasize that electron microscopy cannot distinguish BKV from JC virus [41] (Fig. 2).

The histological diagnosis of BKVAN requires evaluation of a renal biopsy with demonstration and confirmation of the polyomavirus cytopathic changes by IHC and ISH [41]. BKVAN is characterized by the presence of polyomavirus cytopathic changes in the epithelium of the renal tubules and urothelial lining. The infected cells have an enlarged nucleus with a gelatinous basophilic inclusion resulting from the accumulation of the newly formed virions [50]. Confirmation of the polyomavirus infection is usually performed with immunohistochemical stains for the simian virus 40 (SV40) large T antigen (AgT), which identifies all polyomavirus infections due to cross-reactivity between SV40 and both BKV and JCV. Distinction between the different types of polyomavirus requires the use of species-specific antibodies, ISH or in situ PCR. Systematic studies comparing the clinical utility of each method have not been performed [50]. The sections are
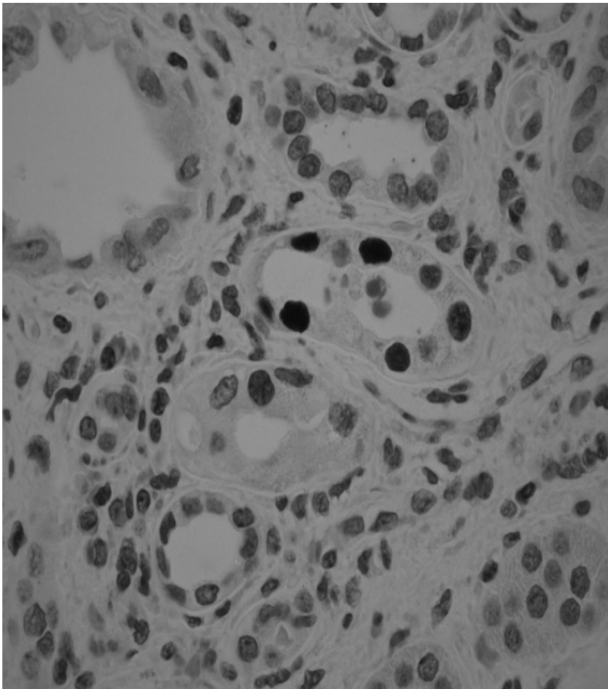
Figure 2
Immunohistochemistry, peroxidase stain, diaminobenzidine as marker, staining for BK polyoma virus with the antibody targeting the SV40 antigen. Note easily detectable, strong nuclear immunoreactivity in tubular cells. (350×), (41).

stained with hematoxylin-eosin and examined by means of light microscopy in order to evaluate the integrity of the tissue before proceeding to molecular analysis, to identify possible pathologic changes, and in particular to search for the presence of morphologic equivalents of cellular polyomavirus infection. In situ hybridization and immunohistochemistry are carried out to define the viral status of the infected tissues The reactions are detected by means of the streptavidin-biotin method and are revealed using diaminobenzidine as a chromogen. In situ hybridization is performed to localize the nucleic acid sequences of BKV and JCV at the subcellular level using commercially available biotinylated DNA probes [51].

For efficient early diagnosis of BKVAN, various molecular approaches are recommended. Quantitative PCR is a non-invasive method clinically useful since it is high sensitive and specific and it supplies quantitative data that allow pharmacological therapy management by clinicians because specific antiviral therapy for BKVAN does not currently exist and the reduction in immunosuppression depend on viral loads in urine and plasma specimens of kidney transplants [32,33,36,52]. Nevertheless it is important to underlie that the relationship between BKV viruria and viremia, the cut-offs and predictive values of

BKV viruria and viremia for the occurrence of BKVAN, are still largely undefined [33]. In fact some literature studies from 2004 to nowadays showed that measurements of BKV viruria and BKV viremia have a different prognostic value for patient's therapeutic response and duration of therapy. In accordance with Drachenberg et colleagues BKV viruria precedes BKV viremia and it is a prerequisite for histologically proven BKVAN because the viral replication within the graft finally leads from viruria to viremia [53]. This hypothesis is also sustained by other Authors that maintained that viremia is not present in patients with low-level/limited viral replication in the urinary tract [34,43,44,52,54]. Moreover, in relation to these Authors, viremia is not useful for screening because of blood inhibitors present in plasma sample. Finally, although analytical and physiological variations may be significant when comparing viral urine load in patients with BKVAN, there is general agreement that repeated values above 10^7 BKV copies per milliliter are associated with BKVAN [32,53]. On the other hand a recent study performed by Basse et collaborators suggested that BKV viremia is a rare event after renal transplantation but it has emerged as the most specific test for BKV associated nephropathy [55]. Some Authors retain BKV viremia as the standard for BKVAN diagnosis since the presence of the virus in the blood represents a significant tissue damage and confirm the renal parenchymal involvement [37,56]. Therefore serial determinations of BK viremia are the best tool to demonstrate resolution of the disease after immunosuppression has been decreased [37,55-58]. Nevertheless, a study carried out by Hymes et colleagues from June 2003 to January 2006 on 20 renal transplant children showed that most patients remained PCR-positive despite reduction of immunosuppression. Moreover they did not identify any one drug as more prevalent among patients with BK viremia [59].

Conclusion
In conclusion, there are several aspects of BKVAN pathology in kidney transplant patients requiring evaluation; it includes BKV transmissibility within kidneys transplanted, target organ effects, risk factors, time frame of reactivation and the best treatment options. Therefore it is essential to understand and to monitor the delicate balance between viral infection, immune regulation in the transplant population and immunosuppressive therapy in order to minimize viral injury and rejection risk to patients with BKV infection. Measuring of BKV DNA in urine and serum is an useful and non invasive tool for early detection and monitoring, nevertheless a combined approach of molecular techniques must be utilized to identify BK virus-associated nephropathy at an early phase facilitating well timed clinical intervention.

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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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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.
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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

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 INVαF strain of *Escherichia coli* using the QIAGEN Plasmid Minikit (QIAGEN, Hilden, Germany). Purified plasmids were subjected to double digestion with *Hin*dIII 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

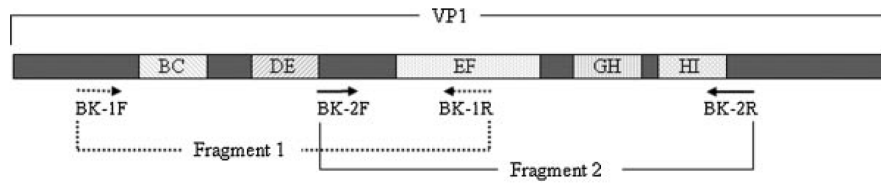[a]Months between renal transplantation and diagnosis of PVAN.

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.

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 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)

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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