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Characterization of JC virus in cerebrospinal fluid from HIV-1 infected patients with progressive multifocal leukoencephalopathy: insights into viral pathogenesis and disease prognosis

Angela Marzocchetti,[1,*] Maurizio Sanguinetti,[2] Simona Di Giambenedetto,[1] Antonella Cingolani,[1] Giovanni Fadda,[2] Roberto Cauda,[1] and Andrea De Luca[1]

[1]*Institute of Clinical Infectious Diseases, Catholic University of Sacred Heart, Rome, Italy*
[2]*Institute of Microbiology, Catholic University of Sacred Heart, Rome, Italy*

***Objectives*. To analyze virological and immunological features of AIDS-related progressive multifocal leukoencepalophathy (PML) and their association to disease prognosis. *Methods*. In HIV-infected patients with virologically confirmed PML, JC virus (JCV) DNA load and levels of Macrophage Chemoattractant Protein (MCP)-1 were determined in cerebrospinal fluid. JCV genotypes, rearrangements and JCV DNA binding sites for cellular transcription factors were analyzed by sequencing the viral VP1 region and regulatory region (RR). *Results*. 45 patients were analyzed: 60% were exposed to highly active antiretroviral therapy (HAART) after PML and 24% before the disease onset. JCV DNA load in cerebrospinal fluid was a strong predictor of patients survival. Lower levels of JCV DNA in cerebrospinal fluid were associated with the following virologic factors: viral genotype 4 ($p = 0.043$), more rearrangements in the RR ($p = 0.046$), duplication of RR block B ($p = 0.028$), and duplication of binding sites for cellular transcription factor NF-1 ($p = 0.060$). In patients with prior antiretroviral exposure there was a trend towards a higher number of binding sites for cellular transcription factors ($p = 0.068$). Lower JCV load was also predicted by exposure to HAART ($p = 0.010$), higher baseline CD4 counts ($p = 0.009$) and higher cerebrospinal fluid MCP-1 levels ($p = 0.036$). In a multiple regression model, MCP-1 levels were independently associated with JCV load. *Conclusion*. HAART leads to a partial immune-mediated control of JCV replication; the virus may tend to escape through the selection of rearrangements in the RR, some associated with enhanced viral replication efficiency, other resulting in multiplication of binding sites for cellular transcription factors.** *Journal of NeuroVirology* (2007) **13,** 338–346.

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Introduction

JC virus (JCV) is an ubiquitous human poliomavirus that causes primary asymptomatic infection during late childhood: up to 80% of the human population is seropositive for JCV worldwide. It establishes a latent infection in the kidneys and probably in other body sites. In immunocompromised patients, such as those with AIDS, cancer or recipients of immunosuppressive therapies, it occasionally reactivates from the site of latency, causing lytic infection of oligodendrocytes and consequently demyelination

*Present address: Division of Viral Pathogenesis, Beth Israel Deaconess Center, Harvard Medical School.

Address correspondence to Angela Marzocchetti, Division of Viral Pathogenesis, Beth Israel Deaconess Center, Harvard Medical School, RE-217, 46 Louis Pasteur Avenue, Boston MA 02215. E-mail: amarzocc@bidmc.harvard.edu

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leading to progressive multifocal leukoencephalopathy (PML) (Brooks and Walker, 1984; Berger and Koralnik, 2005). None of the treatment approaches attempted for PML, including cytarabine, alfa-interferon or cidofovir, has been convincingly shown to confer any benefit on patients survival (Gasnault *et al*, 2001; Marra *et al*, 2002; Geschwind *et al*, 2001). On the other hand, cohort studies have shown that Highly Active Antiretroviral Therapy (HAART) confers an improved survival in about 50% of HIV-infected patients (De Luca *et al*, 2000; Clifford *et al*, 1999; Tassie *et al*, 1999). During antiretroviral treatment, the transactivation of JCV by the HIV tat protein may be reduced and JCV-specific immune responses are improved (Berger *et al*, 2001). Despite this, even with HAART, PML often remains a fatal disease and causes severe chronic disability in survivors (De Luca *et al*, 2001; De Luca *et al*, 1998; Gasnault *et al*, 1999). At disease onset, factors associated with a better survival are higher CD4 counts (Berenguer *et al*, 2003), lower JCV DNA load in the cerebrospinal fluid (Taoufick *et al*, 1998; De Luca *et al*, 1999), lower HIV RNA plasma viral load (Clifford *et al*, 1999), prior exposure to HAART (Gasnault *et al*, 1999), elevated myoinositol in PML lesions on magnetic resonance imaging (Katz-Brull *et al*, 2004) and higher levels of macrophage chemoattractant protein (MCP)-1 in the cerebrospinal fluid (Marzocchetti *et al*, 2005).

The JCV VP1 is the major capside protein and is involved in interactions with cell receptors, antigenic responses and haemagglutination reactions. The sequence analysis of VP1 is employed for the definition of patterns of polymorphism, by which at least 8 different viral genotypes and several subtypes are segregated (Agostini *et al*, 2001). The regulatory region is a 400 bp non coding region that can be divided into 7 distinct sequence sections (A to F) with known base pair length that constitute the so called archetype JCV sequence (Frisque *et al*, 1984). Archetype-like forms with some nucleotides deletions and/or insertions are found in the kidney and urine of healthy and immunosuppressed individuals. The regulatory region amplified from PML brain and cerebrospinal fluid usually shows unique rearrangements generated by point mutations, base blocks insertions and deletions compared to the archetype. It has been proposed that rearranged patterns represent a form of viral adaptation to growth in tissues different from the kidney, the presumed compartment where latency occurs. A classification scheme for the RR has been proposed by Jensen and Major (Jensen and Major, 2001), Each region of the regulatory region contains binding sites for transcriptional cell factors involved in the early and late transcription phases of JCV. As a consequence of the rearrangements of the RR these BS undergo to deletion or enhancement processes that could generate variants able to modulate the viral expression in particular anatomical sites (Reviewed in Vaz *et al*, 2001).

Aims of our study were to analyse the nucleotide organization of JCV strains amplified from cerebrospinal fluid of HIV-1 infected patients with PML treated with HAART or not and to evaluate their correlation with in vivo JCV replication, a marker of disease prognosi.

Patients and methods

Patients selection

HIV-1 infected patients were selected between those undergoing a diagnostic lumbar puncture at the Institute of Infectious Diseases in Rome, Italy, between January 1995 and January 2004. Patients with a virologically-confirmed diagnosis of PML were identified and studied. The diagnosis of PML was based on the detection of JCV DNA in cerebrospinal fluid by polymerase chain reaction (PCR) and the concomitant presence of a compatible clinical and neuroradiological picture (De Luca *et al*, 2000).

All the CSF samples were collected at the moment of diagnosis and all the viro-immunological analyses have been performed on these samples.

Patients' characteristics, treatment history and survival were abstracted from clinical records. HAART treatment was defined as any combination of no less than three antiretroviral drugs including a protease inhibitor (PI). The study protocol was approved by the local Ethics Committees and all patients gave written informed consent.

Virological assays

JCV DNA was extracted from 200 μl of cerebrospinal fluid on resin columns according to the manufacturer's instructions (QIAmp DNA Blood Mini Kit; Qiagen). The viral genome was quantified by using a semi-quantitative limiting dilution nested PCR, with a detection limit of 1.6×10^3 copies/ml of cerebrospinal fluid for diagnostic purpose (De Luca *et al*, 1996). All the samples were retrospectively tested by using the Taq Man real-time PCR technology as described elsewhere (Marzocchetti *et al*, 2005). The detection limit of the real time PCR assay was 500 DNA copies per ml of clinical sample.

HIV-1 RNA levels were quantified in cerebrospinal fluid by RT-PCR using a commercial kit (Ultra sensitive Amplicor HIV-1 Monitor, Roche Diagnostic, Branchburg, NJ) with a detection limit of 20–25 copies/ml. Plasma HIV-1 RNA concentrations were measured using a branched DNA assay with a detection limit of 50 copies/ml (Quantiplex 3.0, BayerHealthCare Diagnostics, CA, USA).

JC viral genotypes and rearrangements

In order to classify viral genotypes we amplified a fragment of 215 bp of the VP1 gene by using JLP15 (nts 1710–1734) and JLP16 (nts 1924–1902) as primers (Agostini *et al*, 1997). In a total volume of reaction of 100 microliters the primers were used at a final

concentration of 25 pmol and 2U of Taq Gold (Applied Bioystems). The amplification protocol was set using 48 cycles using an annealing temperature of of 63°C.

To analyze the regulatory region of the virus we performed a nested-PCR that amplified a fragment of 353 bp (Ferrante *et al*, 2003). The first PCR reaction was carried out in a total volume of 50 μl with 2U of Taq Gold (Applied Biosystems), 10 pmol of each primers JRE1 (nts 4989–5009) and LP2 (nts 537–518) using an amplification protocol of 30 cycles and an annealing temperature of 59°C. For the nested PCR, ten microliters of the amplified product of the first reaction were added to the second PCR reaction. The inner primers RREV (nts 310-291) and RFOR (nts 5085–5104) were used at a concentration of 20 pmol in 30 cycles of amplification with an annealing temperature of 63°C. The amplified products were therefore loaded on a 2% agarose gel electrophoresis, stained with ethidium bromide and the gels were analyzed with Gel Doc 2000 (Biorad, Segrate, Italy). Bands of expected sizes were cut and gel purified with a gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN). Direct nucleotide sequencing of VP1 and Regulatory Region were performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit with 5 to 10 ng/100 bp of PCR product and 3.2 pmol of sequencing primer. Fluorescence based DNA sequence analyses were obtained on an ABI PRISM 377 DNA Sequencer (Applied Biosystem, Foster City, CA). The genotypes classification and homology sequences search were performed using the BLAST (National Center for Bioinformatics, Bethesda, MD, USA) comparing the sequences obtained to those published (Agostini *et al*, 1997; Jensen and Major 2001).

Immunological assays

Macrophage Chemoattractant Protein-1 (MCP-1) levels were measured in cerebrospinal fluid using commercial assays based on quantitative sandwich immune enzymatic techniques (Quantikine, R&D System, Minneapolis, MN, USA), with a detection limit of 10 pg/ml. The instructions given by the manufacturer were followed throughout the procedure. Peripheral blood CD4+ T cell counts were determined by standard flow cytometry.

Statistical analysis

Viral copy concentrations in body fluids and cytokines levels were log-transformed before calculations. Differences between continuous variables were analyzed using the Student's *t*-test, correlations between continuous variables were tested by Pearson's analysis. Survival analysis was performed using Kaplan–Meier curves and Cox's proportional hazards models. All analyses were performed using the Statistica version 6.0 software package (Statsoft, Padua, Italy).

Table 1 Antiretroviral treatment

	PML patients (n = 45)	
Antiretroviral treatment history	Yes	No
NRTI-only before disease onset (%)	22	78
HAART before disease onset (%)	24	76
HAART after disease diagnosis (%)	60	40

PML denotes progressive multifocal leukoencephalopathy, NRTI, nucleoside reverse transcriptase inhibitors; HAART highly active antiretroviral therapy.

Results

Patients' characteristics and immunological features

We enrolled 45 HIV-1-infected patients with confirmed PML, 76% were males, 63% were injecting drug users, and their median age was 38 years (interquartile range [IQR], 32-42). Antiretroviral treatment history is summarized in Table 1. The median baseline CD4 count was 36 cells/μl (IQR, 15-95). The median concentration of MCP-1 in the 45 cerebrospinal fluid samples was 512 pg/mL (IQR 292-780). Thirty-one out of forty-five CSF samples have already been included in our previous paper and therefore should be considered as historical cases (Marzocchetti *et al*, 2005).

Quantitative virologic results

The median HIV-1 RNA level in the cerebrospinal fluid (data available for 16 out of 45 patients) was 2.84 $\log_{10}$ copies/ml (IQR, 2.37-4.21) and in plasma (data available for 39 out of 45 patients) 4.65 $\log_{10}$ copies/ml (IQR, 3.36-5.19). The median JC virus viral load in the cerebrospinal fluid (available in all patients) was 3.30 $\log_{10}$ copies/mL (IQR 3.20-4.30) as measured by the semi-quantitative nested PCR and 4.98 $\log_{10}$ copies/mL (IQR 2.70-6.21) by real time quantitative PCR. There was a positive correlation of JCV DNA levels using the two techniques ($R^2 = 0.13$, $p = 0.033$), but real time PCR results constantly showed higher levels (paired-t-test: mean $\pm$ SD difference $+1.60 \pm 2.29$ $\log_{10}$ copies/mL, $p < 0.001$).

Analysis of JC virus genotypes

Thirteen out of forty-five cerebrospinal fluid samples were amplifiable for the analysis of the VP1 coding region. Each negative sample was tested three times. Samples not amplifiable for VP1 had a significantly lower JCV DNA load as those amplified (mean $\pm$ SD 3.96 ± 1.23 $\log_{10}$ copies/mL versus 7.97 ± 2.24 $\log_{10}$ copies/mL, $p < 0.001$). The nucleotide sequence analysis of this region showed that 33% of the isolates (4 out of 12) amplified from PML patients not treated with HAART before the disease onset were genotype 1a, 42% (5 of 12) were 1b and 25% (3 of 12) were genotype 4.

Interestingly, 2 out of the 3 viral isolates that were classified as genotype 4, had a point mutation (A $\rightarrow$G)

at nucleotide sequence position 1850, that is typically observed in genotype 1b only, so that a viral recombination event between the two variants can be hypothesized.

On the other hand the only virus from a patient who did receive HAART before the diagnosis of PML amplifiable in VP1 was genotype 1a.

Analysis of JC virus Regulatory Region organization

Amplification of the JC virus regulatory region was successful in cerebrospinal fluid samples from 22 out of 45 PML patients.

JC virus RR could not be characterized from 3 patients because there was no cerebrospinal fluid left for the analysis. For the remaining 20 patients, the viral RR region could not be amplified from tested cerebrospinal fluid samples despite three attempts: again, samples which could not be amplified had significantly lower JCV DNA concentrations (mean ± SD 3.84 ± 1.28 $\log_{10}$ copies/mL versus 6.58 ± 2.59 $\log_{10}$ copies/mL, $p < 0.001$). For the amplification of the RR we used a nested-PCR, that could explain why we were able to amplify more RR than VP1 in the cerebrospinal fluid samples.

All the amplified sequences are schematically represented Table 2. Twenty out of 22 regulatory region amplified sequences were type II R, while 2 were type II S or archetype-like (patients number 2 and 15).

As expected, the blocks A, C, E and F were never totally deleted. The B block was completely deleted in 2 out of 5 sequences with prior HAART exposure and in 2 out of 17 without prior HAART exposure while the D block was deleted in 2 of 17 patients without prior HAART exposure.

The different blocks underwent to insertions of various nucleotides length: among the 5 prior HAART-treated patients (median treatment duration 133 weeks, range 21-248) B, C and D had insertions each in 4 cases, while among the 17 patients not exposed to HAART before the disease insertions were observed in A in 1 case, in B in 7, in C in 8, in D and E, each in 6 cases. Duplication of the entire or of a part of the blocks was also observed: in the 5 patients with prior HAART, duplications involved block B in 3 cases, E in 2 cases and C and D each in 5 cases, while among the 17 patients not previously exposed to HAART duplications involved block A in 1 case, B in 8, C in 14, D in 12 and E in 13.

We then decided to calculate a rearrangement score comparing the isolates to the archetype form (A = 36bp, B = 23 bp, C = 55 bp, D = 66 bp, E = 18 bp, F = 69 bp), for every isolate the score was calculated as follows: +1 for deletions present in each block, +1 for insertions present in each block and +1 for each complete block duplication. The mean score of total rearrangements for the amplified sequences was 6.1 (range 1–11): individuals with prior HAART tended to show a higher mean rearrangement score (7.2, as compared to 5.9 in individuals without prior HAART), but the difference was not significant.

Analysis of binding sites for transcriptional control factors

We determined the frequency of occurrence of binding motifs for cellular transcriptional control factors in the regulatory region of cerebrospinal fluid JC viral isolates (Table 3). The most frequent binding sites found were those for NF-1, followed by those for GF-1 and AP-1. PML patients with prior antiretroviral exposure, showed a trend towards a higher number of binding sites for JCV transcriptional control factors compared to those who were naïve for antiretroviral treatment (mean ± SE 5.70 ± 0.42 versus 4.40 ± 0.52, $p = 0.068$).

We then focused our attention on the number of duplications of the up-TAR elements. In 7 of 22 isolates the up-TAR binding motif was absent: 6 of 17 (35%) of no prior HAART and 1 of 5 (20%) of prior HAART exposure; in 5 of 22 there was a single motif: 4 of 17 (23.5%) of no prior HAART and 1 of 5 (20%) with prior HAART, while in 11 of 22 (8/17, 47%, of no prior HAART and 3/5, 60%, with prior HAART) a duplication was found.

JC virus DNA levels in cerebrospinal fluid and PML prognosis

During a median follow up of 60 months, there were 26 (61%) PML-related deaths. At univariable Cox's regression analysis, the PML survival was more accurately predicted by JCV DNA concentration in the cerebrospinal fluid as measured by Real Time quantitative PCR (per $\log_{10}$ higher, hazard ratio [HR] for PML-related death 1.31, 95% confidence interval [CI] 1.11-1.55, $P = 0.001$) as compared to the limiting dilution semi-quantitative PCR (per $\log_{10}$ higher, HR for PML-related death 1.34, 95% CI 0.98–1.82, $P = 0.070$). In a multivariable Cox's model adjusting for use of HAART after the onset of PML, JCV DNA load measured by real time PCR remained independently predictive of survival (per $\log_{10}$ higher, HR for PML-related death 1.30, 95% CI 1.10-1.53, $P = 0.002$).

Correlation of virologic and immunological characteristics with cerebrospinal levels of JC virus

The virologic and immunological characteristics showing a correlation with JCV DNA load measured by real time PCR are summarized in Table 4. The presence of genotype 4 was associated with a lower JCV DNA load. The presence of a higher rearrangement score was correlated with a lower JCV DNA viral load in cerebrospinal fluid (<7 rearrangements, mean ± SE concentration of JCV 7.46 ± 0.77 $\log_{10}$, copies/mL, ≥7 rearrangements, 5.17 ± 0.54 $\log_{10}$ copies/mL, $p = 0.046$). When analyzing each block individually, the duplication of the entire B block, was associated with a lower JCV DNA load (mean ± SE concentration of JCV DNA 5.43 ± 0.69 versus 7.86 ± 0.76 $\log_{10}$ copies/mL, $p = 0.028$). No other block duplication was associated with JCV concentration.

The possible association between the frequency of each of binding sites for cellular transcriptional

Table 2 Distribution of the genotypes and organization of the regulatory region of the JC viral strains from cerebrospinal fluid of HIV-1 infected patients with progressive multifocal leukoencepalopathy (PML)

Patient number	HAART before PML onset	JCV genotype	type	JCV regulatory region organization																													
P1	No	1 B	IIR	A	B	C				D	E												f1	f2		E			f1	F2			
P2	No	1 A	IIS	A	B	C				D	e																			f 2			
P3	No	1 A	IIR	A	B	c				d				b				c			d					E			F1	f2			
P4	No	1 B	IIR	A	B	C				d	E					f1		C			d					e			F1	F2			
P5	No	1 B	IIR	A	B	C				d	E			b				C			d					E			F1	F2			
P6	No	1 B	IIR	A		C			C	d	E							C		c	d					e			F1	F2			
P7	Yes	1 A	IIR	A	B	C				d	e			b				C			d					E			F1	F2			
P8	No	1 A	IIR	A		c				d	E					f1		c			d					E			F1	F2			
P9	No	4*	IIR	A	B									B				C	f2	c	d				c	E			F1	F2			
P10	No	4*	IIR	A	B	C				d	E					f1		C			d					E			F1	F2			
P11	No	1 A	IIR	A	B	C				D	E								f1	f2	d								f1	f2			
P12	No	4	IIR	A	B	C					E			B				c								E			F1	F2			
P13	No	1 B	IIR	A	B	c					e					f1		C								e			f1	F2			
P14	Yes	na	IIR	A	B	C				d				b				c			d					E			F1	F2			
P15	No	na	IIS	A	B	C				D	E																						
P16	No	na	IIR	A	B	C				d	e	d	e		d	e		C			d					E			F1	F2			
P17	No	na	IIR	A	B	c				d	E		f1					c			d					E			F1	F2			
P18	No	na	IIR	A	B	c				d	E		a	B				c			d					E			f1	f2			
P19	Yes	na	IIR	A	B	C				d				b				c			d					E			F1	F2			
P20	Yes	na	IIR	A		c	c	f1	C	d	e							c	f1	e	d					e			f1	f2			
P21	No	na	IIR	A	B	c					e		d	b				c		f1	d					e	d	E	F1	F2			
P22	Yes	na	IIR	A		c												c			d	E	C	d	E			F1	F2			

HAART, highly active antiretroviral therapy; na, not amplifiable. A-B-C-D-E-F1-F2 the nucleotide sequence of the different blocks of the archetypal regulatory region (Ault & Stoner 1993) , a-b-c-d-e-f1-f2 the nucleotide sequence of the different blocks that underwent to point mutations, base block insertions and deletions.

Table 3 Frequency of DNA binding sites for cellular transcriptional control factors

DNA Binding Motif	Number of patients with		
	1 copy	2 copies	>3 copies
Oct-6/tst-1A	18	0	0
Oct-6/tst-1B	1	0	0
NF-1	12	10	0
Pseudo NF-1	8	0	0
SP-1	10	2	0
GF-1	13	8	0
AP-1	10	6	4
AP-1 like	16	0	0
Pentanucleotide	1	0	0
Pentamut	17	0	0

The sequences of each DNA binding sites are the following: Oct-6/tst-1A (5′-TCCTGTATATATAAAAA-3′), NF-1 (5′-TGG CTGCCAGCCA-3′), Pentanucleotide (5′-AGGGAAGGGA-3′), SP-1 (5′-AGGGAGGAGC-3′), GF-1 (5′-GCCAGCCA-3′), AP-1 (5′-TGA GCTCA-3′), AP-1 like (5′-TGTCACCA-3′), Oct-6/tst-1B (5′-GCCAG TAAACAAAGCA-3′), Pseudo NF-1 (5′-TGGAAAGCAGCC A-3′), Pentamut (5′-AGGGAAGGTA-3′), TATA box (5′-TATATAT-3′).

factors and the JCV concentration was explored. NF-1 was the only factor that showed a trend towards an association with lower JC viral load. The number of NF-1 binding sites was negatively correlated with the JCV viral load (NF-1 = 1 mean ± SE concentration of JCV 7.45 ± 0.62 $\log_{10}$, NF-1 = 2 mean concentration 5.42 ± 0.83 $\log_{10}$, $p = 0.060$, $r = -0.41$). No correlation was found between the number of up-TAR elements and the JCV load in the cerebrospinal fluid.

Analyzing the immunologic factors in all study patients we found a negative correlation of the JC viral load by RT PCR with the MCP-1 concentration in the cerebrospinal fluid ($p = 0.036$) as well as with baseline CD4 counts ($p = 0.009$).

HAART exposure prior to PML onset was also associated with lower JCV load ($p = 0.01$). In a multivariable model adjusting for baseline CD4 counts and prior HAART exposure MCP-1 concentration was the only factor independently associated with lower levels of JC viral load (per $\log_{10}$ pg/ml higher, mean difference in JCV DNA concentration −2.62 $\log_{10}$, $p = 0.016$).

Discussion

In the present study we demonstrate that, when using the real time PCR technique for the quantification of the JC virus genome in the cerebrospinal fluid of PML patients, we were able to obtain a better prediction of survival than by using a semi-quantitative nested PCR method. The analytical sensitivity of the assay was 500 DNA copies/ml of cerebrospinal fluid and JCV DNA was detected in all the cerebrospinal fluid samples previously tested with nested PCR. The quantitative JCV DNA detection in cerebrospinal fluid is one of the most sensitive marker for predicting PML progression (Taoufik *et al*, 1998; De Luca *et al*, 1999). After the introduction of HAART, JCV DNA can be cleared from the cerebrospinal fluid with an improvement of the prognosis (De Luca *et al*, 2001; Cinque *et al*, 1998). We showed that our real time PCR was a robust method for measuring the JC viral load at the moment of PML diagnosis in HIV-1 infected patients and that it was a strong predictor patients survival independently from the use highly active antiretroviral treatment.

The analysis of JCV genotypes distribution showed that 77% of amplified strains were subtype 1a or 1b and 23% were genotype 4. While all the JCV genotypes can induce PML, before the introduction of HAART the infection of HIV infected patients with genotype 2, especially 2b, had been associated with a higher risk of developing the disease both in north american and italian cohorts (Agostini *et al*, 1998; Ferrante *et al*, 2001). Unexpectedly, in our study we did not detect any genotype 2 from the cerebrospinal fluid of PML patients but 3 strains were type 4. Of interest 2 out of 3 of the genotype 4 strains contained a point mutation in nucleotide 1850 (A→G) that has been previously reported by Sala *et al* (2001) and is characteristic of genotype 1b. Consistently with our findings, reports from PML series in France and northern Italy show a prevalence of around 20% of genotypes 4 (Sala *et al*, 2001; Ferrante *et al*, 2003). Altogether these findings suggest that JC virus genotype 4 can be associated to PML in a proportion of AIDS patients and that some of these variants

Table 4 Factors associated with cerebrospinal fluid JCV DNA concentration measured by real time PCR (linear regression)

Variables	Number of cases	Mean difference in JCV DNA concentration ($\log_{10}$copies/ml)	P value
Baseline CD4+ counts (per $\log_2$ cells/μl increase)	45	−0.51	0.009
HAART exposure prior to PML onset	45	−1.90	0.010
MCP-1 levels in cerebrospinal fluid (per $\log_{10}$ pg/ml increase)	45	−1.69	0.036
Number of rearrangements in the RR (7 versus < 7)	22	−2.29	0.046
Block B deletion	22	+2.43	0.028
Binding sites for NF-1 (2 versus 1 copy)	22	−2.03	0.060
Genotype 4 (versus other genotypes)	13	−1.78	0.043

PML denotes progressive multifocal leukoencephalopathy, HAART highly active antiretroviral therapy, MCP-1 monocyte chemotactic protein type 1, RR regulatory region, NF-1 nuclear factor type 1.

may result from a recombination of genotype 4 with genotype 1b.

GenBank/EMBL accession numbers for the nucleotide amino acid sequences of the viral isolates present in the paper are the followings:

JCV Regulatory Region:	JCV VP1:
Patient 1: EF 469693	Patient 1: EF 469715
Patient 2: EF 469694	Patient 2: EF 469716
Patient 3: EF 469695	Patient 3: EF 469717
Patient 4: EF 469696	Patient 4: EF 469718
Patient 5: EF 469697	Patient 5: EF 469719
Patient 6: EF 469698	Patient 6: EF 469720
Patient 7: EF 469699	Patient 7: EF 469721
Patient 8: EF 469700	Patient 8: EF 469722
Patient 9: EF 469701	Patient 9: EF 469723
Patient 10: EF 469702	Patient 10: EF 469724
Patient 11: EF 469703	Patient 11: EF 469725
Patient 12: EF 469704	Patient 12: EF 469726
Patient 13: EF 469705	Patient 13: EF 469727
Patient 14: EF 469706	
Patient 15: EF 469707	
Patient 16: EF 469708	
Patient 17: EF 469709	
Patient 18: EF 469710	
Patient 19: EF 469711	
Patient 20: EF 469712	
Patient 21: EF 469713	
Patient 22: EF 469714	

Sequence analysis of the Regulatory Region amplified from 22 PML patients revealed the presence of 20 RR type II R or "classical" rearranged forms and the unexpected finding of 2 archetype-like forms (IIS) with point mutations in the E block but a complete deletion of the F block. In vitro transfection studies have shown that the complete or mutated IIS variant is relatively inactive and it is well known that rearrangements from the archetype form are required for the colonization and active infection of the brain (Daniel *et al*, 1996; Ault *et al*, 1993). Despite this, more recent studies have reported few cases of archetype or archetype-like forms in the cerebrospinal fluid of PML patients. In the paper of Ferrante *et al* (2003) the presence of type II S sequences was associated with better survival, while in Pfister et al (Pfister *et al*, 2001), the archetype form was the sequence in 100% of strains from long term survivors and finally Vaz *et al* (2001cit) reported the finding of 3 of 17 cases of type II S and another 2 of 17 carrying II S with modifications. Our findings, together with the previous reports overcome the JCV dogma that the archetype virus is present in the urine whilst rearranged forms colonize the brain.

Our observations can not confirm an association between the presence of the archetype-like forms and survival, since one of the patients was a progressor and the other a survivor (not shown).

Interestingly, we found a negative correlation between the total number of rearrangements in the RR of the isolates and the JC viral load. This result is in apparent contradiction with two studies, one unable to show any specific genomic markers for JCV neurovirulence in the RR and VP1 loci (Sala *et al*, 2001), the other suggesting that the presence of tandem repeats in plasma and central nervous system RR was associated with poor clinical outcome in patients with PML (Pfister *et al*, 2001). Different results may depend on the limited size of the studies and on the different definitions of the polymorphism analyzed in the RR. When we analysed the number of tandem repeats in the RR we found no association with the JCV DNA load (not shown). In our study, a rearrangement score was determined arbitrarily, since there was no previous consensus in the literature. Based on the assessment that there is an evolutionary gradient from archetype to rearranged forms going from the urine to the blood and the cerebrospinal fluid, we decided to assign a score to each mutation present in the amplified strain compared to the archetype usually found in the urine. A limitation of our study could be the use of direct bulk sequencing of the amplified PCR products instead of cloning, by which we could have identified minority RR sequences (Ciappi *et al*, 1999; Newman *et al*, 1999). Nonetheless, our analysis is representative of the most prevalent viral sequences in the cerebrospinal fluid sample and therefore of the quantitatively most relevant JC virus species.

Noteworthy, we found that the number of B blocks was negatively associated with the JCV DNA concentration The region B is a 23 bp insert that when present in the sequence of the regulatory region, as in the type II S and II R, is responsible for an inefficient transcription and repression of viral activity in vitro (Daniel *et al*, 1996); it is usually conserved but is guaranteed complete only in the type IIS. In the molecular organization of type IIR (A-B-C-D-E-F-B-C-D-E-F) that constitute the majority of our amplified sequences, we would expect to find two B blocks, possibly with several mutations. When in the RR there was the presence of only one block B, JC viral load was higher. Considering the "in vitro" data, we hypothesize that the presence of two B blocks inhibit the JCV replication "in vivo" which might explain the finding of a lower viral load.

Another factor that was associated with a reduction of the JC viral load was the number of biding sites for the cellular transcriptional control factor NF-1. The importance of this motif is underlined by the fact that it was the most represented of all the analyzed binding sites. This binding site plays an important role in the early specific transcription of JCV in the brain and is situated in the C and F blocks of the archetype sequence (Kumar *et al*, 1996, Sumner *et al*, 1996). The NF-1, and specifically the NF-1 class D, is a family of proteins that bind the JCV promoter and is expressed especially in glial cells (Sumner *et al*, 1996). In vitro, the number of NF-1 binding sites is proportional to the level of JCV transcription in glial cell lines (Kumar *et al*, 1996) and in vivo additional NF-1 binding sites are found in patients with fatal outcome (Pfister *et al*, 2001). Surprisingly, our data are in disagreement with what has been

shown however, additional factors may be relevant in modulating viral replication in vivo, in particular antiretroviral treatment and JCV-specific or unspecific immune responses. Therefore, the presence of more NF-1 binding sites in patients with lower JC viral load may represent an inefficient attempt of the virus to escape environmental control. In line with this hypothesis, virus from patients with prior exposure to antiretroviral therapy showed trends towards a higher number of rearrangements in the RR and of binding sites to cellular transcription factors.

In addition, the frequency of the up-TAR sequence which has very high homology with the HIV Tat-responsive elements (TAR) and is contained in the C and E blocks of the archetype (Chowdhury *et al*, 1993) was not correlated with the JC viral load and did not differ between patients who were treated or not with HAART before the PML onset, showing that the selection of this viral element did not seem to play a relevant pathogenetic role in this series.

Overall, interpretation of these observations should be cautious because of the small number of amplified strains, thererefore more extended analyses are required for confirmation.

Finally, we extended our previous finding that JC viral load in the cerebrospinal fluid was negatively correlated with MCP-1 levels in that fluid by adding fourteen new cases (Marzocchetti *et al*, 2005). Lower cerebrospinal fluid levels of JCV were also associated with higher baseline CD4 counts and prior HAART exposure, but the larger sample size allowed to perform a multivariable analysis, that indicated an independent association of MCP-1 with lower JCV concentration in cerebrospinal fluid. This finding suggests that MCP-1 levels in cerebrospinal fluid might be a useful immunologic marker of the control of JC viral replication independently from antiretroviral treatment and immune status.

In conclusion we found a strong correlation between JCV DNA levels in the cerebrospinal fluid and PML-related mortality. Lower JCV concentration was associated with viral genotype 4, a higher number of rearrangements in the RR, the duplication of block B in the RR and the higher number of binding sites for cellular transcription factor NF-1. HAART, higher CD4 counts and, particularly, higher MCP-1 levels were also associated with low JC viral load. We speculate that under antiretroviral treatment JCV replication is reduced as a consequence of immune control and, possibly, reduced HIV-induced replication. Under this condition, JCV may tend to survive through the selection of rearrangements in its regulatory region, specifically attempting to multiply binding sites for cellular transcription factors and loosing sequences associated with the suppression of viral replication.

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