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# A case of a progressive multifocal leukoencephalopathy patient with four different JC virus transcriptional control region rearrangements in cerebrospinal fluid, blood, serum, and urine

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**JC virus (JCV) is the etiological agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS). During the acquired immunodeficiency syndrome (AIDS) epidemic, it was the cause of the death in up to 8% of AIDS patients. The genomic organization of JCV and, in particular, the hypervariability of the transcriptional control region (TCR), a regulatory noncoding region, are well known. Given that the TCR plays a central role in the viral replication of JCV, a crucial role in the determination of the neurotropism and in the pathogenic capabilities of the virus is also suspected. Here the authors describe a case of PML that did not respond to highly active antiretroviral therapy (HAART) therapy. There was a simultaneous presence of JCV strains with four different TCR structures in urine, peripheral blood cells, serum, and cerebrospinal fluid (CSF) samples. These data confirmed that the presence of the archetype TCR is restricted to urine, while also suggesting that the degree of the rearrangement varies and increases from the peripheral blood to CSF.** *Journal of NeuroVirology* (2005) **11,** 51–57.

**Keywords:** cerebrospinal fluid (CSF); JC virus; progressive multifocal leukoencephalopathy (PML); transcriptional control region (TCR) rearrangements

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease caused by the human polyomavirus JC virus (JCV) (Berger and Major, 1999). It asymptomatically infects about 80% of the worldwide population, remains latent in the kidney, and reactivation is possible in severely immunocompromised individuals, for example, during human immunodeficiency virus (HIV) infection (Brooks and Walker, 1984).

JCV has a double-stranded, circular genome that can be divided into two regions, one coding for the early proteins (large and small T antigens) and one coding for the late, structural proteins (VP1, VP2, VP3, and AGNO). These two regions are separated by a noncoding region, the transcriptional control region (TCR), containing the origin and several promoters of the viral replication (Frisque *et al*, 1984). It is characterized by hypervariability, due to duplications and to deletions of nucleotide sequences, with the latter being organized in a system of six blocks, A to F, based on the archetype regulatory sequence, which has been isolated from urine of the healthy population (Yogo *et al*, 1990; Ault and Stoner, 1993).

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Moreover, rearranged forms have been detected in the brain or cerebrospinal fluid (CSF) of PML patients. These forms are characterized by a lack or duplication of the sequence blocks (Agostini *et al*, 1997), which could determine viral tissue tropism (Rentier-Delrue *et al*, 1981; Tada *et al*, 1989).

## Case report

This case report concerns a 30-year-old man, an intravenous drug user (IVDU), who had been HIV positive since 1989, with CD4+ counts <100/$\mu$l at the moment of HIV detection, and also hepatitis C virus (HCV) positive. The patient was first treated with zidovudine (AZT) and didanosine (DDI). However, he did not comply with the therapy and there was no increase in the CD4+ counts or decrease in HIV RNA copies in plasma. A highly active antiretroviral therapy (HAART) was started in March 2001, with administration of zidovudine (AZT), lamivudine (3TC), didanosine (DDI), and lopinavir/ritonavir (LPV/r). Despite the new treatment, CD4+ cell counts remained low (14/$\mu$l) and plasmatic HIV RNA was high (103523 copies/ml). The patient developed wasting syndrome, oropharyngeal candidiasis, reflux esophagitis, and lipodystrophy. He exhibited neurological symptoms, including difficulty in maintaining the standing position, in walking and talking, but there were no cognitive alterations. The magnetic resonance imaging (MRI) examination performed in T1- and in T2-weighted images showed cerebellar lesions (Figure 1). The patient died 1 year after the onset of the neurological symptoms.

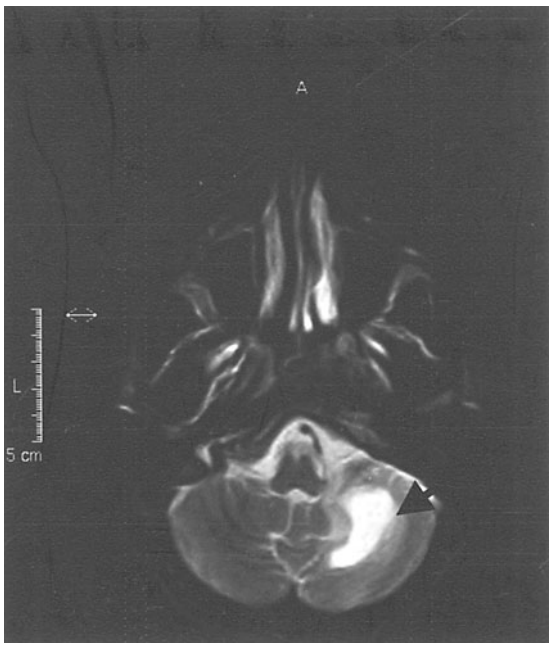



**Figure 1** Diffuse and focal white matter hyperintensity on T2-weighted MRI involving left middle cerebellar peduncle and cerebellar lobe, pons, and mesencephalon on the right side.

PML diagnosis was confirmed by the detection of JCV DNA in the CSF, using both the classic polymerase chain reaction (PCR) and real-time PCR, which were conducted by amplifying the highly conserved viral region belonging to the LT-antigen gene. In addition, the late gene coding for the major capsid protein (VP1) and the noncoding transcriptional control region were amplified from CSF and also from peripheral blood (PB), serum, and urine, and analyzed by automatic sequencing.

## Results

JCV DNA belonging to the highly conserved LT-antigen viral region was found in all the body fluid samples collected from the patient (CSF, peripheral blood, serum, and urine), and in particular, we detected 8048200 copies of viral DNA in the CSF, 4307 copies/$\mu$l in the serum, and 1272 copies/$\mu$l in the peripheral blood. The genotyping of the JCV strain, based on the point mutation analysis of VP1, showed the unique presence of type 2b in the CSF and urine. Type 2b is a subtype that is already known to be overrepresented in Italian PML patients (Ferrante *et al*, 2001).

The archetype structure of the TCR was found in the urine, whereas three different TCR rearrangements were found in the PB, serum, and CSF, with each amplified product having different molecular weights and thus readily detectable by gel electrophoresis analysis (Figure 2). The TCR amplified from the PB showed an almost complete deletion of 56-bp belonging to block D, which is not frequently retained in the viral genome isolated from PML brains (Ault and Stoner, 1993). It also had a short 10-bp insertion, belonging to block F and carrying the DNA sequence of the AP1, which can strongly contribute to enhancement of the expression of the JCV LT-antigen (Sadowska *et al*, 2003), and a complete duplication of the 23-bp block B and consequently of the Sp1 binding sites. Following right after it, there was a partial duplication of the 55-bp block C, with duplication of the TAR binding site and that of cre. The deletion of block D was also maintained in the TCR form

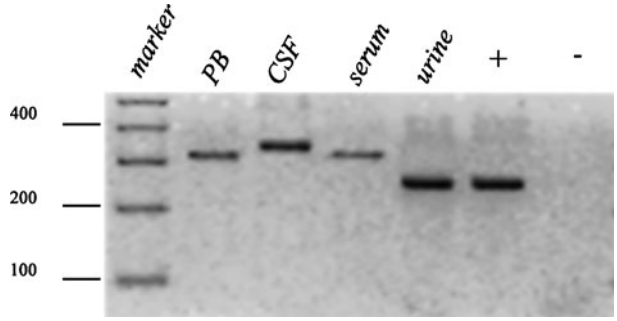



**Figure 2** Agarose gel analysis of the TCR fragments amplified by PCR from PB, CSF, serum, and urine. PCR controls are identified by + (DNA extract from HJC2 cells) and − (no template).

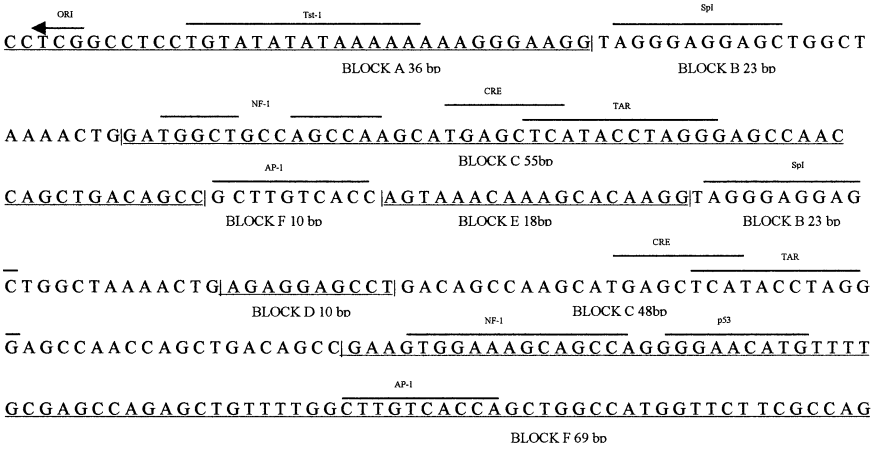
amplified from the serum, as was the duplication of block B (containing Sp1 binding site) and an insertion of 10-bp belonging to block F and carrying a fragment of the NF-1 binding site, whereas the 55-bp block C (containing cre, NF-1, and TAR binding sites) was completely duplicated. Finally, we could sequence the rearranged form amplified in the CSF and it could be defined as a long duplicate because it contains a duplication of block C, a deletion of block D, and a complete duplication of block E (Yogo and Sugimoto, 2001). The two identical 96-bp duplications were divided by the 10-bp insertion derived from block F, which was also present in the TCR amplified from the serum. As frequently observed by others (Ault and Stoner, 1993), the junction of blocks C and D and the junction of blocks D and E are the most frequent targets of strand breakage, resulting in the deletion of block D (Figures 3 and 4).

## Discussion

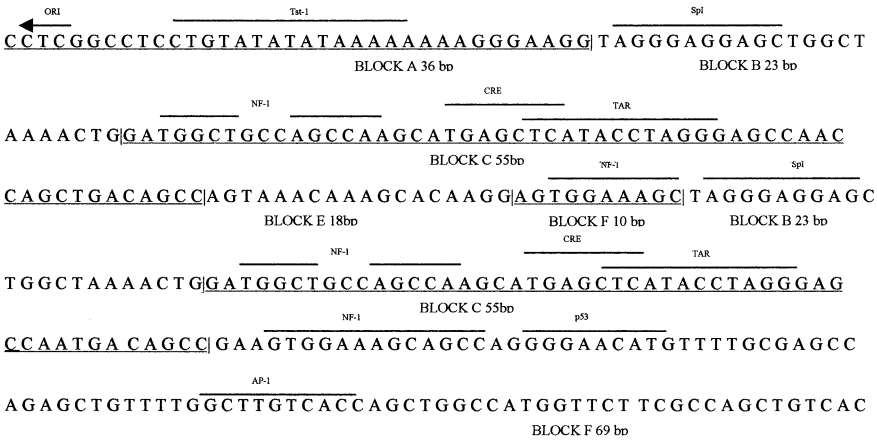
The archetypal TCR, isolated from the urine of patients, contains a single copy of the promoter-enhancer and is frequently detected also in the urine and kidneys of immunocompetent as well as immunosuppressed individuals. Our data represent the first observation of four different TCR rearrangements, defined by direct sequencing, in different body fluid samples collected from one PML patient. PML cases presenting JCV strains in CNS samples and the kidney with different genomes in


**Figure 3** Structure of the transcriptional control region (TCR) of JCV DNA amplified from urine, PB, serum, and CSF of an HIV+, PML+ patient, compared with the archetype form (CY) and with a PML form (Mad-1). The promoter elements are indicated on the sequences form. *(Continued)*

**Peripheral Blood**




**Serum**




**CSF**


**Figure 3** (Continued)

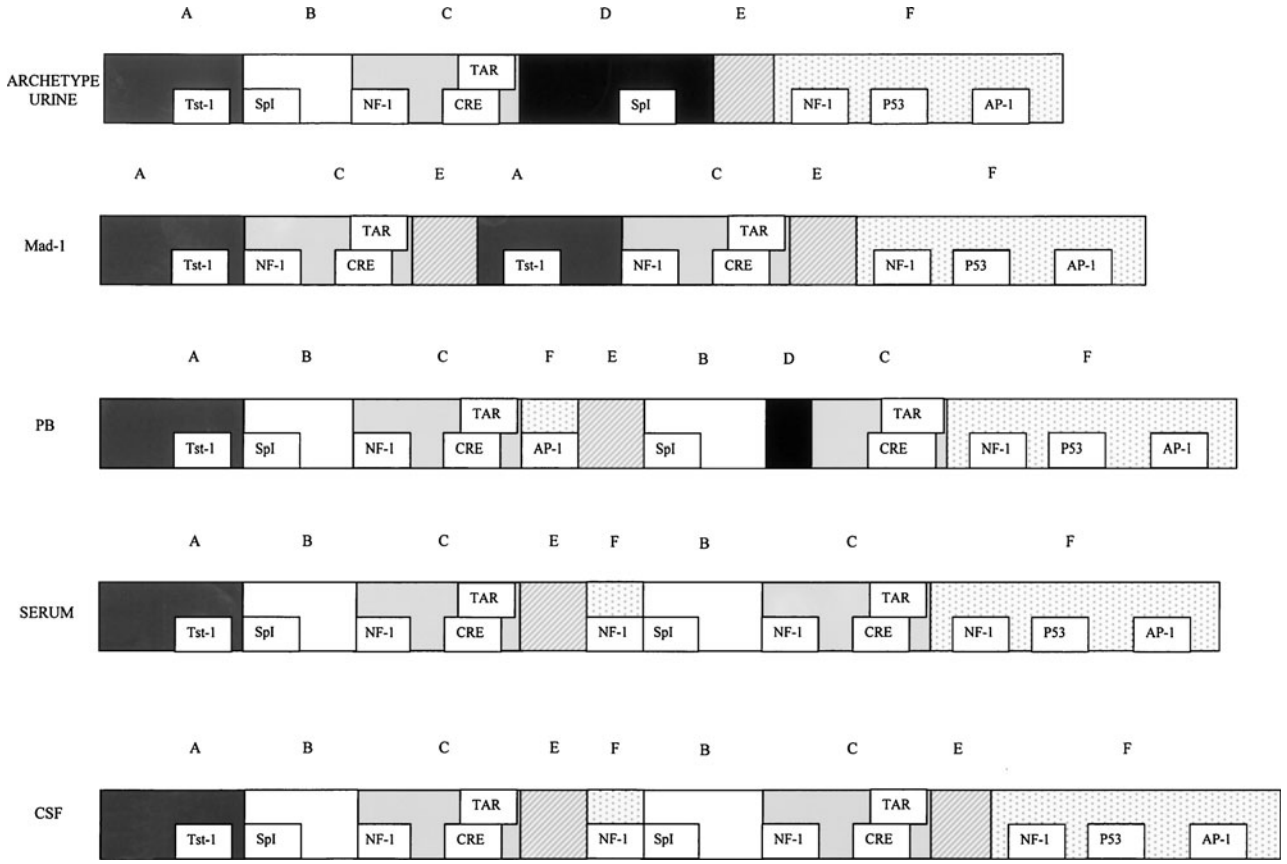



**Figure 4** Schematic structure of the archetypal JCV sequence, Mad-1, and the four different amplified TCR rearrangements, showing blocks A to F and the main promoter binding sites.

the promoter region have already been described (Ault *et al*, 1994; Newmann and Frisque, 1997; Elsner and Dorries, 1998), but the simultaneous presence of different TCR variants has been reported only after cloning of the PCR products (Pfister *et al*, 2001). Showing an evolutionary gradient from the archetype to rearranged TCR, from the urine to the CNS, through the lymphocytes and serum, these results support the previously suggested hypothesis (Yogo *et al*, 1990; Ault and Stoner, 1993; Iida *et al*, 1993), according to which TCR rearrangements found in the serum, PB, and CSF are generated in the host from the kidney-derived archetypal form by deletions and duplications (Flaegstad *et al*, 1991). In contrast with a recent study (Fedele *et al*, 2003), we are proposing that the rearrangements might arise at the moment of the passage from the kidney into the lymphocytes and that subsequent casual rearrangements might also generate new variants with altered tissue tropism and pathogenic capabilities, which could allow JCV to penetrate central nervous system (CNS) cells. This hypothesis is supported by *in vitro* studies that have demonstrated that the JCV strain with rearranged TCR replicates in cell cultures more efficiently than the strain bearing an archetype organization (Daniel *et al*, 1996).

It is generally believed that the presence of viral DNA in serum is due to the release of viral particles from infected cells and thus can be seen as a marker of viral replication (Soldan *et al*, 1997). The presence in our case of two different TCR rearrangements in the PB and in the serum could suggest that though already rearranged, the TCR in PB cells is part of a non-replicating virus, whereas the differently rearranged TCR form in the serum belongs to viral particles generated after active replication.

The lack of the D block carrying an SpI binding site in the rearranged forms found in the samples collected is replaced by the duplication of block B, which also carries an SpI site. The positive influence of SpI on the replication of the viral genes has already been demonstrated, for it is able to maintain CpG islands in active genes in a methylation-free state (Macleod *et al*, 1994; Graff *et al*, 1997). In fact, the long duplicate form found in the CSF of the patient described here carries four consensus NF-1 binding sites, which are well known to bring about highly increased transcriptional activity of the promoter (Amemiya *et al*, 1992; Tamura *et al*, 1998), and resulting in the most pathogenic rearrangement (Figure 4). In addition, it has been demonstrated that NF-1 binding sites are involved in the ability of JCV to

replicate with high efficiency in glial cells (Amemiya *et al*, 1992). The complete duplication of block C also leads to the duplication of the upTAR element, which is important for HIV-1 Tat enhancement of the JCV late promoter, in synergy with Pur$\alpha$, especially in human glial cells (Krachmarov *et al*, 1996). A correlation between the presence of tandem repeats in the CSF and the poor clinical course of the patient can also be suggested here, as rearranged forms are often found in CNS samples collected from patients with fast progressing PML (Pfister *et al*, 2001; Ferrante *et al*, 2003).

On the whole, the data obtained from the study of this PML case lead us to propose that the nonpathogenic archetype form infecting the kidney generated the highly pathogenic CSF variant, through evolution occurring in lymphocytes and serum. As suggested previously, this variant may have enhanced replication, infected the oligodendrocytes, and caused PML (Pietropaolo *et al*, 2003).

## Methods

DNA was extracted from the body fluid samples collected from the patient. Commercial kits were used, according to the manufacturer's instructions, as previously described (Pagani *et al*, 2003). To avoid cross-contamination of the samples, extractions from each sample were performed one at a time, in a clean, dust-free area using sterile and disposable materials.

Amplifications were performed using various sets of primers, specific for different genomic regions of JCV: JC1 and JC2 (outer), PEP1 and PEP 2 (inner), which amplify the highly conserved LT-coding region (Ferrante *et al*, 1995); JRE1 and LP2 (outer), RFOR and RREV (inner), which amplify a 353-bp fragment belonging to the JCV TCR; and JLP15 and JLP16, which amplify a 215-bp fragment of the VP1 gene (Pagani *et al*, 2003).

To perform nucleotide sequence analysis of the VP1 and TCR fragments, PCR products were added to a mixture containing 4 $\mu$l of Ready Reaction Premix 2.5×, 2 $\mu$l of BigDye Sequencing Applied Biosystems 5× buffer, 3.2 pmol of either forward or reverse primer, and water up to a final volume of 20 $\mu$l. The cycle sequencing was performed using the GeneAmp PCR System 9700, according to the following protocol: initial denaturation at 96°C for 1 min, then 25 cycles with a first step at 96°C for 10 s, a second step at 50°C for 5 s, and the last rapid thermal ramp to 60°C for 4 min. Finally, 5 $\mu$l of the purified product underwent electrophoresis on an ABI PRISM 310 Genetic Analyzer. Sequence homology searches were performed using BLAST at NCBI (USA), according to Agostini *et al*. (1997) for JCV genotyping and Ault and Stoner (1994) for JCV TCR rearrangements.

The viral load of JCV DNA was determined using the TaqMan Q-PCR strategy. Amplification and detection were performed using an ABI PRISM 7000 Sequence Detection System. The real-time PCR assay used forward (5′-GAG TGT TGG GAT CCT GTG TTT TC-3′) and reverse (5′-GAG AAG TGG GAT GAA GAC CTG TTT-3′) primers and the fluorescent TaqMan probe (5′-6-FAM-TCA TCA CTG GCA AAC ATT TCT TCA TGG C-TAMRA-3′) (PE Applied Biosystems, Cheshire, United Kingdom) to amplify and detect a 54-bp amplicon in the JCV large T antigen region.

A standard curve of the cycle threshold (Ct) values was generated using serial 10-fold dilutions of an external JCV standard. Each standard, each sample, and one negative control were analyzed in triplicate; 10 $\mu$l of the sample were added to 40 $\mu$l of the reaction mixture consisting of 25 $\mu$l of Universal Mastermix (PE Applied Biosystems) and of primers and probe, at a concentration of 900 nM and 200 nM, respectively. The cycling parameters were: 50°C for 2 min, 95°C for 10 s, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

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