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in
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TITOLO:

INDAGINI SIEROLOGICHE E MOLECOLARI SULLA TRASMISSIONE MATERNO-FETALE DELLA INFEZIONE DA JV VIRUS E BK VIRUS E SIGNIFICATO CLINICO DELLE MUTAZIONI DI REGIONI GENOMICHE VIRALI

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INTRODUZIONE

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I polyomavirus (PVs) umani sono virus altamente diffusi nella popolazione mondiale sia adulta che infantile[1] e rivestono un ruolo fondamentale nella patogenesi di alcune importanti patologie a carico di pazienti immunocompromessi, come la Leucoencefalopatia Multifocale Progressiva (PML) in pazienti con AIDS, la Cistite Emorragica in pazienti trapiantati di midollo osseo o la Nefropatia Associata a Polyomavirus (PVAN) in trapiantati renali[2-5].

A tutt'oggi molti aspetti dell'infezione umana da parte di questi virus rimangono da chiarire; uno degli aspetti più controversi risulta essere la modalità di trasmissione con cui i PVs si diffondono nella popolazione umana, in quanto l'infezione primaria ad essa associata non presenta una fase acuta chiaramente riconoscibile[6].

Approfondire le conoscenze su un aspetto sicuramente importante della storia naturale dei PVs umani, come le loro modalità di trasmissione, risulta critico per poter comprendere al meglio la patogenesi delle importanti patologie umane di cui rappresentano il principale agente etiologico e per poter approntare una efficace terapia e prevenzione.

I Polyomavirus

I polyomavirus (PVs) appartengono alla famiglia delle *Polyomaviridae*, anche se storicamente venivano classificati come genere appartenente, assieme a quello dei *Papillomavirus*, alla famiglia delle *Papovaviridae*[6]. L'infezione virale può avvenire in forma *litica* –con distruzione della cellula bersaglio e liberazione della progenie virale- o *abortiva* –cioè con espressione parziale delle proteine virali, in assenza di distruzione cellulare- con potenzialità di trasformazione oncogenica in vari tipi cellulari (infatti il loro nome deriva dalla fusione di "Poly" e "oma" proprio a sottolineare questa loro caratteristica[7]. Attualmente sono noti diversi PVs in grado di infettare su larga scala molte specie tra i mammiferi in modo relativamente specie-specifico tra cui topo, criceto, scimmia e anche l'uomo.

L'uomo è l'ospite naturale di cinque PVs; i primi a essere scoperti e per tanto i più studiati a tutt'oggi risultano essere il Polyomavirus hominis 1 e 2, meglio conosciuti con l'acronimo, rispettivamente, di BK virus (BKV) e di JC virus (JCV) derivante dalle iniziali dei pazienti in cui sono stati isolati per la prima volta nel 1971. BKV è stato isolato per la prima volta nelle urine di un paziente con trapianto renale che aveva sviluppato nel periodo post-operatorio una stenosi ureterale[8]. Mentre JCV è stato isolato partendo da oligodendrociti (cellule del sistema nervoso centrale produttrici di mielina) di un paziente con morbo di Hodgkin in terapia immunosoppressiva, che aveva sviluppato Leucoencefalopatia Multifocale Progressiva (PML)[9], una patologia fatale del

sistema nervoso centrale caratterizzata da demielinizzazione sottocorticale, multifocale della sostanza bianca talora estesa alla sostanza grigia cerebrale, che si manifesta in un contesto di immunodeficienza[10]. Recentemente sono stati scoperti tre nuovi PVs in grado di infettare l'uomo: KI (Karolinsk Institute) e WU (Washington University)isolati in escrezioni respiratorie, e Merkel cell polyomavirus isolato un anno fa e descritto come probabile agente eziologico del carcinoma a cellule di Merkel[11].

Il Simian virus 40 (SV-40) è uno dei virus più studiati, di cui si conosce sia l'intero genoma, sia il suo modo di interagire con la cellula ospite; sebbene il suo ospite naturale sia rappresentato dalle scimmie, SV-40 può infettare anche l'uomo. Sembra sia 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 una volta nell'uomo sia stato poi in grado di infettare nuovi individui[12].

Mentre per BKV e JCV è stato ormai accertato il loro ruolo in diverse patologie umane, per quanto riguarda SV-40 sono ancora in corso studi; osservazioni epidemiologiche su popolazioni accidentalmente infettate da vaccini antipoliomielite non hanno evidenziato apparenti legami tra la presenza del virus e l'insorgere di specifiche patologie. Recenti osservazioni hanno ipotizzato che esso possa prendere parte al processo di cancerogenesi umana specie per ciò che riguarda l'insorgenza di mesoteliomi maligni[13,14].

Strutturalmente i PVs (vedi **figura 1**) presentano un capside a simmetria icosaedrica delle dimensioni di 40-45 nm, privo di envelope, formato da 3 tipi di proteine: viral protein 1 (VP1), viral protein 2 (VP2) e viral protein 3 (VP3). VP1 rappresenta la proteina capsidica maggiore in quanto da sola costituisce circa l'80% dell'intero capside; essa si associa in 72 pentameri che costituiscono l'intera superficie esterna del capside, e pertanto è la diretta responsabile dell'interazione con le molecole di superficie della cellula bersaglio. VP2 e VP3 invece costituiscono insieme il restante 20% del capside associandosi tra loro in complessi che ne formano la superficie interna[15].

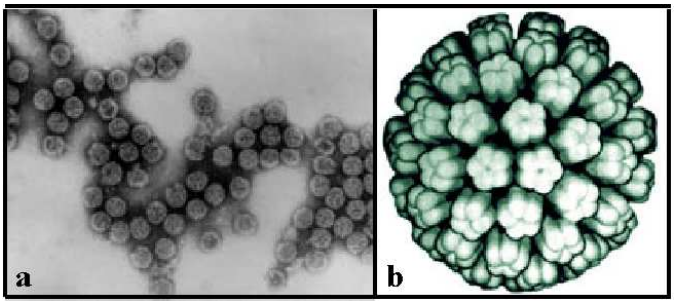
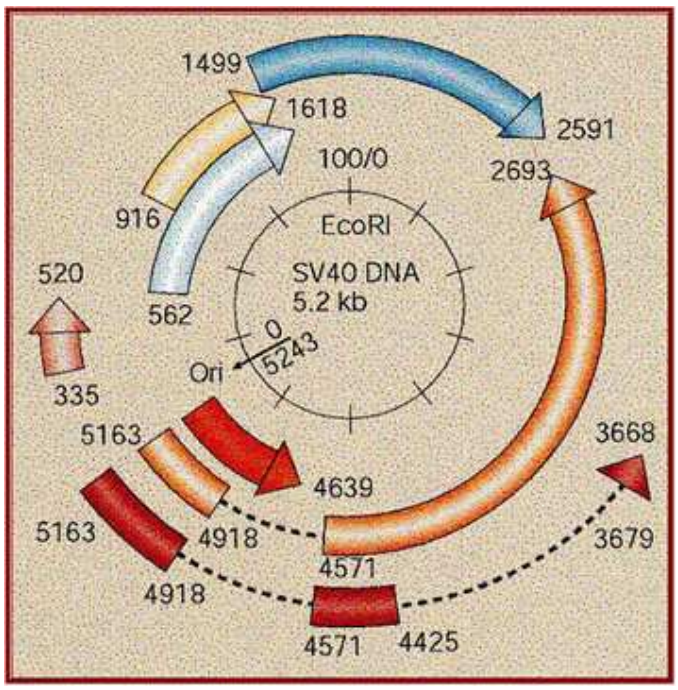


Figura 1: A) Fotografia al microscopio elettronico del PV di scimmia SV-40 (Ingrandimento 10000); **B)** Rappresentazione grafica 3D della struttura esterna dei PV rappresentata dall'envelope formato nello strato esterno solo dalla VP1.

Il genoma virale dei PVs consiste in una singola molecola di DNA, a doppio filamento, delle dimensioni di circa 5300 paia di basi (bp), che si trova all'interno del capside associata con quattro istoni (H2A, H2B, H3 e H4) provenienti dalla cellula eucariotica infettata; il DNA e gli istoni sono complessati sotto forma di cromatina a costituire quello che spesso viene definito un mini-cromosoma virale. Il genoma dei tre PVs precedentemente citati (BKV, JCV ed SV40) presenta una elevata omologia di sequenza (i PVs umani hanno una omologia del 75%, mentre se li confrontiamo con SV40 scende al 70%) ad indicare una comune origine nell'evoluzione[14,15].

Funzionalmente il genoma dei PVs (vedi **figura 2**) viene suddiviso in tre regioni.

La **regione precoce** (Early region, nota anche come regione LT) di 2400bp, che codifica due proteine non strutturali: una fosfoproteina nucleare definita "**large tumor (T) antigen**" (**TAg**) e una proteina citoplasmatica ricca in cisteine, definita "**small tumor (t) antigen**"(**tAg**). Tali proteine sono le prime ad essere espresse durante l'ingresso del virus nella cellula e sono perciò indicative della presenza virale. TAg riveste un ruolo critico nel ciclo replicativo del virus in quanto contribuisce attivamente alla sua regolazione; infatti promuove la progressione nel ciclo cellulare del cellula ospite inducendo l'entrata in fase S, contribuisce al reclutamento del macchinario replicativo di cui il virus è sprovvisto, prende parte attivamente alla replicazione del DNA come fattore di iniziazione del processo di sintesi e come elicasi. TAg svolge inoltre un importante ruolo nello sviluppo di neoplasie in animali da laboratorio, attraverso l'interazione con svariate proteine cellulari che ricoprono il ruolo di importanti oncosoppressori o regolatori del ciclo cellulare, tra cui la proteina 53 (p53) e la proteina associata al retinoblastoma (pRb). Il ruolo di tag invece è poco noto, sembra svolgere un'attività di supporto nei confronti di TAg[15].



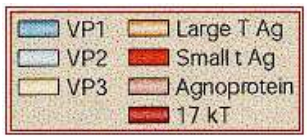


Figura 2: Struttura del genoma del PV SV-40. Come si può notare dall'immagine partendo dall'origine (ORI) i geni del virus si dividono in due gruppi: quelli rappresentati dalle frecce orientate in senso orario sono i geni della regione tardiva; mentre quelli rappresentati dalle frecce orientate in senso antiorario sono i geni della regione precoce.

La **regione tardiva** (Late region) di 2300 bp, che viene espressa efficacemente solo durante la replicazione virale, codifica per le proteine capsidiche del virus **VP1**, **VP2** e **VP3** e per una proteina a funzione ignota, definita appunto, **agnoproteina** (tale proteina sembra svolgere un ruolo nell'assemblaggio del virione prima del suo rilascio all'esterno della cellula)[7,14]. Queste due regioni genomiche, codificanti, sono, dal punto di vista genomico stabili, ovvero le mutazioni nella sequenza di basi nucleotidiche che le compongono sono rare e generalmente coinvolgono singole basi nucleotidiche[15]. La proteina VP1 sembra svolgere un ruolo cruciale nell'influenzare la capacità del virus di infettare con diverso tropismo le cellule bersaglio, in quanto riconosce e lega specificamente molecole bersaglio sulla superficie cellulare, permettendo l'intenalizzazione della particella virale[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] .La proteina VP1, è costituita da 362 aminoacidi (regione codificante di circa 1086 nucleotidi) e viene suddivisa in 5 loops: BC, DE, EF, GH e HI. 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[19]. Successive analisi hanno inoltre evidenziato che VP1 di BKV e JCV è in grado di legare selettivamente oligosaccaridi leganti all'N-terminale una molecola di acido sialico presenti sulla membrana cellulare[18,20] (**Figura 3** e **4**). JCV e BKV vengono inoltre classificati sulla base di variazioni nucleotidiche della regione codificante per la VP1. In particolare JCV viene suddiviso in 8 genotipi (definiti **tipo1**, **tipo 2**, **tipo 3**, **tipo 4**, **tipo 5**, **tipo 6**, **tipo 7, tipo 8**), loro volta suddivisi in numerosi sottotipi, in base alla sequenza nucleotidica della regione di 164 bp compresa tra il nucleotide 1736 e il 1900[21]. Analogamente anche BKV è stato diviso in quattro genotipi denominati **gruppo I**, **gruppo II**, **gruppo III** e **gruppo IV** in base alla sequenza nucleotidica di 69 bp compresa tra i nucleotidi 1744-1812[22]; i genotipi così identificati di BKV corrispondono ai sierotipi identificabili nelle diverse popolazioni (il concetto di sierotipo riflette la risposta immune specifica dell'ospite verso epitopi antigenici di differenti strutture), mentre per quanto riguarda JCV questa sovrapposizione di classificazione non è possibile in quanto la regione tardiva del suo genoma non mostra una variazione di sequenza sufficiente a generare epitopi antigenici sierologicamente distinguibili[23].

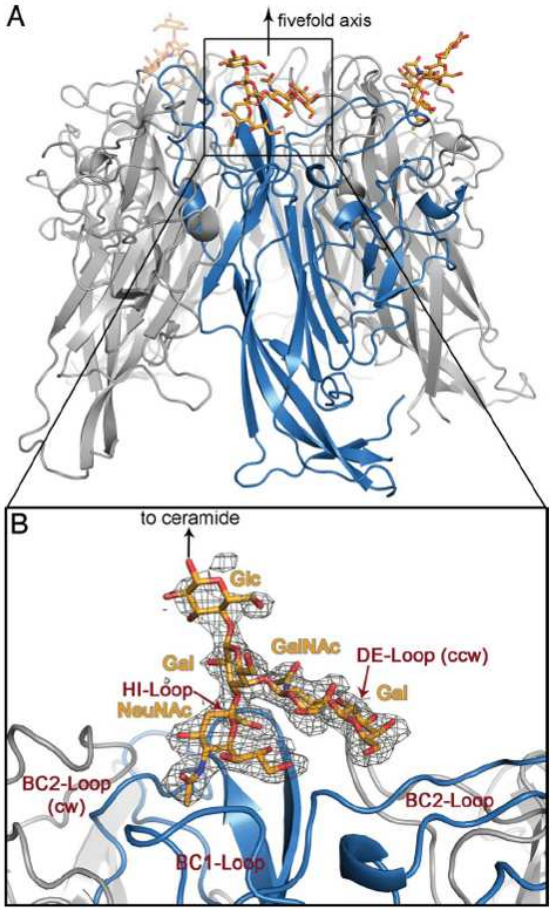


Figura 3: Struttura del complesso VP1 del PV SV-40 e il suo ligando naturale. **A)** Struttura completa; le 5 catene monometriche di VP1 sono rappresentate come nastri con uno dei monomeri evidenziato in blu,gli altri sono in grigio; il ligando è rappresentato come struttura a bastoncino, in arancione sono evidenziati gli atomi di carbonio, in rosso quelli di ossigeno e in blu quelli di azoto. La molecola ligando si lega alla sommità del pentamero di VP1 che corrisponde alla superficie capsidica. **B)** Particolare del legame.

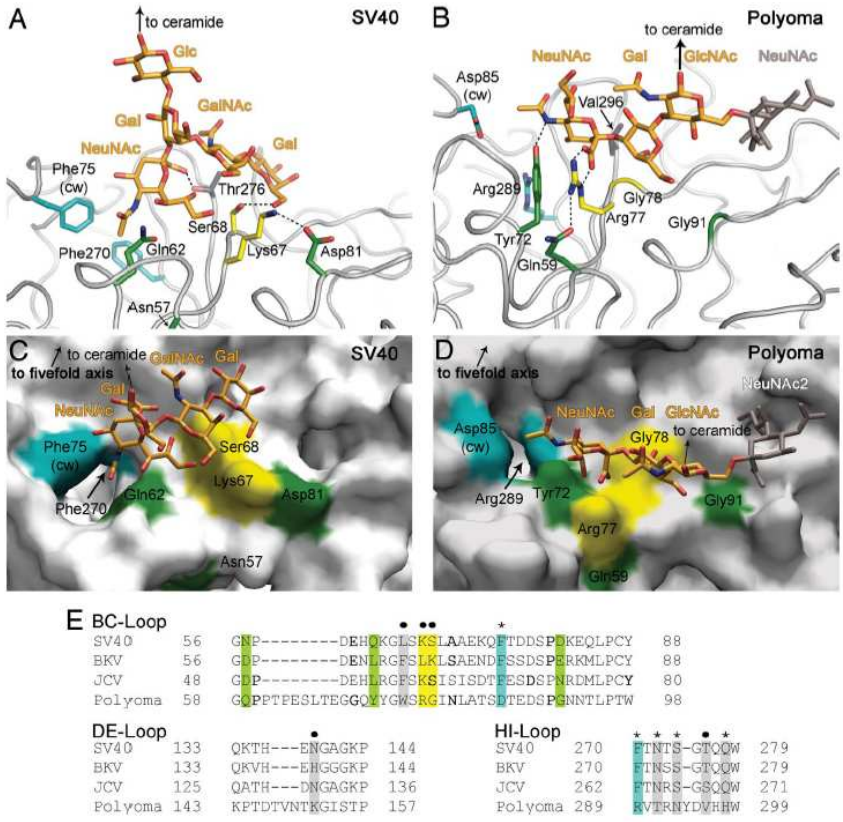


Figura 4: Legame tra SV-40, polyomavirus e il loro ligando naturale in differenti orientamenti e conformazioni. **A e B)** Confronto delle specifiche interazioni tra SV-40 (A) e polyomavirus (B) e il loro ligando. **C e D)** Conformazione dei siti di legame di VP1 in SV-40 (C) e in polyomavirus (D). **E)** Allineamento della sequenza aminoacidica di VP1 di SV-40, BKV e JCV. L'asterisco identifica i residui identici tra SV-40, BKV e JCV; il punto identifica i residui identici in almeno due dei tre polyomavirus.

La **regione di controllo trascrizionale** (TCR region) di circa 400 bp, non codificante, è posta tra la regione precoce e quella tardiva, e svolge un importante ruolo nel controllo della regolazione della sintesi e della replicazione virale. Contiene le **origini di replicazione** (**ORI**) e gli elementi di **promoter/enhancer** di entrambe le regioni codificanti, permettendo la trascrizione dei geni precoci su uno dei filamenti in una direzione, e quella dei geni tardivi sul filamento complementare in direzione opposta[24]. All'interno di questa regione sono anche presenti siti di legame per diversi fattori trascrizionali, tra i quali i più importanti sono: **Sp1** proteina endogena dell'ospite coinvolta nel differenziamento cellulare, il cui ruolo principale sembra essere quello di mantenere libere da metilazione le isole CpG mantenendo così attiva la trascrizione; **Nuclear factor 1** (**NF1**) proteina dell'ospite che media le reazioni infiammatorie ed immunologiche in risposta a vari stimoli, mutazioni a questo livello sembrano interferire con la trascrizione dei geni tardivi; **TAg** proteina di origine virale che agisce attraverso un meccanismo di controllo negativo, infatti inibisce l'attività del promotore precoce; **Pur**α proteina che controlla la replicazione e la trascrizione del DNA, in particolare promuove la trascrizione dei geni precoci e viene inibita da TAg[25]. La regione TCR, a differenza di quelle precedenti, è caratterizzata da una ipervariabilità mutazionale dovuta a inserzioni di singoli o molteplici nucleotidi, duplicazioni o delezioni. In contrasto con quanto appena detto sono state trovate un limitato numero di sequenze di TCR altamente conservate definite ***archetipi***; queste sequenze rappresentano i genotipi dei PVs realmente circolanti nella popolazione umana e derivano dalla co-evoluzione con l'ospite (prova di ciò ne è il fatto che è possibile usare JCV come marker per ricostruire le migrazioni umane). L'ipotesi è che dalle sequenze archetipo presenti in un individuo si originano le sequenze ricombinanti a causa della ipervariabilità della regione, allo scopo di adattarsi alle condizioni presenti nell'ospite modificando il tropismo cellulare, il potere infettivo, l'aggressività e l'abilità replicativa virale[26]. Infatti la regione regolatoria sembra controllare la trascrizione cellulo-specifica del DNA virale come è stato osservato negli studi di Sock et al.[27] dove è emerso che la TCR di JCV aumenta notevolmente la trascrizione nelle cellule gliali in coltura in confronto alle cellule non gliali; inoltre, in altri studi è stato osservato che le sequenze riarrangiate mostrano un'attività differente da quelle archetipo, che può essere sia più alta sia più bassa[28]. Allo scopo di classificare più agevolmente i genotipi di JCV, *Ault* e *Stoner*[29] (vedi **figura 5**) divisero arbitrariamente la sequenza archetipo individuata da *Yogo et al.*[30], definita Mad1, in cinque blocchi definiti **A**(25bp), **B**(23bp), **C**(55bp), **D**(66bp), **E** (18bp) e **F**(69bp) in base alle sequenze che mancavano o duplicavano se confrontate con altri archetipi .

Analogamente anche la regione TCR di BKV fu divisa in cinque blocchi di sequenza definiti da *Yoshiike e Takemoto*[31] **O**(124bp), **P**(68bp), **Q**(39bp), **R**(63bp) ed **S**(63bp), basandosi su una sequenza archetipo (BKV-WW) .

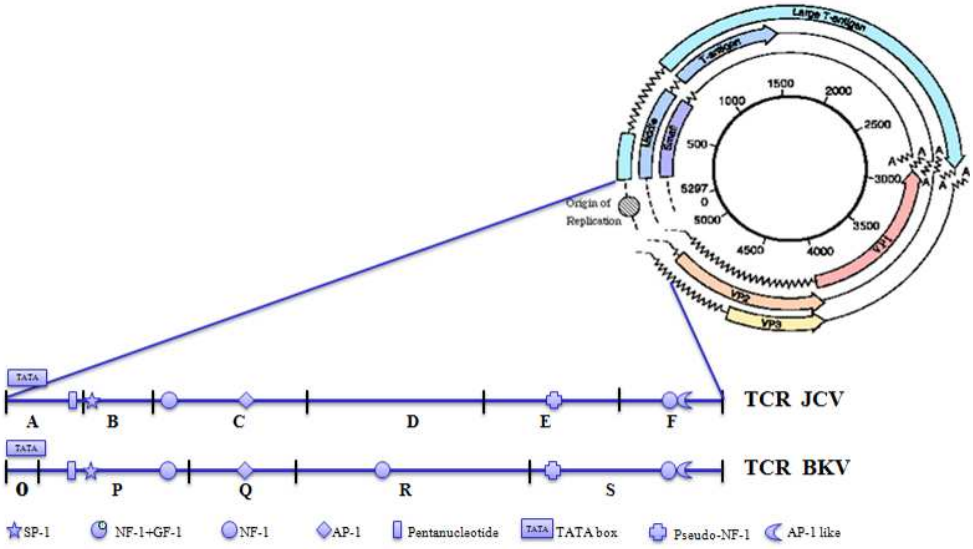


Figura 5: Divisione in blocchi di sequenza della regione regolatoria (TCR) in base al legame di fattori trascrizionale della cellula ospite, rispettivamente di JCV e di BKV.

I PVs umani sono virus ubiquitari; infatti circa il 60-80% degli adulti possiede anticorpi della classe IgG diretti contro BKV e JCV[1]. Si ritiene che l'infezione primaria avvenga durante l'infanzia e decorra in forma asintomatica od oligosintomatica (blande infezioni a livello respiratorio con disturbi per lo più simil-influenzali), autolimitante. Successivamente il virus rimane latente riattivandosi in seguito a immunodepressione, soprattutto se dovuta ad un deficit di linfociti T.

La patogenesi dell'infezione da PVs non è stata ancora definitivamente chiarita. L'ipotesi più probabile prevede una trasmissione per via aerea[32], la successiva moltiplicazione a livello dell'apparato respiratorio con conseguente viremia transitoria. Per via ematogena, veicolato dai linfociti T, il virus raggiunge vari siti di latenza attualmente identificati in oligodendrociti del sistema nervoso centrale, cellule tubulari renali, urotelio[1], tessuto stromale tonsillare , linfociti B e loro precursori[32].

I virus aderiscono alla membrana citoplasmatica della cellula ospite tramite la proteina capsidica VP1[33], vengono internalizzati mediante vescicole endocitotiche e quindi trasportati al nucleo dove, previa fusione delle vescicole alla membrana nucleare, sono liberati nelle cisterne perinucleari (vedi **figura 6**). La sintesi del DNA virale necessita dei prodotti trascritti nella fase precoce, in particolare del TAg, che funziona da attivatore trascrizionale ed è richiesto per il

completamento del ciclo replicativo del virus e per iniziare la trascrizione dei geni tardivi. In particolare, una volta privato di rivestimento capsidico, il virus inizia la trascrizione degli RNA messaggeri della regione precoce ad opera della RNA polimerasi dell'ospite; i trascritti vengono tradotti nel citoplasma. La trascrizione della regione tardiva consente la produzione delle proteine che entreranno a far parte del capside ed assumeranno pertanto il ruolo di determinanti antigenici. Le particelle virali, una volta assemblate nel nucleo, liseranno la cellula ospite acquisendo la capacità di iniziare un nuovo ciclo infettivo.

L'infezione della cellula può essere di due tipi:

- Infezione **produttiva o litica** nelle cellule permissive, con formazione di particelle virali complete a cui consegue la morte cellulare e la liberazione di particelle virali infettanti.
- Infezione **abortiva o non produttiva** nelle cellule non permissive: si suppone che Tag si leghi a proteine cellulari, come quelle prodotte dai geni onco-soppressori quali p53 e pRB[34] che vengono inattivate. Solitamente questo effetto si manifesta per pochi giorni, dopodichè il genoma virale viene rilasciato dalla cellula che ritorna a possedere le caratteristiche normali.

A volte il genoma virale può integrarsi in modo casuale nel DNA della cellula ospite che assume caratteri trasformati. Tale processo sembra essere coinvolto nell'insorgenza delle neoplasie. L'integrazione del genoma virale potrebbe inoltre provocare la comparsa sulla superficie cellulare di antigeni virali, riconosciuti dal sistema immunitario con conseguente danno cellulare immunomediato (per reazione crociata) ed essere quindi causa di malattie autoimmunitarie (sclerosi multipla, lupus eritematoso sistemico, artrite reumatoide).

Come già accennato l'infezione primaria può decorrere in forma asintomatica o esordire con lieve sintomatologia delle prime vie aeree. Generalmente negli individui immunocompetenti BKV e JCV non causano quadri clinici di significato patologico ed una riattivazione virale si può occasionalmente riscontrare, mediante citologia urinaria, durante la gravidanza, negli anziani, nei pazienti affetti da neoplasie e trattati con chemioterapia, nei diabetici, nei soggetti dializzati[35].

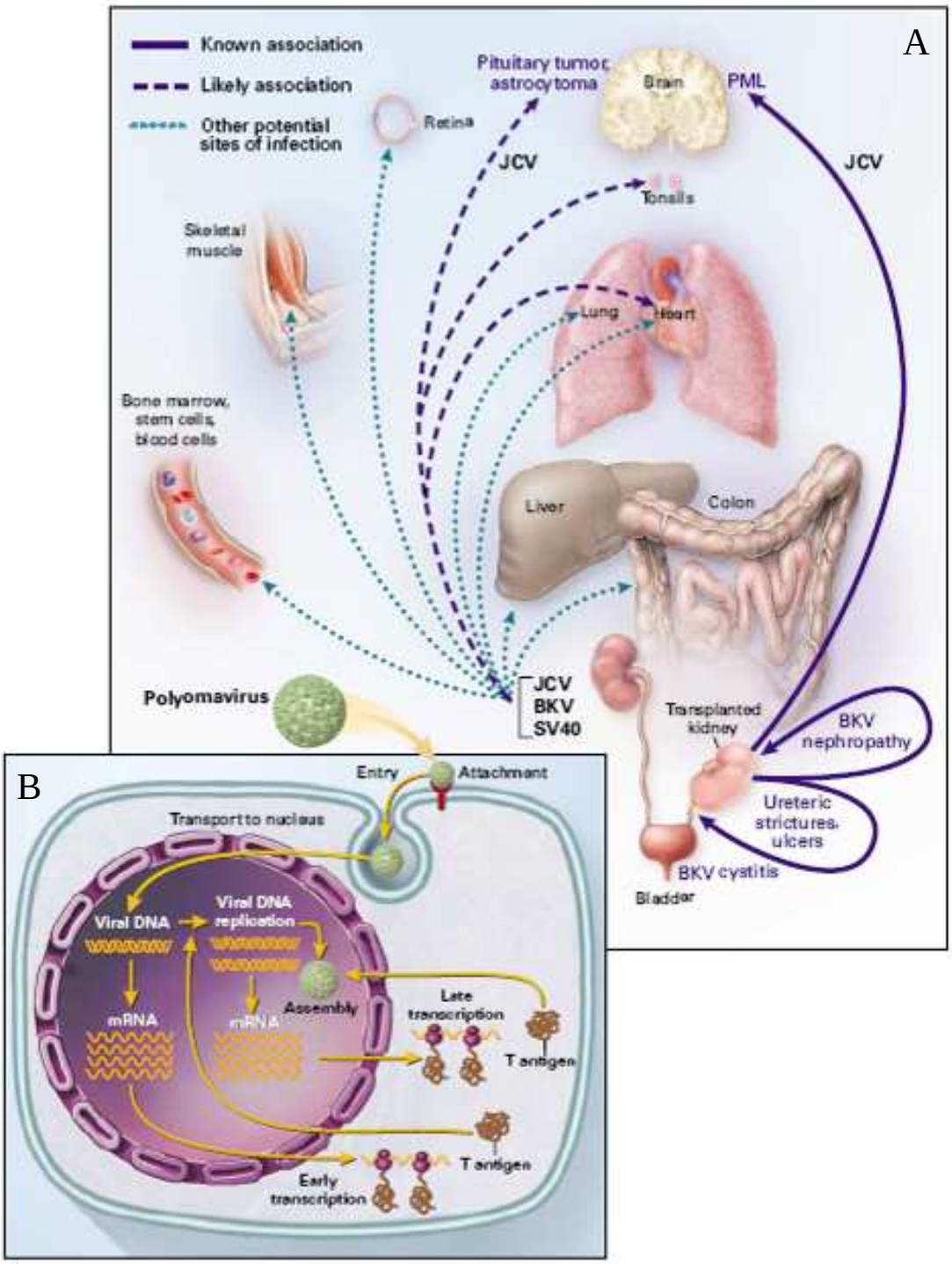


Figura 6: A) Principali siti di latenza noti dei PVs umani, JCV e BKV, e di SV-40 all'interno dell'organismo umano. **B)** Ciclo replicativo dei PVs. FASE 1 Il virus interagisce con i recettori di membrana della cellula ospite; FASE 2 in seguito all'interazione tra il virus e il recettore di membrana inizia il processo endocitico con cui il virus penetra nella cellula ospite; FASE 3 il virus ormai all'interno della cellula si dirige verso il nucleo; FASE 4 il virus interagisce con i fattori trascrizionali endogeni per poter iniziare la fase di trascrizione genica; FASE 5 l'mRNA virale prodotto migra nel citosol; FASE 6 le proteine virali generate nel citosol tornano nel nucleo della cellula ospite; FASE 7 nel nucleo le proteine virali possono o far partire un nuova fase replicativa del genoma virale (FASI 8 e 9) oppure riassemblarsi intorno alla molecola di dsDNA virale per generare nuovi virioni (FASE 10), che attraverso la via esocitica vengono liberati nell'ambiente esterno (FASE 11,12 e 13).

Le manifestazioni cliniche da PVs si riscontrano prevalentemente nei pazienti affetti da deficit dell'immunità cellulo-mediata, in particolare a carico dei linfociti T, come avviene in corso di AIDS[2,3], di trapianto allogenico di midollo osseo[4] e di trapianto renale[5].

Allo stato attuale delle conoscenze possiamo affermare che JCV sia sicuramente l'agente eziologico della PML[36], mentre BKV è attualmente riconosciuto come agente eziologico delle cistiti emorragiche in pazienti riceventi trapianto di midollo osseo eterologo[37] e della cosiddetta nefropatia da PVs che colpisce una piccola percentuale di pazienti portatori di trapianto renale.

Nonostante il largo numero di studi effettuati, specie recentemente, sussistono ancora alcuni dubbi su vari aspetti della storia naturale dell'infezione virale, sulle modalità di trasmissione dell'infezione e infine su siti di latenza virale, oltre a quelli già noti del sistema nervoso centrale e dell'apparato uropoietico.

Nefropatia associata a PVs

La nefropatia associata a PVs (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[38]. Tale patologia è causata in prevalenza da infezione-riattivazione di BKV[39], anche se di recente è stato riportato un caso di possibile coinvolgimento di JCV[40]. 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[41] (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)[38]. 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[42]. 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[38]:

- **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%)[43]. 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.

Le possibili vie di trasmissione dell'infezione da PVs umani

La modalità con cui i PVs si diffondono all'interno della specie ospite rimane, di sicuro, uno degli aspetti più oscuri e di difficile studio della loro storia naturale, non solo per quanto riguarda i PVs umani, ma anche per i PVs infettanti altre specie come il Polyomavirus murino (*Murine Polyoma virus* (MuPyV[44] o SV-40[45]. Per quanto riguarda le possibili vie di trasmissione dei PVs umani BKV e JCV sono state fatte varie ipotesi:

- **Trasmissione per via aerea:** dato che l'infezione primaria dei PVs sembra avvenire a livello delle vie respiratorie superiori, si ipotizza che individui infettati in forma asintomatica possano

rilasciare nell'ambiente le particelle virali tramite fini gocce di aereosol, oppure tramite gocce di saliva. A conferma di questa ipotesi sono stati riportati casi in cui sono stati isolati i virus partendo da lavaggi e spazzolati provenienti della gola di individui immunocompromessi[46], e soprattutto nel tessuto tonsillare di bambini con ricorrenti patologie respiratorie[47]. Si deve però aggiungere che, nonostante sia stato individuato il DNA virale, non è stato possibile dimostrare l'infettività dei virus isolati transfettandoli in linee cellulari suscettibili.

- **Trasmissione urinaria:** è noto da studi effettuati su campioni autoptici e chirurgici che rene e vie escretrici urinarie rappresentano importanti siti di latenza di JCV e BKV e alcuni studi rilevano un aumento della viruria di BKV e JCV in condizioni di deficit immunologici transitori o persistenti[48,49]. E' stato perciò ipotizzato che una delle vie con cui le particelle virali vengano propagate e trasmesse da individuo ad individuo sia tramite la loro escrezione nelle urine[50].
- **Trasmissione oro-fecale:** alcuni studi hanno messo in evidenza la possibilità che i PVs possano utilizzare come sito d'entrata il tratto gastrointestinale tramite l'ingestione di cibi e acqua contaminati. Questa ipotesi viene supportata dal ritrovamento dei genomi virali in tessuti provenienti dall'apparato gastrointestinale[46]. Inoltre è stato recentemente riportato che è stato possibile individuare DNA di BKV analizzando il materiale proveniente dai rifiuti urbani in concentrazioni relativamente elevate (10^1-10^3 particelle virali per 4 ml di rifiuti)[51].
- **Trasmissione tramite organo trapiantato:** costituisce una possibile via di trasmissione nell'eventualità in cui si trapianti un organo di latenza per i PVs, come per esempio il rene, da un individuo sieropositivo ad uno sieronegativo[52].
- **Trasmissione verticale:** quest'ultima via di trasmissione è stata proposta da alcuni autori sulla base di osservazioni epidemiologiche che evidenziano sia la presenza di IgG anti JCV e BKV nel sangue di soggetti in età pediatrica[53], sia la presenza di materiale genomico di SV-40 all'interno di tumori pediatrici[54]. In particolare è stata proposta la **trasmissione per via trans-placentare,** anche se non si esclude la possibilità di trasmissione nel periodo perinatale**.** L'ipotesi della trasmissione trans-placentare viene supportata sia da studi epidemiologici, che hanno messo in evidenza la presenza di IgG anti JCV e BKV nel sangue di cordone ombelicale in neonati sani[55], sia da studi sperimentali effettuati sulla possibilità di trasmissione trans-placentare di analoghi Polyomavirus nel topo (*Murine Polyoma virus* (MuPyV))[44] e nel criceto (SV-40)[56] dimostranti il passaggio di virioni attraverso il cordone ombelicale durante l'infezione virale acuta. Tale ipotesi viene, inoltre, supportata dall'osservazione che durante la gravidanza, verosimilmente in seguito a mutate condizioni immunologiche ed ormonali, si evidenzia con discreta frequenza una riattivazione dell'infezione da PVs[57]. Ciò è dimostrato dalla escrezione urinaria delle cosidette decoy cells, cellule uroteliali con tipiche inclusioni intranucleari, espressione morfologica di proliferazione

virale. Altri autori tuttavia, basandosi sull'utilizzo di metodiche sierologiche, non sono riusciti a dimostrare con certezza la possibilità di una significativa trasmissione trans-placentare dell'infezione[58,59], pertanto tale modalità di trasmissione è tuttora controversa.

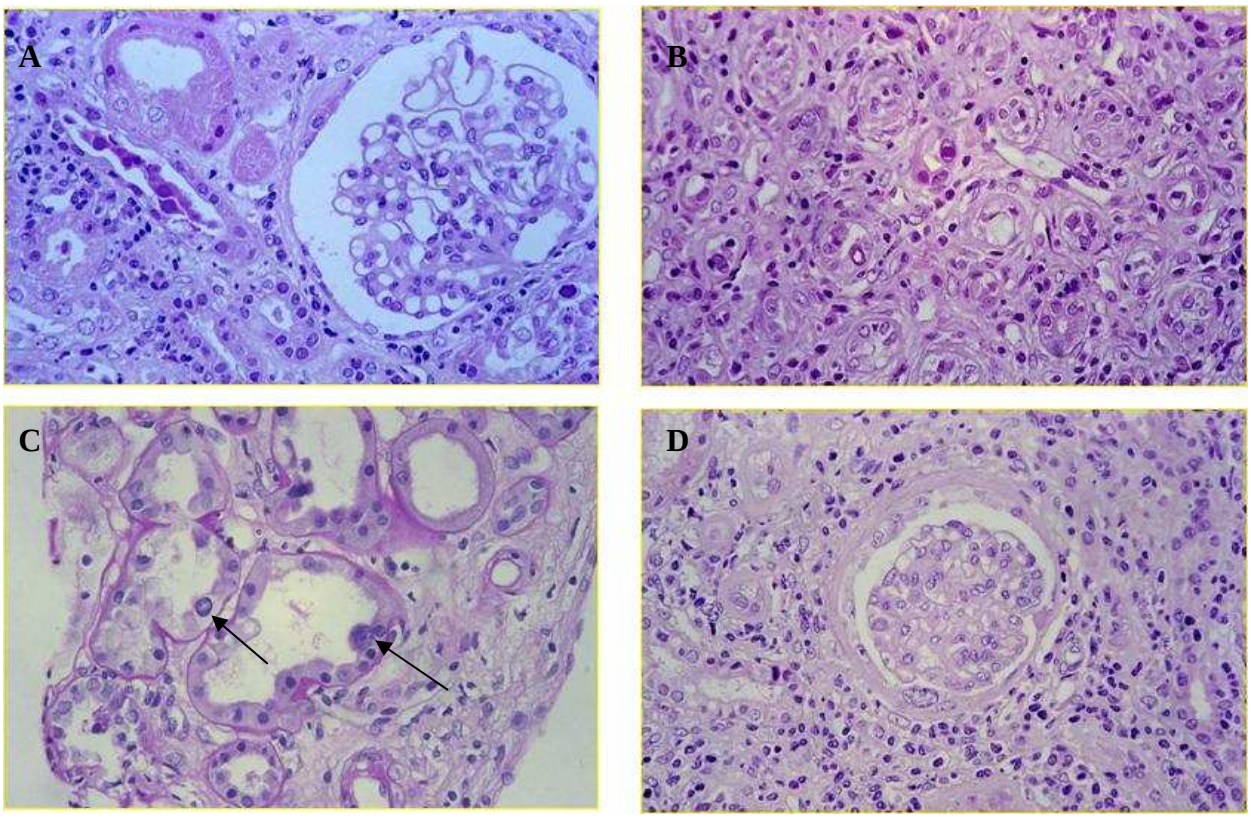


Figura 7: Biopsie renali di soggetti con nefropatia polyomavirus-associata: A,B,C) Varie tipologie di inclusi virali in cellule tubulari renali (frecce) e D) in cellula parietale di capsula di Bowman (freccia).
Ematossilina-Eosina, Ingrandimenti originali: 250x

SCOPO DELLA TESI

Durante il periodo di dottorato la candidata ha seguito due differenti progetti di ricerca, volti entrambi a chiarire alcuni punti oscuri dell'infezione da polyomavirus umani, ma in contesti differenti.

Il primo progetto di ricerca ha riguardato l'analisi di sequenza dell'intera regione codificante la proteina capsidica VP1 di BKV in soggetti trapiantati renali allo scopo di identificare eventuali mutazioni aminoacidiche che possano influire sulla patogenicità virale. Nello specifico è stato condotto uno studio, in collaborazione con il Laboratorio di Patologia e di Ricerca Traslazionale del Ospedale "San Giuseppe" di Milano, su una casistica di pazienti trapiantati renali afferenti al Centro Trapianti di Rene dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità" che hanno sviluppato nel corso degli anni PVAN istologicamente dimostrata e, per confronto, su una popolazione di pazienti con infezione da Polyomavirus ma che non hanno sviluppato PVAN (gruppo di controllo).

Il secondo progetto di ricerca, invece, è stato volto a chiarire la possibile trasmissione verticale dei polyomavirus umani BKV e JCV e del polyomavirus di scimmia SV-40. Allo scopo di investigare la trasmissione trans-placentale è stato condotto uno studio di carattere osservazionale su una popolazione non selezionata di donne con gravidanza fisiologica in fase terminale (37° settimana di gestazione), afferenti alla I divisione di Ostetricia e Ginecologia dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità", e i rispettivi neonati utilizzando metodiche molecolari qualitative per identificare ed eventualmente caratterizzare i PVs presenti. In una fase successiva, allo scopo di valutare la trasmissione verticale (inclusa la via trans-placentare), lo studio è stato esteso a tutti i tre trimestri gestazionali e al primo mese di vita dei bambini. Nello specifico, è stata presa in esame una nuova casistica costituita da una popolazione di donne con gravidanza sia fisiologica che patologica afferenti alla I divisione di Ostetricia e Ginecologia dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità" e i rispettivi bambini afferenti al reparto di Pediatria dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità". In questo studio sono state utilizzate metodiche molecolaria sia qualitative che quantitative per identificare e caratterizzare i PVs presenti, inoltre in collaborazione con Laboratorio di Microbiologia del Dipartimento di Pediatria della "Johns Hopkins" University School of Medicine di Baltimora, sono stati determinati i livelli sierologici di immunoglobuline specifiche contro i BKV e JCV sia nelle donne in corso di gravidanza che nei rispettivi bambini durante il primo mese di vita.

RISULTATI

PUBBLICAZIONE 1

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

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

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

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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

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biologic properties of the virus in vivo in the ability to induce tumors in mice (Freund et al., 1991a; Bauer et al., 1995) and in vitro in virus hemagglutination properties, propagation in cell cultures, and capsid integrity (Freund et al., 1991b; Dugan et al., 2007). Given the importance of the outer loops of BKV-VP1, it has been proposed that amino acid changes within this protein may be associated with an increase in the pathogenic potential of this virus and therefore may contribute to the development of PVAN. To this purpose, Baksh et al. and Rhandawa et al. analyzed the VP1 subtyping region of allograft biopsies from PVAN patients, showing a strong genetic instability and suggesting a possible implication of VP1 amino acid changes for evasion to the host immunity (Baksh et al., 2001; Randhawa et al., 2002). A recent study reported frequent mutations within the BC and DE loops of BKV isolates from renal transplant patients but did not find any correlation between these amino acid substitutions and viruria (Krautkrämer et al., 2009).

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

Materials and Methods

Urine samples and DNA purification

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

None of the patients enrolled in the study experienced graft loss due to viral infection, although one PVAN patient showed disease progression leading to renal fibrosis. In this study, 15 urine samples (one for each BKV-positive patient enrolled), collected when the allograft biopsy for PVAN diagnosis was performed, were investigated. An informed consent form was signed by each patient at the time of collection. DNA for molecular analysis was extracted from 200 μl of urine using the commercial kit Nucleospin RNA virus (Macherey Nagel, Düren, Germany).

Standard PCR for VP1 amplification

In order to amplify the genomic region encompassing the five external loops of VP1, two standard PCRs were performed with two different sets of primers: BK-1F [5′-AGTGCCAAAACT-ACTAATAAAAG-3′, nucleotides (nt) 1,632–1,654]/BK-1R (5′-CTGGGCTGTTGGGTTTTTAG-3′, nt 2,121–2,102) and BK-2F (5′-GAAAACCTATTCAAGGCAGTAA-3′, nt 1,988–2,009)/BK-2R (5′-AAATTGGGTAAGGATTCTTTACA-3′, nt 2,470–2,448). As shown in Figure 1, BK-1F/BK-1R and BK-2F/BK-2R amplified two partially overlapping fragments: fragment 1, delimited by BK-1F and BK-1R, was 489 bp in length, while fragment 2, delimited by BK-2F and BK-2R, was 482 bp in length (Fig. 1). The two amplifications were carried out in a total volume of 50 μl, containing 20 pmol of forward and reverse primer, 0.6 mM dNTPs, 1.5 mM $MgCl_2$, and 2 U of Euro Taq Polymerase (EuroClone, Pero, Italy) in the presence of 1× Reaction Buffer supplied by the manufacturer. A sample of 2, 5, or 7 μl of DNA extracted from urine were added to the PCR mixture. The two amplifications were performed running the same protocol in a GeneAmp PCR System 9700 (Applied Biosystems): an initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C, 30 sec extension at 72°C, and a final extension step at 72°C for 7 min.

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

Molecular cloning and sequencing of PCR fragments

The products of amplification were cloned using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. After the transformation process, the plasmid DNA was extracted from the INVα'F strain of *Escherichia coli* using the QIAGEN Plasmid Minikit (QIAGEN, Hilden, Germany). Purified plasmids were subjected to double digestion with *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.

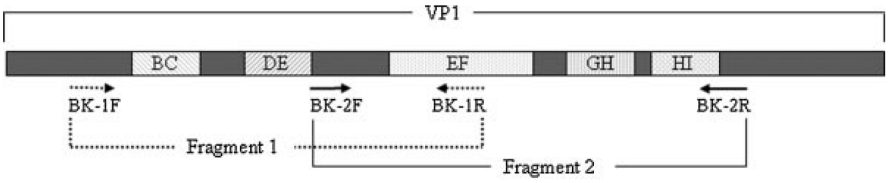

Fig. 1. Target sites of primers BK-1F, BK-1R, BK-2F, and BK-2R within the VP1 region. The use of these sets of primers ensures amplification of all of the outer loops of VP1, together with their neighboring regions.

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.

TABLE 2. BKV isolates used to obtain consensus sequences of the complete VP1 region for genotypes I, II, III, and IV

Genotype	Isolate	Accession number	References
I	DUN	NC_001538	Seif et al. (1979)
I	Gardner	Z19534	Jin et al. (1993)
I	Dik	AB211369	Nishimoto et al. (2006)
I	WW	AB211371	Nishimoto et al. (2006)
I	JL	AB211370	Nishimoto et al. (2006)
I	MT	AB211372	Nishimoto et al. (2006)
I	MM	V01109	Yang and Wu (1979)
II	SB	Z19536	Jin et al. (1993)
II	ETH-3	AB 263916	Zheng et al. (2007)
II	GBR-12	AB 263920	Zheng et al. (2007)
III	AS	M23122	Tavis et al. (1989)
III	KOM-3	AB211386	Nishimoto et al. (2006)
IV	RYU-3	AB211389	Nishimoto et al. (2006)
IV	THK-8	AB211390	Nishimoto et al. (2006)
IV	TW-3	AB211391	Nishimoto et al. (2006)
IV	KOM-2	AB211387	Nishimoto et al. (2006)
IV	FIN-2	AB 260033	Ikegaya et al. (2006)

Statistical analysis

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

Results

Two partially overlapping fragments, encoding the five external loops of BKV-VP1, were amplified from the urine of PVAN patients and controls. The amplified regions were cloned, and at least five positive clones for each PCR fragment were sequenced. Substitutions that were detected in all or most of the clones were considered to be originally present in the urine samples, while those rarely detected were considered to be artifacts introduced by PCR or cloning (Eckert and Kunkel, 1990). However, it should be pointed out that if an heterogeneous population of BKV isolates is present in a given patient, rarely detected substitutions may also be linked to the amplification of less represented viral strains. These viral strains are therefore likely to be missed by using this approach.

Distribution of BKV genotypes

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

Identification of amino acid substitutions within the VP1 region

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

A total of 8 and 18 mutations were identified in the PVAN patients and controls, respectively. The two groups shared four amino acid variations: D77E, E82D within the BC loop and D175E, V210I within the EF loop. The frequency of the mutations detected ranged from 1 to 4 for the PVAN group and

TABLE 3. Genotypes, viral load, and amino acid substitutions in the VP1 region of BKV isolates from urine of PVAN patients

			Amino acid substitution[b]		
Patient	Genotype	Viral load[a]	BC loop	DE loop	EF loop
1	I	6.06E+07	K69R	—	D175E, V210I
2	I	6.26E+08	E82D	H139N	D175E, V210I
3	I	7.20E+08	D75N, E82D	H139N	D175E, V210I
4	IV	1.78E+06	D77E	—	—
5	IV	6.30E+08	—	—	—
6	IV	N/A	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

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

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

TABLE 5. Frequency of amino acid substitutions detected in the VP1 region of BKV isolates from urine of PVAN patients and controls

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

[a]Amino acid numbers are those of Dunlop strain (Seif et al., 1979).

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

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

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PUBBLICAZIONE 2

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

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

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

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

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

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

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VIROLOGY

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.

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

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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.

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MATERIAL AND METHODS

Sample collections

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

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

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

Urine samples

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

Blood samples

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

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

PCR assay

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

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

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

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

Region	Name	Position	Sequence
LT			
Outer	PM1+	*4022–4045	5′-TCT TCT GGR YTA AAR TCA TGC TCC-3′
	PM1−	*4550–4572	5′-TTW TAG RTK CCA ACC TAT GGA AC-3′
Inner	PM2−	*4388–4411	5′-GGT AGA AGA CCC YAA RGA CTT TCC-3′
	JC+	†4086–4108	5′-ATA TTA TGA CCC CCA AAA CCA TG-3′
	SV+	‡4291–4314	5′-ATA ATT TTC TTG TAT AGC AGT GCA-3′
	BK+	*4059–4085	5′-GAA TGC TTT CTT CTA TAG TAT GGT ATG-3′
TCR BKV			
Outer	BKTT1	*5106–5133	5′–AAG GTC CAT GAG CTC CAT GGA TTC TTC C-3′
	BKTT2	*630–657	5′–CTA GGT CCC CCA AAA GTG CTA GAG CAG C-3′
Inner	BRP1	*82–101	5′–TTG AGA GAA AGG GTG GAG GC-3′
	BRP2	*339–358	5′–GCC AAG ATT CCT AGG CTC GC-3′
TCR JCV			
Outer	JRE1	†4989–5009	5′-CCT CCC TAT TCA GCA CTT TGT-3′
	LP2	†518–537	5′-TGC GGC ACC CAT GAA CCT GA-3′
Inner	RFOR	†5085–5104	5′-GCC TCC ACG CCC TTA CTA CT-3′
	RREV	†291–310	5′-CAG AAG CCT TAC GTG ACA GC-3′

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

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

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

Direct DNA sequencing

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

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

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

RESULTS

Cytology

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

Molecular biology

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

None of the umbilical cord blood samples contained polyomavirus DNA.

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

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

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

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

Urine samples

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

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

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

Blood samples

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

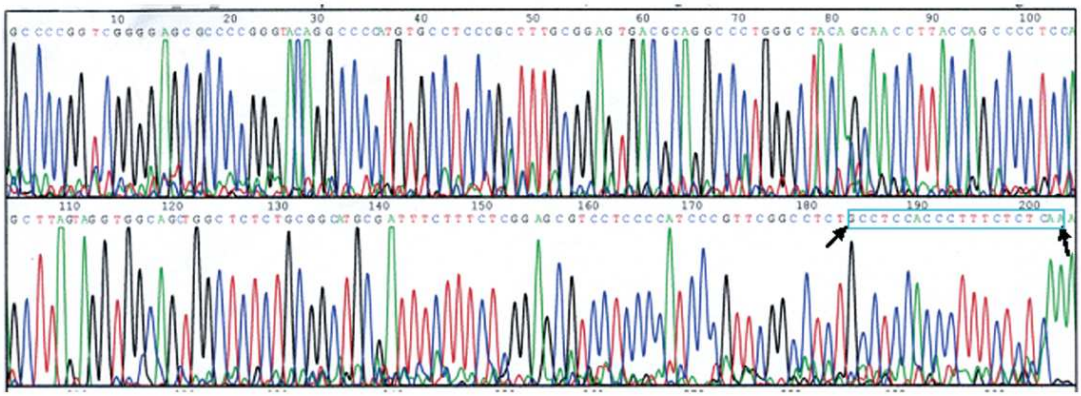

FIG. 1 Electropherogram showing a 20 bp insertion (arrows) of BKV transcription control region in human chromosome 20, isolated from blood sample. The *x* coordinate corresponds to the scan number throughout the time of data collection. The *y* axis is a relative height scale for peak intensity. Traces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

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

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

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

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

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

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

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PUBBLICAZIONE 3

Nello studio precedente non si sono avute evidenze di trasmissione materno-fetale dei PVs umani presi in esame (BKV e JCV) durante la fase terminale di gravidanza, inoltre l'infezione/riattivazione di tali virus sembra essere rara, almeno nel periodo gestazionale investigato.

Lo scopo del presente lavoro è verificare ed approfondire la frequenza di infezione/riattivazione dei PVs umani BKV e JCV e l'eventuale trasmissione verticale dell'infezione stessa estendendo l'analisi al primo, secondo e terzo trimestre di gravidanza e al primo mese di vita dei rispettivi neonati. Lo studio è stato condotto su una popolazione di 19 donne con gravidanza fisiologica e patologica arruolate presso la I Divisione di Ostetricia e Ginecologia dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità"di Novara, e sui rispettivi neonati seguiti dal reparto di Pediatria dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità"di Novara. Durante questo studio sono stati raccolti e analizzati mediante metodiche molecolari sia qualitative che quantitative (nested-PCR e quantitative-PCR) per la ricerca di genoma virale: A) 57 campioni di urina e 57 campioni di siero provenienti dalle donne in gravidanza (19 campioni di urina e siero per ogni trimestre analizzato), B) 19 campioni di siero fetale provenienti dal cordone ombelicale prelevati al momento del parto, e C) 38 campioni di siero, 38 campioni di secrezioni delle prime vie aeree e 26 campioni di urina appartenti ai neonati, raccolti durante le visite ambulatoriali di routine ad una settimana e aun mese di vita. Inoltre, in collaborazione con il Laboratorio di Microbiologia del Dipartimento di Pediatria della "Johns Hopkins" University School of Medicine di Baltimora, sono stati condotti studi immunologici su tutti i campioni di siero, per la determinazione di immunoglobuline dirette contro specifici antigeni virali mediante metodiche di diagnostica immunologica (VLP-ELISA).

I risultati di tale studio ci consentono di affermare che: 1) la trasmissione verticale dell'infezione da BKV e JCV non rappresenta una via prioritaria nella popolazione umana; 2) nelle donne in gravidanza sussiste una elevata frequenza di IgG anti-JCV e/o -BKV, a dimostrazione di una elevate diffusione della infezione da questi virus nella popolazione generale; 3) la riattivazione della infezione -evidenziata dalla identificazione di IgA e IgG specifiche anti- JCV e -BKV- è un evento relativamente frequente, ma che sembra essere limitato al solo compartimento uro-genitale, senza coinvolgimento sistemico; 4) l'infezione primaria non si manifesta frequentemente, mai nella nostra casistica e solo occasionalmente in altre casisitiche più numerose; 5) la trasmissione degli anticorpi madre-figlio avviene in modo passivo, ciò è confermato dai livelli decrescenti dei titoli anticorpali ad un mese di vita del neonato.

VERTICAL TRANSMISSION SEEMS NOT A FREQUENT MODE OF SPREAD FOR JC AND BK POLYOMAVIRUSES IN HUMANS.

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INTRODUCTION

The human polyomaviruses (PVs) BK Virus (BKV) and JC Virus (JCV), are widely diffuse in the worldwide[1-4]. Since their discovery in 1971[5,6] the pathogenic role of BKV and JCV in immune deficiency conditions has been well documented: JCV causes progressive multifocal leucoencephalopathy in AIDS patients, whereas BKV is the etiologic agent of the so called polyomavirus-associated nephropathy, that occurs in renal transplanted patients[7-10]. The primary infection by JCV and BKV usually occurs during childhood with only slight respiratory symptoms[11] thereafter, they persist latently in several organs, but mainly in urogenital system[12], brain[13] and also in circulating leucocytes[14]. Reactivation of both viruses is common and it is frequently associated with asymptomatic viruria[15-17]. While the natural history of infection is well established, the way of transmission of both BKV and JCV is still not known with certainty. Several hypothesis have been proposed, such as respiratory[18,19], oral-fecal[20], or urinary route of transmission[2,12,21]. Moreover, also on the base of the high frequency of PV infection in childhood, a few authors have investigated the possibility of a vertical way of transmission[16,23-28], already demonstrated for other animal homologue polyomaviruses, as the murine (MuPV)[29] and Simian polyomavirus (SV-40)[30], but with conflicting results.

In a previous paper[31], we did not find any evidence of trans-placental transmission of JCV and BKV in a population of 300 unselected pregnant women and their offspring. However, the study was only partially conclusive due to the evaluation was performed during the 3rd trimester of pregnancy only, by the search for viral genome in maternal urine, blood and in umbilical cord blood by qualitative molecular methods. Moreover, in that paper, serologic investigation of both PVs in mothers and newborns was not performed.

To better evaluate the possibility of vertical transmission (including trans-placental and/or maternal-fetal ways) of JCV and BKV, the present study was extended over the 1st, 2nd and 3rd trimester of pregnancy in 19 unselected pregnant women. Moreover, their newborns were evaluated during the first month of life. All the samples were investigated by means of qualitative and quantitative molecular techniques in order to search for BKV and JCV genome. In addition serological data were obtained by means of VLP-ELISA test.

MATERIAL AND METHODS

Case studies

The study was performed on pregnant women and their respective newborns followed at the Obstetrics and Gynecology Unit of Azienda Ospedaliero-univeristaria Maggiore della Carità (Novara, Italy) between May 2008 and Agust 2009.

The criteria of selection included: 1) Peripheral blood (PB) and urine samples taken at routine follow-up in each one gestational trimester for the mothers and PB samples at one week and one month of life for the babies; 2) Umbilical cord blood samples taken immediately after deliveries, and 3) Nasopharyngeal secretion samples taken at one week and one month of life for the babies. Moreover, when possible, urine samples were additionally taken from the babies at one week and one month of life.

Using these strict criteria, 19 cases were included in the study.

Informed consent was obtained from each patient and the study design was approved by the medical ethics committee of the Azienda Ospedaliero-universitaria Maggiore della Carità.

A total of 57 urine and PB samples from mothers, 19 umbilical cord blood samples, 38 pediatric PB samples, 38 nasopharyngeal secretion samples and 26 pediatric urine samples were collected.

Urine samples were concentrated by means of centrifugation at 700 g for 10 minutes. Maternal urine samples were divided in two aliquots: one was submitted to cytological analysis, the other was stored at -20°C for molecular analysis. Conversely, all the urine samples from newborns, were used for molecular analysis due to scarcity of material.

All blood samples (PB and UCB) were centrifuged at 700 g/min for 10 min, after which the serum was separated and stored in 1.5 mL tubes at -20°C. The sera subdivided in two aliquots and used both for molecular and serological analysis.

Nasopharyngeal secretions were collected from all the newborns by means of Dacron fiber tip swab. and immediately resuspended in 400µL of PBS and submitted to DNA extraction for molecular analysis.

Cytological analysis

Five mL of the urine pellets, obtained from the first centrifugation, were then cytocentrifuged at 250 g/min for 10 min onto two slides, which were both fixed in 95% ethanol, stained with Papanicolau stain, and cytologically examined in order to identify the presence of decoy cells (DCs) indicating active PV replication[32].

DNA extraction and multiplex nested- PCR for LT amplification

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

In order to amplify the LT regions, a multiplex nested-PCR (n-PCR) was performed directly on the urine samples (as proposed by Agostini et al.33) and on the DNA extracted from all serum and oro-pharyngeal 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 SV40+ (an amplified fragment of 135 bp) as inner primers, all simultaneously mixed in a single reaction, in order to distinguish the different members of the Polyomavirus genus (**Table 1**). The samples were amplified in a total volume of 25 µL containing 1.25U GoTaq® Flexl DNA polymerase (Promega, Madison, WI, USA) in the presence of 1x Green GoTaq® Flexl buffer (Promega, Madison, WI, USA), 4 mM MgCl2 (2 mM for the inner PCR), 0,2 pmol/µL of each primer (Roche Diagnostics, Milan, Italy), 0.2 mM dNTPs (Promega, Madison, WI, USA), and 10 µL serum and salivary DNA, 2.5 µL 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 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 DNase/RNase-free water (Bioline, London, UK) was used as the negative control; the positive controls were DNA extracted from PML brain tissue (for JCV), from renal tissue from a subject with histologically proven BKV nephropathy (for BKV) and from an SVG cell line culture (for SV40). The sensitivity of the multiplex nPCR was estimated by amplifying serial dilutions of a JCV-positive sample (data not shown).

Quantitative Real Time-PCR for BKV and JCV

The viral load of samples that tested positive for the PVs genome amplification by means of multiplex n-PCR, was determined by quantitative real-time PCR assay (q-PCR). Specific separated reactions targeting a conserved region of LT gene were settled for BKV and JCV (Primers and probes sequences used were shown in **Table 2**). Both reactions were performed in a final volume of 25 µL containing a 1x Taqman Universal PCR Master Mix (Applied Biosystem, Foster City, CA, USA), 900 nM of each primer, 200 nM probe and 5 µL of extracted nucleic acid. Thermal cycling was carried out on a 7500 Fast Real Time-PCR System (Applied Biosystem, Foster City, CA, USA) according to the following steps: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, at the end of which fluorescence was read.

Each sample was analyzed in triplicate, and each run contained a negative control (DEPC-treated DNase/RNase-free water (Bioline, London, UK)). Standard curves for quantification of BKV and JCV were constructed using serial dilutions of plasmids containing the whole BKV genome (1,5x105 copie/µL (Tebu-bio, Columbia, MD, USA)) and JCV genome (1,1x105 copie/µL (Tebu-bio, Columbia, MD, USA). The detection limit for this assay was determined to be 5 copies/reaction. Data were expressed as logarithm 10 copies of viral DNA per microliter of sample.

Serological analysis

BKV-specific antibodies will be measured in serum samples using a virus like particle (VLP)-based ELISA assay. Briefly, 96-well Maxisorb microtiter plates (Nunc) are coated with purified VLP protein (20 ng /well) followed by treatment with a polyvinyl alcohol containing blocking solution. Serum samples (diluted 1:100 for detection of IgA and IgM and 1:200 for detection of IgG), are allowed to react on the antigen coated plates, and antigen bound immunoglobulin is detected with peroxidase conjugated antibodies to human IgG, IgA, or IgM (Southern BioTech, Birmingham, AL).

The IgA and IgM measurements are performed after an initial blocking step with 15% goat anti-human IgG (Sigma-Aldrich, St. Louis, MO). Color development is initiated by addition of substrate, and optical density (OD) is measured in an automated microtiter plate reader. Positive and negative control sera, sensitivity controls, and reproducibility controls are included in each run. Runs where replicate serum values fall outside the expected coefficient of variation are repeated. Results are recorded both as an OD value, and as a categorical variable (seropositive or seronegative) based on cut off points determined from the distribution of reactivity of samples from young children after excluding outliers.

Statistical analysis

Statistical analysis was performed using XLSTAT 09 software program. Results were presented as geometrical mean±standard deviation, or as median with range, as appropriate. Fisher exact test was run to determine statistical significance as appropriate and statistical results with a P value less than 0,05 were considered significant (95% confidence interval).

RESULTS

Clinical Data

The clinical data of the 19 women included in the study are shown in **Table 3**.

Mean age was 28,5±1,146 years (median: 29 years; range: 20 – 36 years) at the first control (1st trimester of pregnancy). All but two pregnancy were physiologic; the case 8 developed a steatohepatitis during the pregnancy whereas the case 17 received renal transplant and took immunosuppressive drugs during all the pregnancy .

Gestational age at deliveries ranged from 37th to 41st weeks (median: 40th weeks); 13 deliveries (68,4%) were eutocics, whereas six distocics (31,58%), the latter were resolved by cesarean deliveries (five cases) and vacuum extraction (one case).

The babies were nine females and ten males, the average weight at birth was 3396±1,153 gr (median: 3370 gr; range: 2780-4330 gr); all but one had natural feeding.

All the babies were well, four of them (cases 3, 7, 9 and 13) complained jaundice that quickly resolved spontaneously.

Cytological analysis

No decoy cells were identified by cytological examination of the 57 urine samples from pregnant women, despite a careful search. Cytologic analysis of the urine samples from the babies was not performed due to the paucity of samples.

Molecular Analysis

Multiplex n-PCR

Genome of PVs was detected in 20 of 57 (35,1%) urine samples, from 8 pregnant women (42,1%). One of these had only one positive sample, two had two positive samples and in five cases all the samples were PV positive. Notably, in all the cases, the type of PV was stable in the samples taken at different time of pregnancy. On respect to the type of PV isolated, JCV genome was identified in four cases (21%), BKV in three (16%) , and the co-infection BKV- JCV in one case only (**Figure 1**).

The **Figure 2** shows the frequency of excretion and type of PV identified in urine samples from the eight PVs positive pregnant women**.** The overall percentage of urine samples that tested positive for PV genome did not change significantly over the three controls. However, the analysis of the single cases, disclosed a variability of genome detection during the three trimester of pregnancy. In detail, BKV positivity was identified in three cases (1, 2, 7): the case 1 over the three controls, in the case 2 during 2nd and 3rd control and in the case 7 in 1st control only. JCV positivity

was identified in four cases (3, 4, 9, 18): cases 3, 4 and 9 over the three controls, and case 18 during the 1st and the 3rd control. One case (13) was positive for both BKV and JCV in all the controls (**Table 4**).

Multiplex n-PCR performed on PB samples of pregnant women and on all the samples from newborns (UBC, PB, nasopharyngeal secretion and urine samples) was negative in every cases.

Viral Load

The median peaks and range of detection for JCV and BKV in urine of pregnant women were calculated during each trimester of pregnancy (**Figure 3**). The median peak of JCV was 3,5 $\log_{10}$ copies/μL (range: 1,5-4,8 $\log_{10}$ copies/μL), in the 1st trimester, significantly decreased in the 2nd (1,7 $\log_{10}$ copies/μL (range: 0-3,7 $\log_{10}$ copies/μL), and raised in the 3rd trimester (3,73 $\log_{10}$ copies/μL (range: 2,2-4,6 $\log_{10}$ copies/μL). Conversely, the median peak of BKV constantly increased during the pregnancy: it was 1,7 $\log_{10}$ copies/μL (range: 0-2,8 $\log_{10}$ copies/μL), in the 1st trimester, 2,3 $\log_{10}$ copies/μL (range: 0-3,8 $\log_{10}$ copies/μL), in the 2nd and 3,5 $\log_{10}$ copies/μL (range: 0-4,7 $\log_{10}$ copies/μL) in the 3rd trimester.

The **Figure 4** shows the pattern of BKV and JCV viral load in urine over the three trimester of pregnancy in each pregnant women.

The JCV viral load decreased in four cases in the 2nd trimester and tested negative in one case (**fig 4a**) but interestingly, it raised again in the last control in all the cases.

Conversely, the pattern of BKV viral load (**Figure 4B**) was more unpredictable. Viral load of cases 1 and 13 slightly increased over the 1st and 2nd control but more significantly in the 3rd trimester; the case 7 tested positive only in the 1st control, whereas the case 2, was negative in the 1st trimester, and positive in the 2nd and 3rd trimester.

Serological Analysis

Data from VLP-ELISA test in serum samples of pregnant women are summarized in **Figure 5**. The overall prevalence of anti-PV IgG antibodies was 89% (17/19); in detail, 9 cases (47%) had anti-BKV IgG, one case (5%) anti-JCV IgG and 7 cases (37%) had anti- BKV and JCV IgG,. For practical purpose, the latter were considered once BKV-specific IgG and once JCV-specific IgG.

The seroreactivity to JCV was stable over the time during the three controls in all the cases, but the OD values of anti-JCV IgG decreased during the three trimesters (**Figure 6A**); indeed, the median OD values for anti-JCV IgG were: 1,674 OD (range: 0,646-2,0665 OD) in the 1st trimester, 1,467 OD (range: 0,731-2,229 OD) in the 2nd , and 1,012 OD (range: 0,433 - 2,049 OD) in the 3rd

trimester. Of note, anti-JCV antibodies IgM and IgA were also detected in case 13 over the three controls, whereas they were found only in 2nd and 3rd trimester in the case 3.

The seroreactivity was also stable for BKV over the time, in all but two cases (case 8 and 4), where anti-BKV IgG were detected only in the 2nd and 3rd trimester respectively. The median OD values for anti-BKV IgG (**Figure 6B**) increased during the 2nd control from 0,8465 OD (range: 0-2,51OD) at the 1st control, to 1,33025 OD (range: 0-2,45 OD), and afterward decreased to1,0645 OD (range: 0,1815- 2,0545 OD) at the 3rd control. Interestingly, anti-BKV IgA were also detected in 5 cases over the three controls, one of these tested positive also for anti-BKV IgM; in another case anti-BKV IgM was detected in the 3rd control (case 4).

The seroreactivity to BKV and/or JCV obtained from serum samples of newborns was specular to that found in the mothers. The median OD values for anti-JCV and anti- BKV IgG decreased during the first month of life but without a total vanishing. Median OD levels were: a) anti-JCV IgG: 0,7765 OD (range: 0,4955- 2,017 OD) at delivery; 0,76075 OD (range: 0,1715-1,9525 OD) at one week of life and 0,482 OD (range: 0,2395-1,267OD) at one month; b) anti-BKV IgG: 1,6725 OD (range: 0,5515-2,255OD) at delivery; 1,7255 OD (range: 0,337-2,2295 OD) at one week of life and 1,04 OD (range: 0,1635- 1,919 OD) at one month.

The case 4 showed a particular serological condition. Seroreactivity to both anti -BKV IgM and IgG was found in the 3rd trimester of pregnancy, whereas the serum sample of the newborn started to be seroreactive from one week, and maintained positivity in one month control.

JCV and BKV viruria in seroreactive pregnant women

The frequency of viruria in the groups of JCV and BKV seroreactive pregnant women was different. As shown in the **figure 7**, 62.5% (5 cases of 8)of pregnant women JCV seroreactive showed viruria, in comparison with only 25% (4 cases of 12)of the BKV seroreactive group; however the difference did not reach a statistical significance (P=0,0994).

The **figure 8**, compares the median OD value of anti-JCV and BKV IgG in pregnant women with and without viruria. In the JCV pregnant women the median OD value was higher (1,656; range: 0,809-2,229) for the group with viruria than in the group without viruria (1,232; range: 0,433- 1,809); conversely the median anti-BKV OD values in pregnant women with viruria was lower (0,886; range: 0,485-1,967) as compared to the group without viruria (1,153; range: 0,1815-2,51).

Viral load of JCV and BKV in urine were compared with OD values of anti-JCV and BKV IgG in serum samples over the three controls during pregnancy. As shown in the **figure 9A** either JCV viral load in urine and OD values of anti-JCV IgG decreased between 1st and 2nd control,

whereas an increase of viral load and decrease of anti-JCV IgG was found between 2nd and 3rd trimester of pregnancy. The pattern of BKV viral load in urine and OD values of anti-BKV IgG had a similar trend; the **figure 9B** shows a constant increase of both values over the three controls.

TABLES

TABLE 1: Multiplex n-PCR primers

REGION	NAME	POSITION	SEQUENCE
OUTERN	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'

Wobble position IUB code: Y=C/T; R=A/G; W=A/T; K=G/T;
* BKV Dunlop strain GenBank Acession Number: V01108;
°JCV Complete genome GenBank Acession Number: J02226;
** SV40 complete genome GenBank Acession Number: J02400.

TABLE 2: Q-PCR primers and probes

PV	NAME	POSITION	SEQUENCE
BKV	BKV for	*4986-5012	5'-ATT CAT TCT CTT CAT TTT ATC CTC GTC-3'
	BKV rev	*5078-5049	5'-AAT CTT CCC TTA ATG AGA AAA GCT TAT TTA -3'
	BKV probe	*5016-5046	5'-FAM-CCC TTT GTC AGG GTG AAA TTC CTT ACA CTT C-TAMRA-3'
JCV	JCV for	°4299-4321	5'-GAG TGT TGG GAT CCT GTG TTT TC -3'
	JCV rev	°4352-4375	5'-GAG AAG TGG GAT GAA GAC CTG TTT-3'
	JCV probe	°4323-4350	5'-FAM- TCA TCA CTG GCA AAC ATT TCT TCA TGG C-TAMRA -3'

* BKV Dunlop strain GenBank Acession Number: V01108;
°JCV Complete genome GenBank Acession Number: J02226.

TABLE 3: Clinical data

CASE	MOTHERS DATA			NEWBORNS DATA			
	AGE	**PATOLOGY**	**DELIVERY**	**GA/SEX**	**WEIGHT**	**PATOLOGY**	**FEEDING**
1	31	none	CD	41^{+1}/F	4050 gr	no one	maternal
2	36	none	ED	ND/M	3500 gr	no one	maternal
3	32	none	CD	37/F	3430 gr	jaundice	maternal
4	25	none	ED	41/M	4060 gr	no one	maternal
5	28	none	ED	40/M	3480 gr	no one	maternal
6	34	none	ED	40/F	3130 gr	no one	maternal
7	29	none	ED	ND/F	3370 gr	jaundice	maternal
8	29	steatohepatitis	CD	37/F	2840 gr	no one	maternal
9	27	none	ED	39/M	3130 gr	jaundice	maternal
10	30	none	ED	40^{+3}/M	3080 gr	no one	maternal
11	28	none	VE	41/F	2800 gr	no one	maternal
12	31	none	ED	41/M	3970 gr	no one	maternal
13	27	none	ED	40/M	4300 gr	jaundice	maternal
14	27	none	ED	40/F	3050 gr	no one	maternal
15	20	none	ED	40/F	3270 gr	no one	maternal
16	31	none	ED	40/M	3540 gr	no one	maternal
17	32	renal transplant recipient	CD	38/F	2780 gr	no one	maternal
18	25	none	CD	40/M	4330 gr	no one	maternal
19	24	none	ED	40/M	3040 gr	no one	maternal

ND= Not Determined; ED= Eutocic Delivery; CD= Cesarean Delivery; VE= Vacuum Extraction; GA= Gestational Age (weeks+ days); M= Male; F=Female.

TABLE 4: Results of multiplex n-PCR on the urine samples of the eight pregnant women tested positives for PVs genome.

CASE	1st TRIMESTER	2nd TRIMESTER	3rd TRIMESTER
1	BKV	BKV	BKV
2	NEG	BKV	BKV
3	JCV	JCV	JCV
4	JCV	JCV	JCV
7	BKV	NEG	NEG
9	JCV	JCV	JCV
13	BKV/JCV	BKV/JCV	BKV/JCV
18	JCV	NEG	JCV

FIGURES

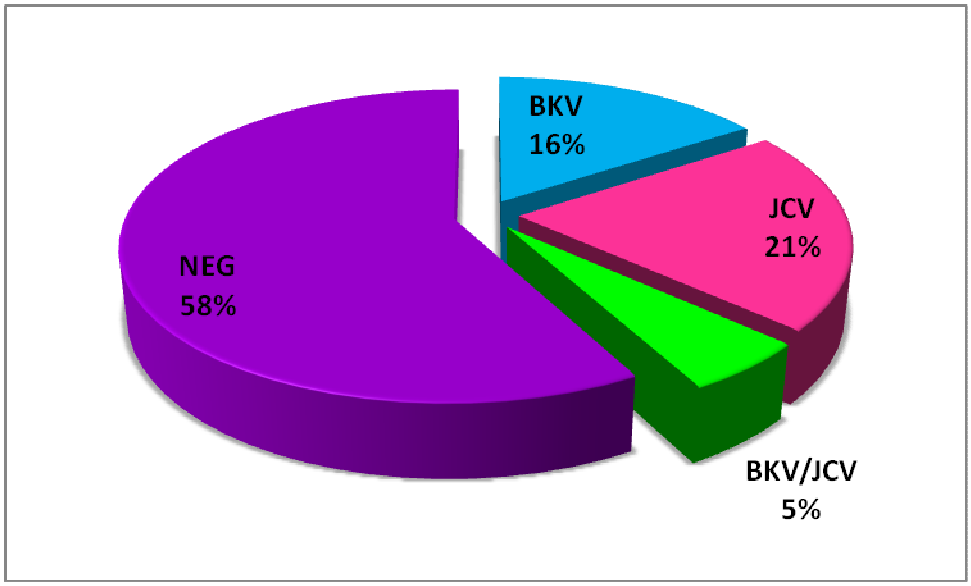

Figure 1: Distribution of PVs urinary excretion among women during pregnancy.

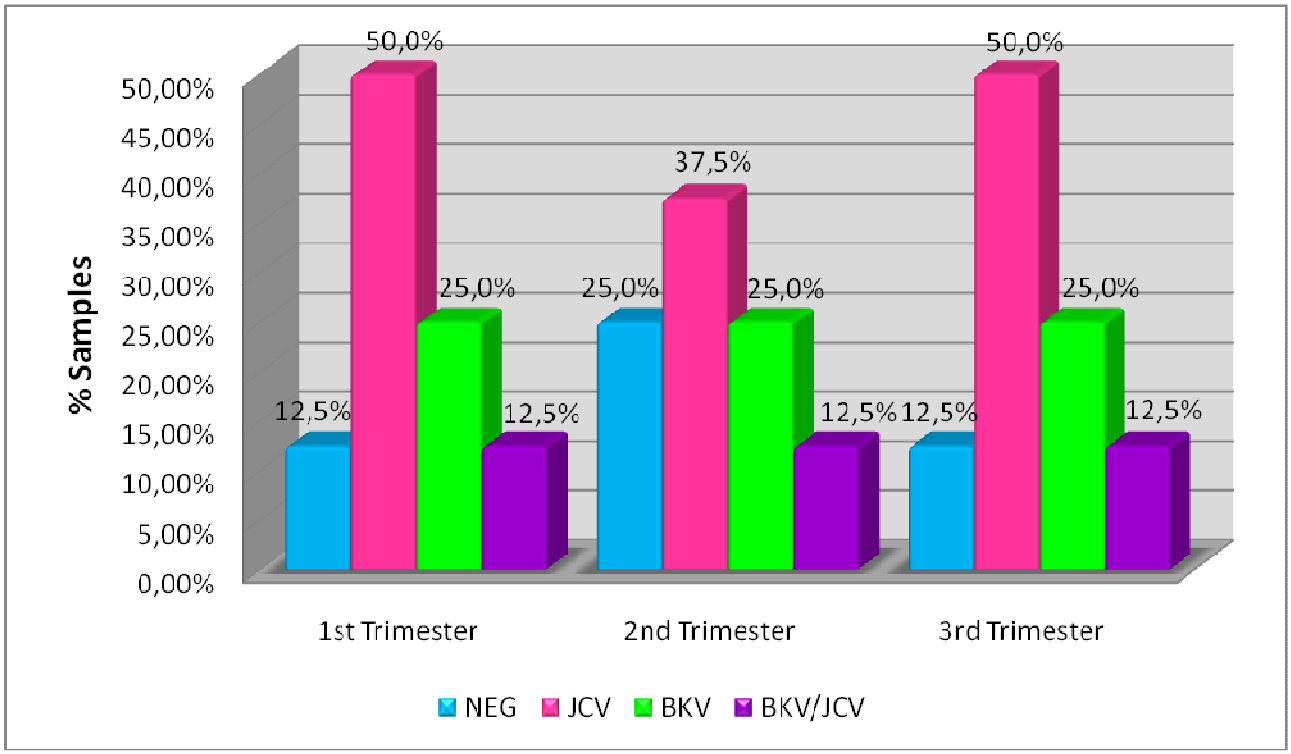

Figure 2: Frequency of excretion and type of PV identified in urine samples from the eight pregnant women that tested positive for PV genome by multiplex n-PCR in the 1st, 2nd and 3rd trimester of pregnancy.

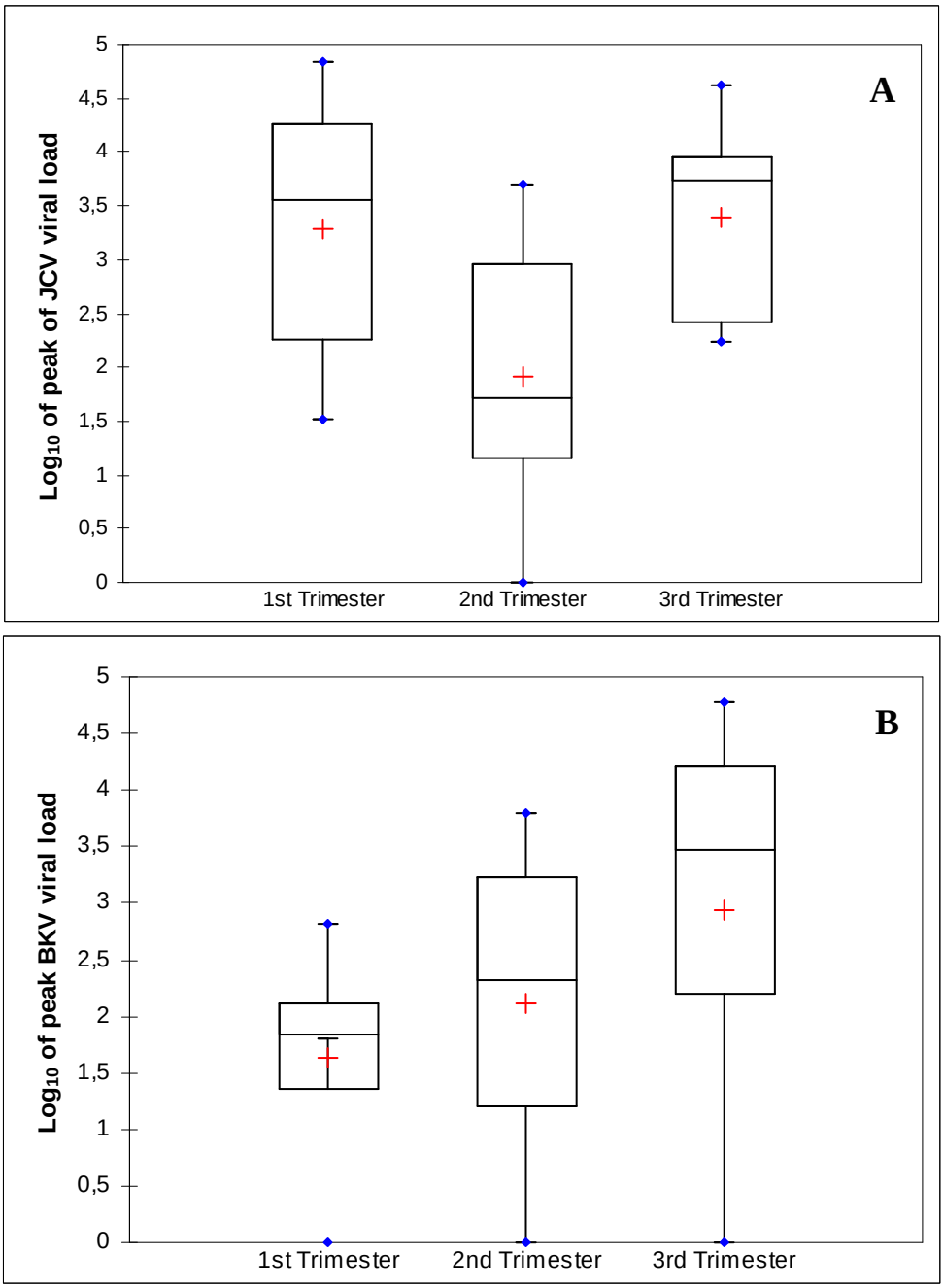

Figure 3: A) Box plots of log_{10} peak JCV viral load in pregnant women during the three gestational period. **B**) Box plots of log_{10} peak JCV viral load in pregnant women during the three gestational period. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.

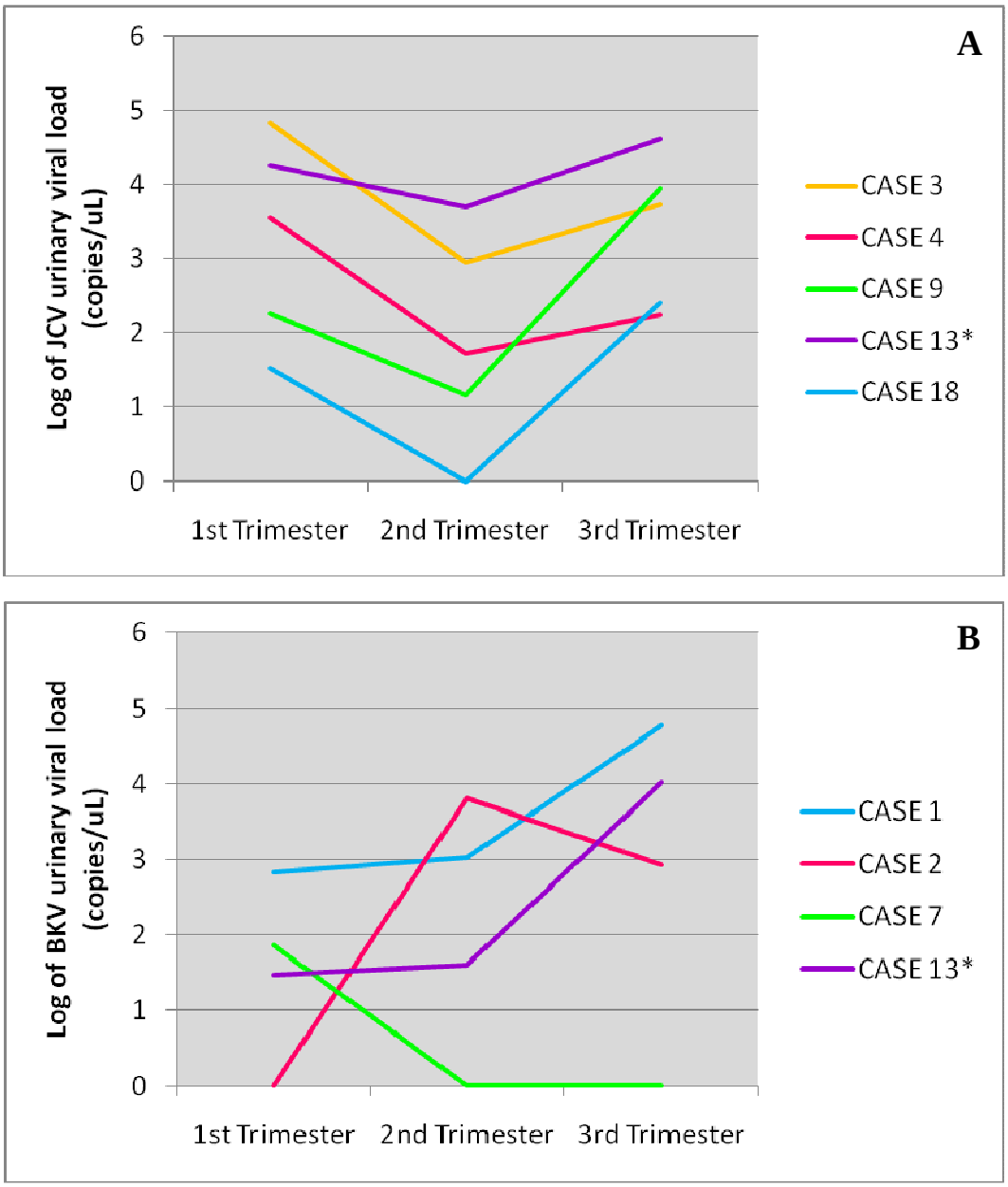

Figure 4: A) Pattern of JCV viral loads in pregnant women during the three trimesters of gestation. **B**) Pattern of BKV viral loads in pregnant women during the three trimesters of gestation. Data are shown as $\log_{10}$PVs copies/µL. No viruria is represented using a value of 1 PVs copy/µL of urine.
* Case co-infected by BKV and JCV.

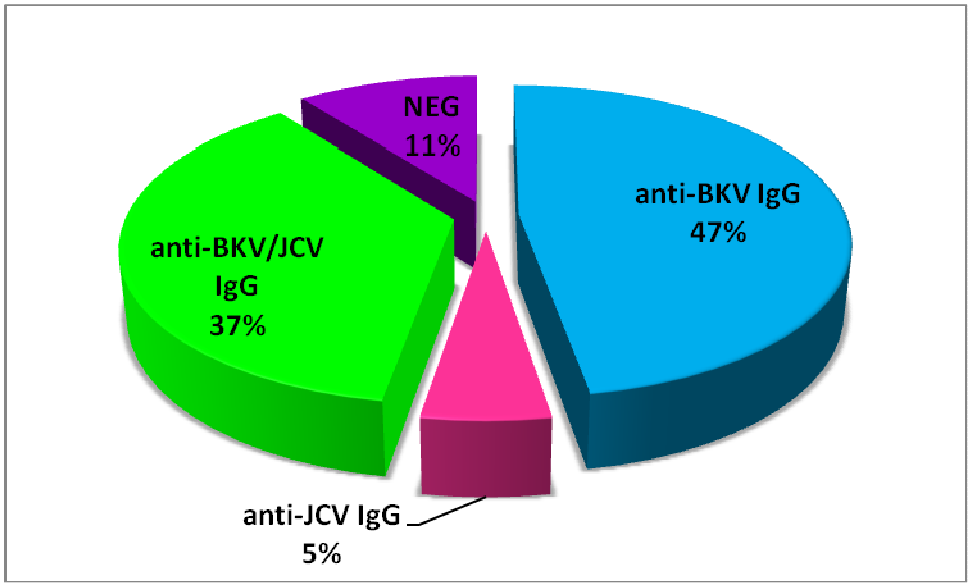

Figure 5: Prevalence of specific anti-BKV and/or anti-BKV IgG among the pregnant women.

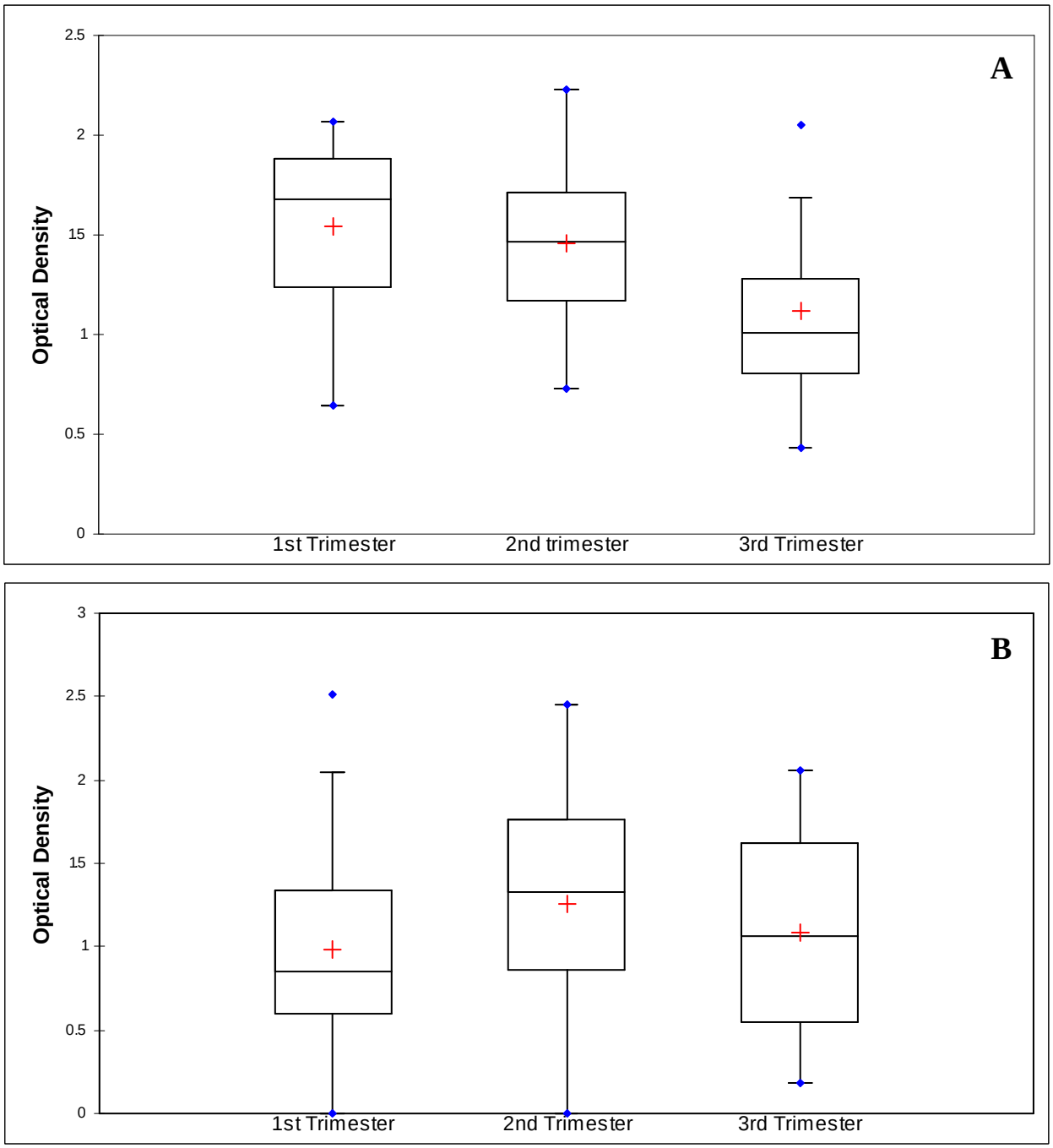

Figure 6: A) Box plots of OD anti-JCV IgG value for pregnant women during the three gestational period. **B**) Box plots of OD anti-BKV IgG value for pregnant women during the three gestational period. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.

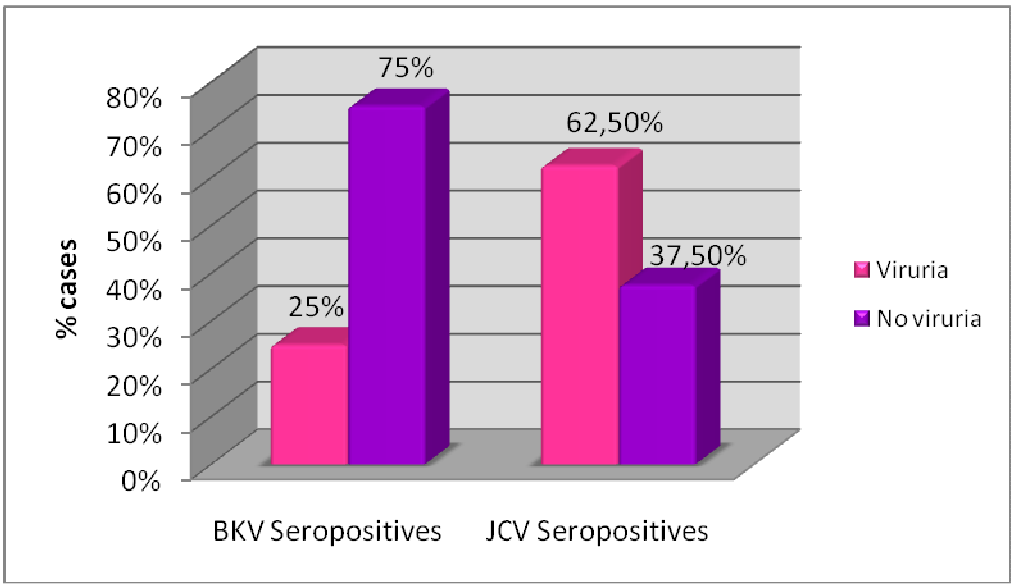

Figure 7: Frequency of BKV and JCV viruria in BKV and/or JCV seropositive women during pregnancy.

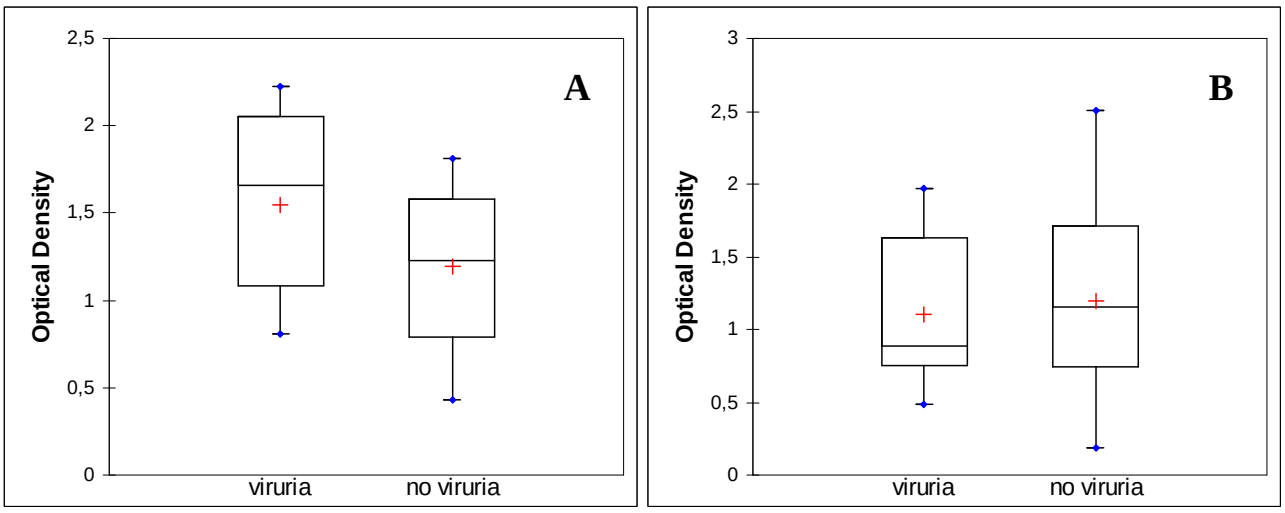

Figure 8: A) Box plots of OD anti-JCV IgG value for pregnant women with and without JCV viruria. **B**) Box plots of OD anti-BKV IgG value for pregnant women women with and without JCV viruria. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.

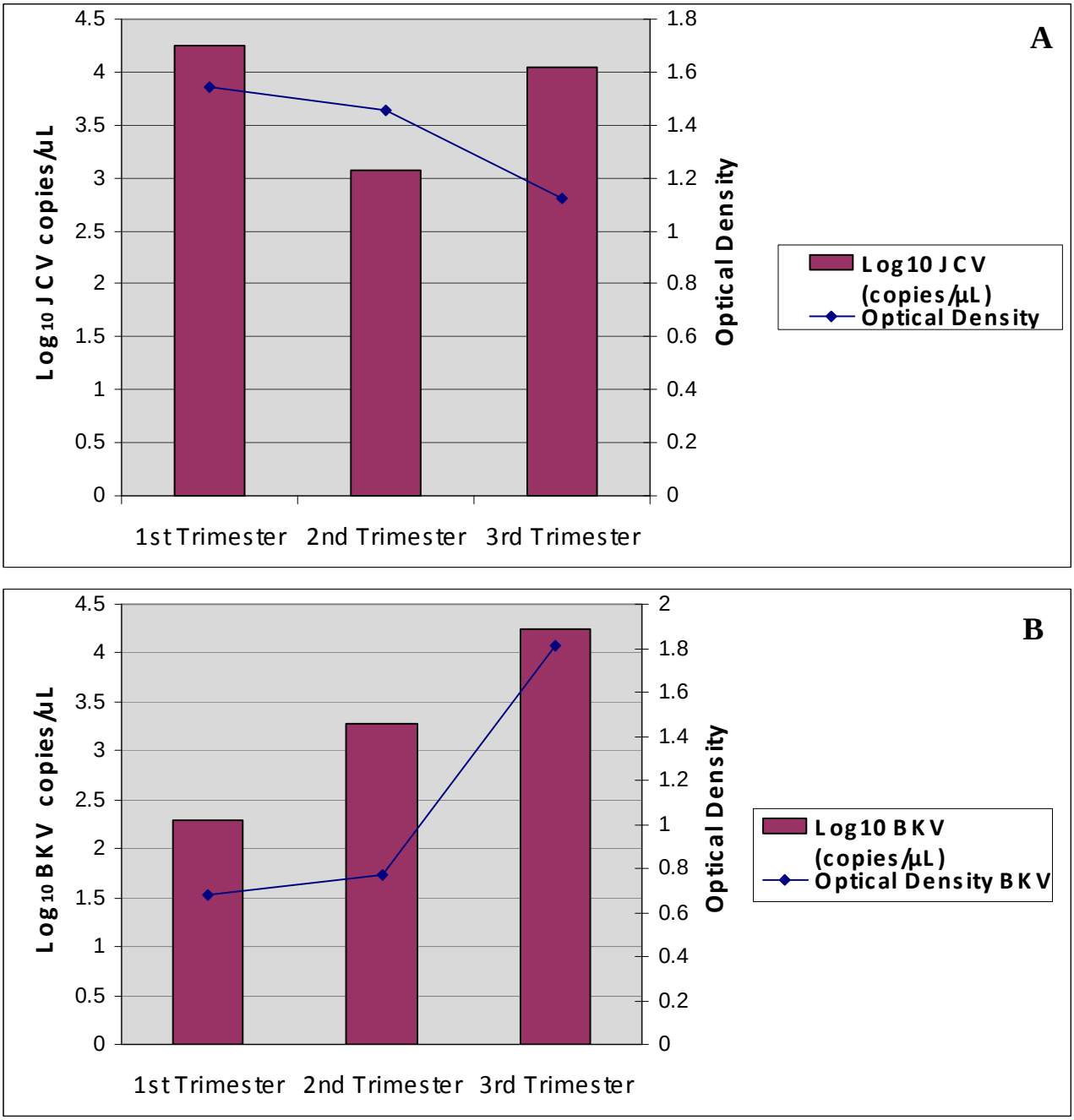

Figure 9: A) Mean JCV viral loads in urine and mean OD anti-JCV IgG values durinf pregnancy in JCV seropositive women. **B**) Mean BKV viral loads in urine and mean OD anti-BKV IgG values durinf pregnancy in BKV seropositive women.

DISCUSSION

Vertical transmission of DNA and RNA virus among humans and animals has been well documented. The modes of viral transmission include passage across the placenta over the pregnancy, during birth-related processes, soon after birth, or also in combined ways. The overall rates of such mode of transmission can be quite different among different virus species: i.e. the rates of vertical transmission of varicella zoster virus and human immunodeficiency virus range from 2 to 20% and from 13 to 30% respectively, in comparison with cytomegalovirus in which vertical transmission has been reported in only 0.2% of the cases[34]. The potential for these modes of transmission for members of the *Polyomaviridae* family is controversial.

In our study, we tried to clarify whether vertical transmission of human Polyomavirus JCV and/or BKV could be a mode of spread of one or both viruses, and, possibly, to indicate its frequency. Therefore, we performed, to the best of our knowledge, the first molecular and serological-based study in a population of pregnant women and their offspring, the last followed up to one month of life.

The main result of this study was that in our population no evidence of vertical transmission could be found. The persistence of BKV and JCV-specific IgG in 89% of maternal sera reflects what it was previously reported in sero-epidemiologic studies of the adult population[1] and by Shah et al.[26] that reported BKV-specific IgG prevalence in 91% of pregnant women.

Seven cases (35%) showed evidence of BKV (70%) and JCV (30%) reactivation, as demonstrated by the acquisition of IgG and/or IgA specific antibodies during pregnancy, and this reactivation persists until delivery in all the cases. This finding is similar to that reported by Gibson et al.[27], but quite different from that of Shah et al.[26], that observed BKV reactivation in 5% of their cases. In that study, however the sera were collected several months distance each one, and a transitory reactivation in between could not be detectable. The significance of this reactivation in pregnancy is controversial. In our cases, median OD levels (data not shown) for both JCV and BKV-specific IgA and IgM were low, near to the cut-off positivity level, therefore, systemic viral reactivation was quite unlikely. McCance and Mims[35], have found in mice infected immediately after birth with murine polyoma virus, that pregnancy reactivates local infection in kidneys but not in other organs or in the fetus. In contrast, primary viral infection acquired during pregnancy could be followed be transplacental passage of murine polyomavirus as reported by McCance and Mims[36] and Zhang et al.[29]. The first hypothesis could not be excluded, although in our study there were no evidence of urinary reactivation of PV, indeed no decoy cells were identified and viral load in urine does not correlate with presence of BKV and JCV specific IgA or IgM.

Finally, the rates of reactivation of BKV were higher than JCV in the present study in contrast with results obtained by Gibson et al.[27].

In our study we found no evidence of primary infection by JCV or BKV but this occurrence is only rarely reported in other studies concerning human PVs in clinical setting[24-26].

Umbilical cord blood samples, blood samples taken at one week and one month of life from the babies clearly indicated that BKV and JCV specific immunity are passively transmitted to the newborn. It was demonstrated by: a) absence of viral genome in all the samples from newborn and babies and b) decrease of median OD value of BKV and JCV specific antibodies from umbilical blood samples to blood samples taken at one month of life where they tend to disappear. These data confirm early studies by Shah et al.[26], Borgatti et al.[24], and Coleman et al.[25], that were performed in largest series of pregnant women.

Molecular analysis identified PV genome in urine samples from less than 50% of pregnant women, whereas blood samples from newborn and babies as well as urine and nasopharyngeal secretion were negatives.

These data confirm what was found in a previous paper[31] where molecular-based study performed on 300 pregnant women yield no evidence of viral genome detection in blood samples from mothers and umbilical cord, but with PV identification in urine.

The comparative analysis between viral load in urine and median OD values for JCV and BKV specific IgG seems evidence differences in viral excretion for JCV and BKV. The BKV viruria increased parallel with IgG antibodies during the pregnancy, whereas JCV viruria was almost stable but median OD level of IgG decreased. These findings, not previously studied in literature in clinical setting, could be related to different viral kinetic between JCV and BKV during pregnancy in analogy to what reported in other more severe immune deficiency conditions, such as renal transplanted or AIDS subjects.

In this paper, sequence analysis of transcriptional control region and viral capsidic protein of BKV and JCV was not carried out. Recently, Patel et al.[30], demonstrate that different strains of SV40 as produced by TCR rearrangements, had different ability to produce transplacental infection However, in a previous study we did not find a significant rearrangements in BKV and JCV isolated from urine samples of pregnant women.

The small number of cases included in our study could be a bias in drawing definite conclusions; however either molecular and serologic data seem denied that vertical transmission could be a frequent way of BKV and JCV spread in human population. Since we have not found primary infection by BKV or JCV we are not able to exclude that this occurrence during pregnancy could be a serious risk of congenital transmission for these viruses.

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CONCLUSIONI

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A conclusione dei nostri studi, condotti con metodiche molecolari e siero-immunologiche, si può affermare che l'infezione da BKV e JCV pur essendo estremamente diffusa nella popolazione generale, viene verosimilmente contratta durante l'infanzia o l'età adulta, mentre l'infezione congenita –sia essa transplacentare, peripartum o perinatale- non risulta dimostrabile e dunque non rappresenta una via prioritaria di diffusione del virus.

L'analisi di sequenza della regione genomica tardiva VP1 di BKV, da noi valutata nella sua totalità in soggetti portatori di trapianto di rene con e senza nefropatia BKV-associata non ha evidenziato mutazioni significativamente correlate con lo sviluppo della patologia renale. Tuttavia sono state identificate mutazioni che sembrano più specificamente presenti nei soggetti con nefropatia BKV-associata. Ciò potrebbe suggerire che l'analisi di sequenza possa risultare uno strumento utile nella identificazione di ceppi virali a maggiore patogenicità. Un recente lavoro (citato come voce bibliografica N°56) ha inoltre suggerito che mutazioni di regioni genomiche virali specifiche di SV40 possano associarsi a maggiore propensione per questo virus ad associarsi a trasmissione verticale in criceti. Ciò ci stimolerà ad ulteriori investigazioni per valutare il significato clinico di varianti virali che occorrono nella popolazione generale.

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ALTRE PUBBLICAZIONI

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.**

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

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

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

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

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

* IgA indicates immunoglobulin A.

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

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

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

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

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

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

MATERIALS AND METHODS

Patients

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

Samples

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

Urine Samples.—The urine samples were concentrated by

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

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

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

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

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

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

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

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

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

Molecular Biology

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

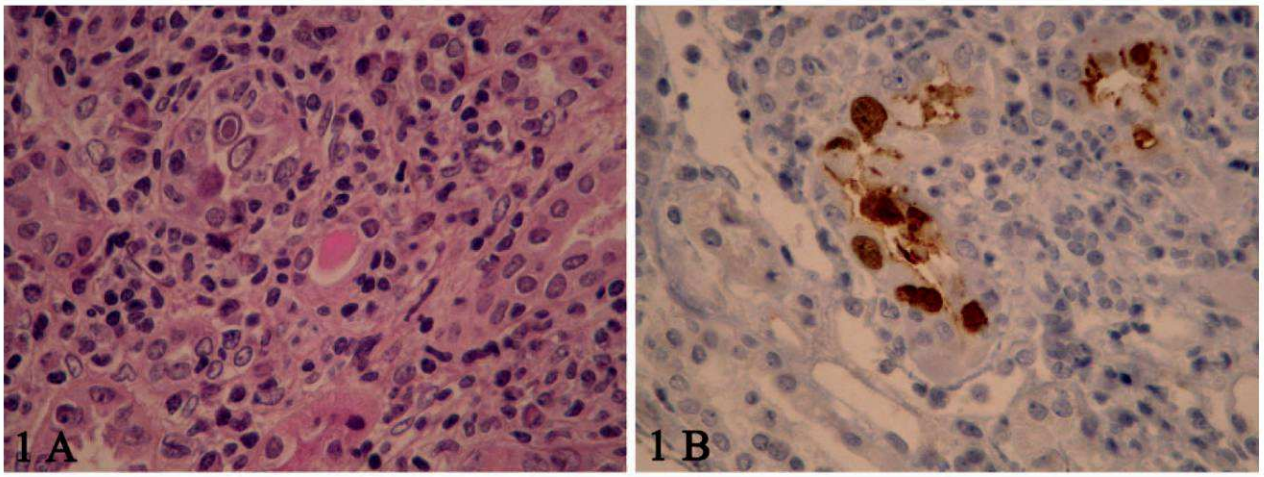
DNA was extracted from the serum samples using commercial columns (Nucleospin virus, Macherey-Nagel, Germany) with a silica matrix and high DNA-binding capacity.

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

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

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

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



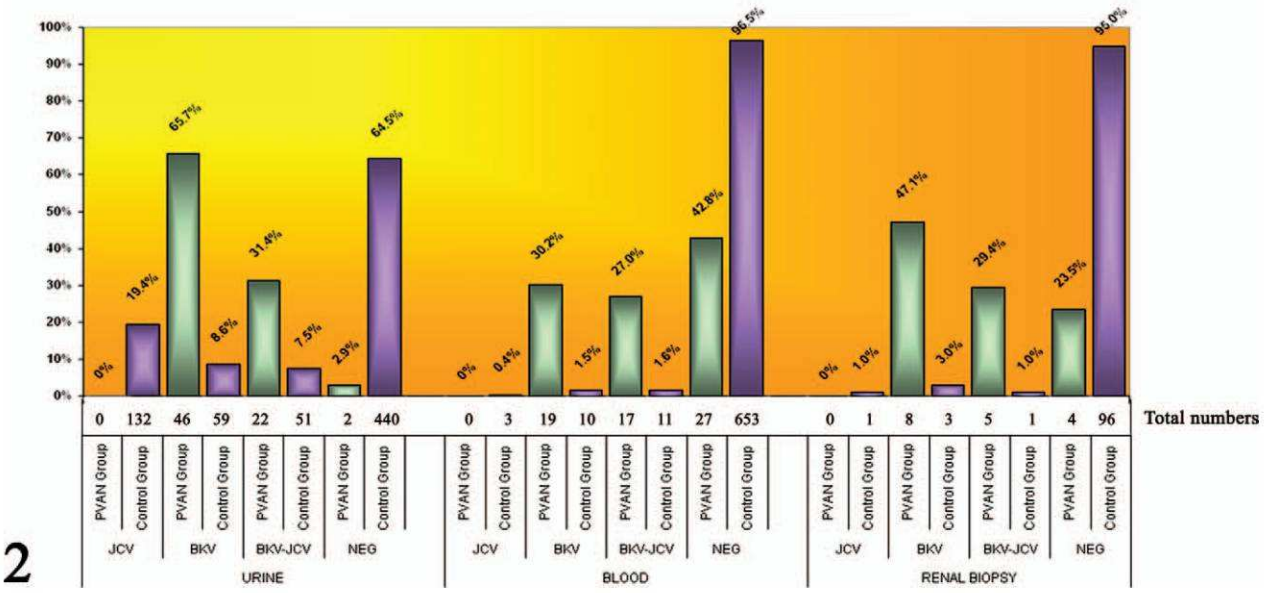


Figure 1. *Histologic and immunohistochemical findings of BK virus nephropathy. A, Type 2 viral inclusions within epithelial tubular cells and dense, mixed, interstitial inflammatory infiltration. B, Strong reactivity of viral inclusions–bearing tubular cells by using anti–simian virus 40 large T antibody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

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

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

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

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

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

Statistical Analysis

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

RESULTS

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

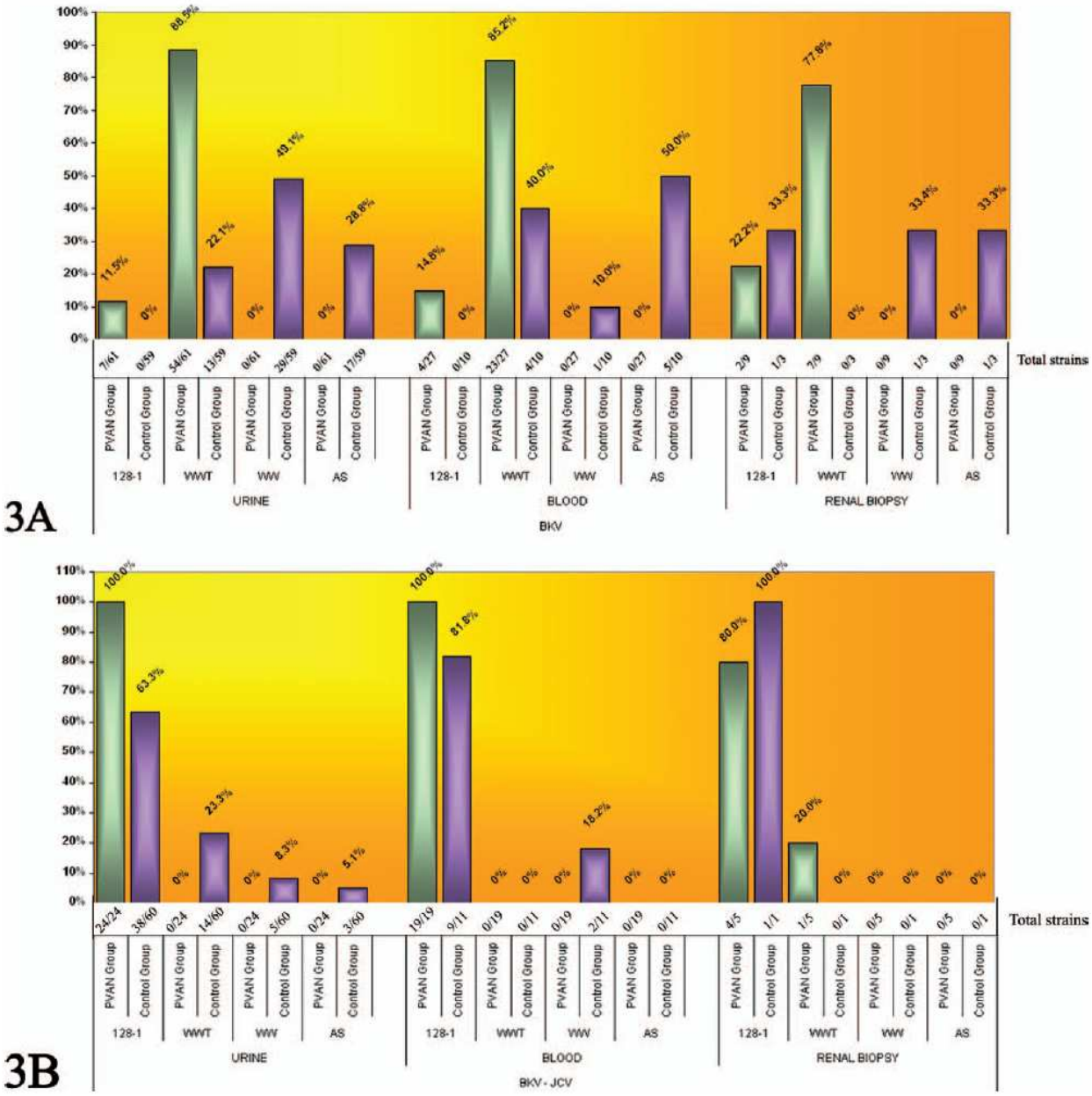


Figure 3. *Distribution of BK polyomavirus (BKV) strains in urine, blood, and renal biopsy samples showing A, BKV infection alone or B, BKV and JC virus (JCV) coinfection. PVAN indicates polyomavirus-associated nephropathy.*

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

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

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

The control patients showed a wider range of BKV

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

COMMENT

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

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

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

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

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

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

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

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

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

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

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

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

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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

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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 immunohistochemical 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 autocleaved 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 for40 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)

	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
A																							
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			
I (MM)																						GAA	
I (GS)																						GAC	
II (SB)	GAT					TAT					ACT				GCC		GAC					GAC	AAA
III (AS)	GAT					TAT		CAG	CAC						GCC		GAG					GAC	
IV (IV)	AAT	GAC				TAT			AGA		ACT			ACT	GCC		GAG					GAC	
IV (MG)	AAT	GAC				TAT			AGA		ACT			ACT	GCC		GAC					GAC	
	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,
B																							
	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)																						Asp	
II (SB)	Asp					Tyr					Thr				Ala		Asp					Asp	Lys
III (AS)	Asp					Tyr		Gln	His						Ala		Glu					Asp	
IV (IV)	Asn	Asp				Tyr			Arg		Thr			Thr	Ala		Glu					Asp	
IV (MG)	Asn	Asp				Tyr			Arg		Thr			Thr	Ala		Asp					Asp	

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

Genotype[a]	Subtype[a]	No. of patients: BKV nephropathy group	No. of patients: 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.

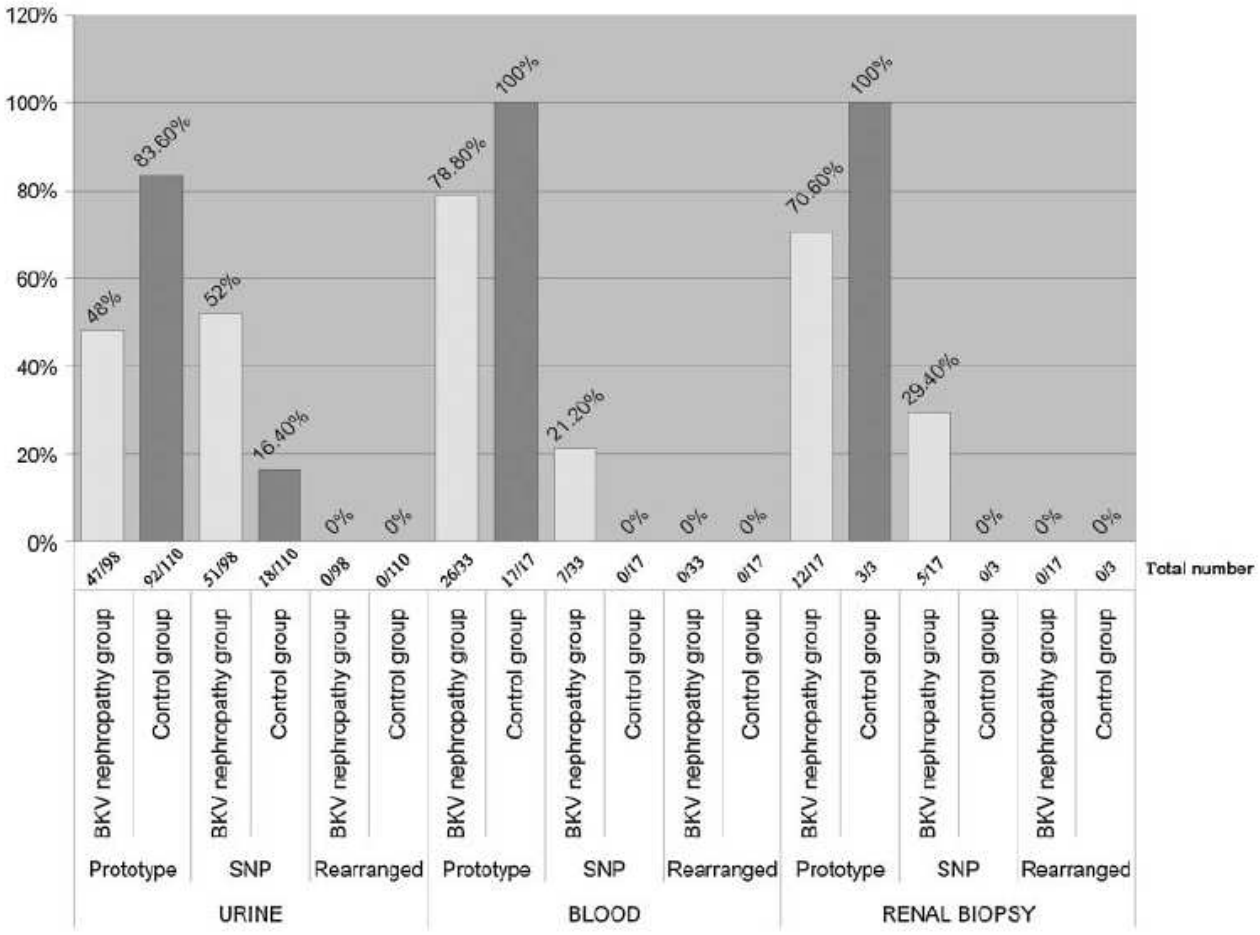

Fig. 1. Diagrammatic representation of BKV genomic mutations in Viral Protein 1 region detected in urine, blood, and renal biopsy samples from BK Virus nephropathy and control group. First line SNP, single nucleotide polymorphism.

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

A

		Amino acid positions														
Patient	Sample	Asn → Lys 62	Asp → Asn 62	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					3	1					1	
#5	Urine	6											9	1		4
	Blood	1											1			1
#6	Urine				4		3									
	Blood				1		1									
	Renal biopsy				1											
#8	Blood						1									

B

		Amino acid positions																
Patient	Sample	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		
#2	Urine										1							
#3	Urine						3											
#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

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

(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]. 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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TEACHING CASES

Primary vulvar Ewing's sarcoma/primitive neuroectodermal tumor in a post-menopausal woman: A case report

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ABSTRACT

Ewing's sarcomas/peripheral primitive neuroectodermal tumors (ES/pPNETs) are high-grade malignant neoplasms rarely found outside the skeletal system. Only 12 cases of vulvar ES/pPNET have so far been reported, all involving children or women of child-bearing age. We describe the case of a 52-year-old woman who was admitted to our hospital for the local excision of a 4 cm vulvar mass, originally thought to be a Bartholin's gland cyst. It was subsequently found to consist of small round cells positive for anti-CD99 antibody, thus suggesting a diagnosis of ES/pPNET. The demonstration of EWSR1 gene translocations by means of fluorescent *in situ* hybridization excluded small-cell carcinoma, squamous cell carcinoma of the small type, Merkel cell carcinoma, and lymphoblastic lymphoma. After surgery, the patient received six cycles of polychemotherapy and radiotherapy; she is still alive and well after 1 year of follow-up. Our findings underline the crucial role of molecular biology techniques in the differential diagnosis of small round cell tumors in these unusual locations.

Introduction

Ewing sarcomas/peripheral primitive neuroectodermal tumors (ES/pPNETs) are high-grade malignant neoplasms that typically arise within the skeletal system [1,2], although extra-skeletal sites [3,4] such as the chest wall, lower extremities, and the paravertebral region have been observed in about 15% of the cases [4]. Female genital tract involvement is rare, but ovarian, uterine cervix and corpus, and vaginal localizations have been reported in individual cases or small patient series [4–19].

A primary ES/pPNET of the vulva was first described by Scherr et al. [9], and only about 12 cases have been reported so far, all involving children or adult women of child-bearing age (Table 1). We describe and discuss the differential diagnosis of the first case of an ES/pPNET primarily arising in the vulva of a post-menopausal woman, whose diagnosis was confirmed by fluorescence *in situ* hybridization (FISH). As ES/pPNETs are histologically indistinguishable from other small cell tumors potentially affecting the vulva, the crucial role of diagnostic techniques, such as FISH and molecular biology, is underlined.

A 52-year-old woman, para 0, was admitted to the hospital of Borgomanero, Italy, with a vulvar mass. Her past medical history was unremarkable. A physical examination revealed a palpable, painless, and fixed nodule (about 4 cm in diameter) located in the lower third of the right labium major, and extending into the vestibule. Pelvic examination of the uterus and adnexae was negative. Given the suspicion of a Bartholin gland cyst, the patient underwent local excision of the mass with negative macroscopic margins. Her post-operative course was regular: computed tomography of the abdomen and pelvis, chest radiography, and a bone scan showed no evidence of metastases, and the results of all routine clinical laboratory tests remained within the normal range.

After surgery, the patient received six cycles of first-line polychemotherapy with vincristine, adriamycin, and ifosfamide, alternated with ifosfamide and etoposide, and then a total of 44 Gy radiotherapy to the pelvis and vulva, with a 16 Gy boost to the vulva. Six months later, a physical examination detected nothing of note, and chest radiograph and pelvic and abdominal contrast computed tomography showed the absence of any mass or adenopathy.

Twelve months after diagnosis, she is alive and well without any clinical signs of a local recurrence or metastasis.

Materials and methods

The tumor specimen was sampled, fixed in 10% neutral formalin, and embedded in paraffin, after which 4-μm thick

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Table 1
Clinical and pathological features of the primary EWS/pPNETs of the vulva reported in the literature.

Authors (Ref.)	Age (years)	Site	Size (cm)	Therapy	Molecular confirmation	Follow-up (months)
Scherr et al. [9]	10	Left labium major	6.5	Surgical excision	None	NA
Vang et al. [12]	28	Right labium minor	0.9	Surgical excision+CT	RT-PCR	AW, 18
McCluggage et al. [17]	40	Right labium minor	3	Surgical excision+CT	FISH	AW, 12
McCluggage et al. [17]	20	Right labium	6.5	NA	FISH	Died, pulmonary metastasis
McCluggage et al. [17]	19	Vulva, NOS	4	Surgical excision+CT	RT-PCR+FISH	NA
Takeshima et al. [13]	45	Right labium minor	3	Surgical excision	None	AW 36
Moodley and Jordaan [16]	26	Right labium major	5	Surgical excision+RT/CT	None	NA
Nirenberg et al. [10]	20	Right labium major	12	Surgical excision+ RT+CT	None	Died 18
Lazure et al. [14]	35	Vulva, NOS	20	Surgical excision+CT	RT-PCR	AW 7
Paredes et al. [11]	29	Left vulva	5	Surgical excision+RT/CT	None	AW 8
Habib et al. [8]	23	Vulva, NOS	Not reported	Surgical excision	None	NA
Fong et al. [18]	17	Vulva, left	Two fragments 0.7 and 2.1	Surgical excision+CT	RT-PCR	AW 12
Boldorini et al. (this study)	52	Right labium major	4	Surgical excision+CT/RT	FISH	AW 12

CT: chemotherapy; NA: not available; AW: alive and well; RT-PCR: reverse-transcription polymerase chain reaction; FISH: fluorescent *in situ* hybridization; RT: radiotherapy.

sections were stained in hematoxylin–eosin and periodic-acid Schiff (PAS) with and without diastase digestion.

Immunohistochemistry was performed using an automated staining machine (LabVision Corporation, Bio-Optica); the slides were incubated for 1 h with the following primary antibodies: CD99 (clone HO36-1.1, dilution 1:50, Cell Marque), vimentin (clone AB2, dilution 1:600, Neomarkers), CK20 (clone Ks20.8, dilution 1:200, Biocare), CK7 (clone OB-TL12/30, dilution 1:150, Biogenex), cytokeratin (clone AE1/AE3, dilution 1:200, Neomarkers), epithelial membrane antigen (Clone AB3, dilution 1:200, Dako), CD45 (clone LCA88, dilution 1:80, Biogenex), desmin (clone D33, dilution 1:50, Neomarkers), synaptophysin (clone SNP 88, dilution 1:80, Biogenex), and chromogranin (clone LK2H10+PHE5, dilution 1:150, Biocare). The subsequent reactions were performed using the Novolink Polymer Detecting System kit (Novocastra, Menarini) and incubation with universal secondary antibody, and the signal was visualized using 3,3′-diaminobenzidine hydrochloride.

FISH analysis was performed using the commercial Vysis® LSI® EWSR1 (22q12) Dual Color Break Apart Rearrangement Probe (Abbott Molecular), which consists of two FISH DNA probes: the first is labeled in SpectrumOrange, flanks the 5′ side of the EWSR1 gene, and extends inward into intron 4; the second is labeled in SpectrumGreen and flanks the 3′ side of the EWSR1 gene. The most common chromosomal translocation, t(11;22) (q24;q12), and variants t(21;22) (q22;q12), t(7;22) (p22;q12), and t(2;22) (q33;q12) are all identified by the same signal pattern, which consists of one signal fusion and two separate green and orange signals within the nuclei of neoplastic cells. The cells were detected using a fluorescent microscope (Axioplan 2 Imaging, Zeiss). About 80 neoplastic cells were selected, and the images were captured by means of dedicated software (Axiovision 4, Zeiss).

Results

Macroscopic examination showed a non-encapsulated brownish mass with a smooth surface and a maximum diameter of 4 cm; it was fleshy on the cut surface, and free of necrosis.

Microscopically, the tumor seemed to be located just beneath the squamous epithelium of the vulva, and consisted of relatively small round cells with ill-defined borders, scanty cytoplasm, and hyperchromatic nuclei with small nucleoli (Fig. 1). The mitotic count was 6 × 10 high power fields, and necrosis was frequent. No Homer–Wright rosettes were identified.

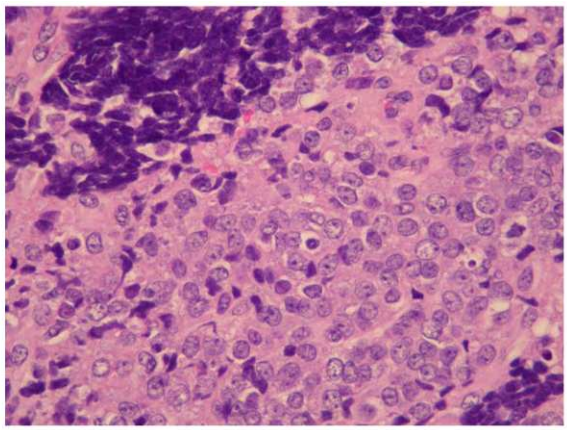

Fig. 1. The micrograph shows the classical characteristics of a small round cell tumor: perfectly round nuclei, with small and inconspicous nucleoli and scanty cytoplasm. Hematoxylin–eosin; original magnification 250 ×.

PAS staining showed cytoplasmic granules in a small percentage of the neoplastic cells, which were diffusely immunoreactive for CD99 (Fig. 2) and cytokeratin AE1/AE3, and focally for vimentin, epithelial membrane antigen, and neurofilaments; CK20, CK7, desmin, synaptophysin, and chromogranin were negative.

FISH analysis identified the typical pattern of chromosomal translocation involving the EWSR1 gene in about 70% of neoplastic cells (Fig. 3).

A diagnosis of primary vulvar ES/pPNET was made on the basis of the microscopic, immunohistochemical, and FISH data.

Discussion

Two main categories of PNETs are currently recognized on the basis of their cell origin and location: central PNETs arise from the neural tube and involve the brain and spinal cord, whereas peripheral PNETs arise from the neural crest and involve soft tissue and bone. ES, pPNETs, and small-cell tumors of the thoracopulmonary region (Askin tumors) are all members of the Ewing family of tumors (EFT), and characterized by similar phenotypical and molecular features: round small-cell morphology, immunohistochemical positivity to anti-CD99 antibody, and

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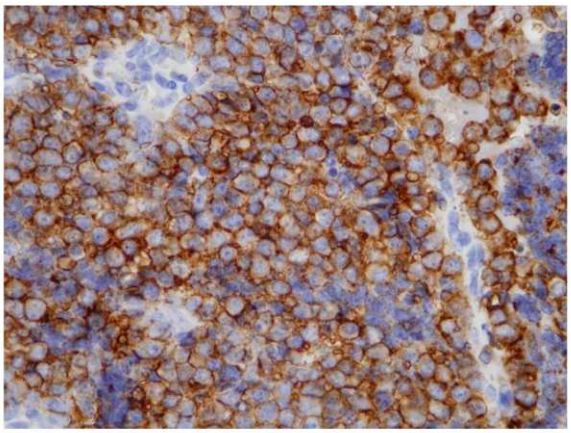

Fig. 2. The neoplastic cells showed strong and diffuse anti-CD99 antibody immunoreactivity along the cytoplasmic membranes. Anti-CD 99 antibody, slight hematoxylin counterstain; original magnification 250 × .

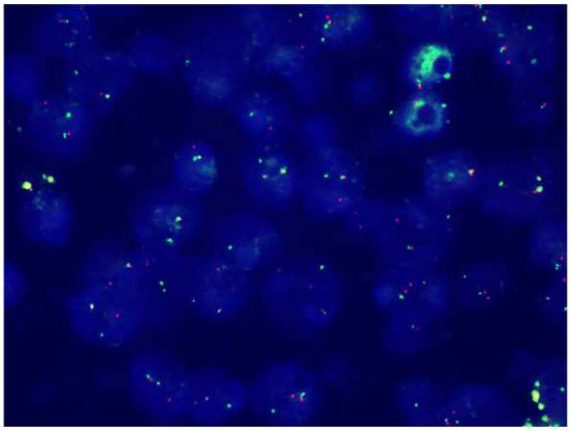

Fig. 3. Fluorescent *in situ* hybridization showing the typical pattern of a chromosomal translocation involving the EWSR1 gene: one signal fusion and two separate green and orange signals can be seen in most of the nuclei of the neoplastic cells. Dual Color Break Apart Rearrangement Probe, DAPI stain; original magnification 1000 × .

the expression of EWS/ETS gene fusion, which has been identified in virtually all cases.

Extra-skeletal ES/pPNETs have been reported in various sites, the most frequent being the chest wall, lower extremities, and the paravertebral region. However, they have only rarely been found in the female genital tract.

As shown in Table 1, the mean age at the time of the initial diagnosis of previous cases is 26 years (median 23, range 10–45), but our patient was older and post-menopausal. Therefore, excluding small round blue-cell tumors of children, such as rhabdomyosarcoma and neuroblastoma, the main differential diagnoses that were taken in account were primary or metastatic small-cell carcinoma, poorly differentiated squamous cell carcinoma of the small type, non-Hodgkin lymphoblastic lymphoma, malignant melanoma, and Merkel cell carcinoma, all of which share a similar morphology of round neoplastic cells with a high mitotic index. In addition, as a number of them (small-cell carcinomas and lymphomas) may stain positively for anti-CD99 antibody, a diagnosis of extra-skeletal ES/pPNETs has to be supported by the RT-PCR or FISH demonstration of the presence of the EWSR1 fusion transcript and/or gene translocations. These techniques can be regarded as "gold standards", which raises the question as to whether such tumors can be diagnosed only on the basis of their classic morphology and CD99 positivity.

Like McCluggage et al. [17], we believe that molecular confirmation is necessary when the tumor has unusual morphological features, is located in an unusual site, or (as in our case) develops in a patient falling outside the usual age group, but it is worth noting that only six of the published cases of vulvar ES/pPNET were molecularly confirmed (Table 1).

As only a few cases have been described, it is difficult to predict whether ES/pPNETs of the vulva behave differently from those arising in skeletal or more frequent extra-skeletal sites, and establish the best way of treating them. Table 1 shows that they have so far been treated by means of surgical excision alone (three cases), surgery associated with chemotherapy (five cases), or chemo- and radiotherapy (three cases); the treatment of one case was not described. Follow-up information has been provided in eight cases: two patients died because of the disease; the other six were alive and free of disease 7–36 months after diagnosis.

Our patient underwent surgery, and then received six cycles of first-line polychemotherapy followed by radiotherapy; 12 months after diagnosis, she is alive and well, and shows no signs of a local recurrence or metastasis. These albeit limited data suggest a possibly more favorable prognosis than that reported for more common ES/pPNETs, probably because of the superficial location, early detection, and complete removal of the neoplasm.

In conclusion, our case of an ES/pPNET primarily arising in the vulva of a post-menopausal woman was successfully treated by means of local excision, chemotherapy, and radiotherapy. FISH-based differential diagnosis excluded more frequent age-related tumors of the vulva, such as primary or metastatic small-cell carcinomas, poorly differentiated squamous cell carcinomas of the small type, and Merkel cell carcinomas. We suggest that molecular biology techniques are useful in the differential diagnosis of small round-cell tumors, especially unusual locations.

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