Journal of NeuroVirology, 9(suppl. 1): 42–46, 2003
 ISSN 1355-0284/03 $12.00+.00
DOI: 10.1080/13550280390195405

Taylor & Francis
Taylor & Francis Group

Analysis of JC virus genotype distribution and transcriptional control region rearrangements in human immunodeficiency virus–positive progressive multifocal leukoencephalopathy patients with and without highly active antiretroviral treatment

Pasquale Ferrante,[1,2] Serena Delbue,[2] Elisabetta Pagani,[1] Roberta Mancuso,[1] Angela Marzocchetti,[1] Elisa Borghi,[2] Renato Maserati,[3] Arabella Bestetti,[4] and Paola Cinque[4]

[1]*Laboratory of Biology, Don C Gnocchi Foundation ONLUS, IRCCS, Milan, Italy;* [2]*Department of Biomedical Science and Technology, University of Milan, Milan, Italy;* [3]*Clinic of Infectious Diseases, Policlinico San Matteo, Pavia, Italy; and* [4]*Clinic of Infectious Diseases, San Raffaele Hospital, Milan, Italy*

After the introduction of highly active antiretroviral therapy (HAART), the incidence of many acquired immunodeficiency syndrome (AIDS)-related opportunistic infections, but not of progressive multifocal leukoencephalopathy (PML), has been dramatically decreased. However, it has been shown that about 50% of the HAART-treated PML patients had a significantly prolonged (>6 months) survival time, in comparison to the short (<6 months) survival time of the classical form of PML. In order to verify if a particular genotype or genomic rearrangements of JC virus (JCV) could affect the clinical course of PML, the authors performed nucleotide sequencing of 25 virion protein (VP1) and 18 transcriptional control region (TCR) DNA amplified in the cerebrospinal fluid (CSF) of HAART-untreated PML patients, of 17 HAART-treated PML patients, and in the urine of 23 healthy individuals. In nontreated PML patients, 52% and 44% of amplified JCV were respectively type 1 and type 2, whereas in HAART-treated PML patients, 59% of the amplified JCV were type 1, 23% type 2, and 18% type 4, without differences between long and short survivors. In both groups, the amplified TCR had unique and extensive rearrangements, whereas archetype TCR without rearrangements was detected in all the healthy subjects and in the CSF of two long-survivor PML patients. The data obtained indicate that the introduction of HAART has induced changes in JCV genotype distribution and probably reduced the rate of rearrangements of TCR region among PML patients. *Journal of NeuroVirology* (2003) **9(suppl. 1),** 42–46.

Keywords: HAART treatment; JCV genomic organization; progressive multifocal leukoencephalopathy (PML)

Introduction

The human polyomavirus JC (JCV) is a widespread virus that infects up to 80% of the human population (Chesters *et al*, 1983) and causes in immunosuppressed individuals progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the white matter of the central nervous system (CNS) (Brooks and Walker, 1984).

Before of the introduction of highly active antiretroviral therapy (HAART), PML was one of the most frequent complication seen in acquired immunodeficiency (AIDS) patients, observed with prevalence up to 8%. Moreover, it was a fast progressive fatal disease capable of inducing death in less than 6 months from the time of diagnosis (Berger *et al*,

Address correspondence to Pasquale Ferrante, MD, Laboratory of Biology, Don C. Gnocchi Foundation, Via Capecelatro 66, 20148 Milano, MI, Italy. E-mail: pferrante@dongnocchi.it

This work was supported by grants given to the Don C Gnocchi Foundation, IRCCS, by the Italian Ministry of Health (Ricerca Corrente 2002 and Programma Nazionale AIDS 2001).

Received 25 October 2002; accepted 24 January 2003.

1987; Berger and Major, 1999; Brooks and Walker, 1984).

The introduction of HAART treatment into clinical practice has reduced the frequency of many of the opportunistic infections observed in AIDS patients, but not of PML (Clifford *et al*, 1999; De Luca *et al*, 2000). Moreover, in HAART-treated human immunodeficiency virus (HIV)-positive patients, an important change in the clinical course of PML has been observed: nearly half of PML patients have a slow clinical progression with a survival time of more than 6 months, significantly longer than that observed in the classical form of PML (Albrecht *et al*, 1998; Cinque *et al*, 1998; Miralles *et al*, 1998). Based on this observation, the definition of long- and short-survivor PML patients has become of common use, although a commonly accepted terminology is still under definition (Cinque *et al*, 2003).

This evolution of the clinical course might be due to the partial reconstitution of the immune system observed in HAART-treated HIV-positive patients, but it is unclear if viral aspects related to the JCV genomic organization could also play a pathogenic role (Giudici *et al*, 2000; Miralles *et al*, 2001). To verify this hypothesis, we investigated the genomic organization of the JCV strains amplified in cerebrospinal fluid (CSF) samples obtained from HAART-treated and untreated HIV-positive patients suffering PML, and, as control, in the urine of healthy subjects. Because rearrangements of the transcriptional control region (TCR) could affect JCV replication and thus possibly influence the clinical evolution of PML, we analyzed TCR organization following the regulatory region compass classification scheme (Jensen and Major, 2001). In this innovative classification, the JCV TCR variants are arranged into quadrants according to the insertion of particular sequence blocks and repetition of groups of sequence blocks.

On the basis of the nucleotide sequence of the virion protein (VP1) upstream portion, eight genotypes distributed in various geographic areas of the world (Agostini *et al,* 1996) have been identified, and it has been also suggested that JCV type 2 could have a more pronounced neuropathogenic activity (Agostini *et al*, 1998; Ferrante *et al*, 2001).

In the present study, we have analyzed the distribution of JCV genotypes in a group of long- and short-survivors HAART-treated PML patients and compared with that observed in PML patients studied in the pre-HAART era.

Results

The positivity for JCV of the studied samples was previously verified by searching, by polymerase chain reaction (PCR), DNA belonging to the highly conserved large T antigen (LT) genomic region of the virus in the CSF of PML patients and in the urine of healthy controls. In the CSF of the 21 PML patients studied in the pre-HAART era, we amplified 25 VP1 DNA, because dual infection with both type 1 and type 2 was observed in four of these PML patients.

Table 1 Distribution of the various JCV genotypes in CSF collected from PML patients studied before and after the introduction of HAART treatment and in urine from Italian healthy individuals

		HAART PML		
Genotypes	*Pre-HAART PML*	*Long survivors*	*Short survivors*	*Healthy Italian population*
1	13 (52%)	5 (56%)	5 (63%)	9 (39%)
2	11 (44%)	2 (22%)	2 (25%)	6 (26%)
3	0	0	0	1 (4%)
4	0	2 (22%)	1 (12%)	7 (30%)
Undefined	1 (4%)	0	0	0

As shown in Table 1, in the PML patients studied in the pre-HAART era, JCV type 1 and type 2 were the most frequently detected, representing respectively 52% and 44% of the amplified strains, whereas none of the other JCV genotypes was found. Interestingly in one of the examined CSF, we amplified a nonclassifiable strain, showing a type 1 pattern with a point mutation (G → C) in position 1818, typically observed in type 2.

Among the 17 JCV DNA amplified in CSF collected from HAART-treated PML patients, 10 (59%) were type 1, whereas 4 (23%) and 3 (18%) were respectively type 2 and type 4. The analysis of JCV genotype distribution among the long- and short-survivor PML patients did not show significant differences between the two groups: JCV type 1 was found in 63% of the long and 56% of the short survivor, JCV type 2 was detected in 22% of the long and in 25% of the short survivors, and JCV type 4 was amplified in 22% of the first group and 25% of the second group of PML patients.

The pattern of JCV genotype distribution observed in the HAART-treated PML patients is similar to that found in the healthy controls, among whom one JCV type 3 has been also amplified.

The analysis of TCR region organization (Table 2) showed that among the PML patients not treated with HAART, only repeated regulatory regions were detected and particularly 10 repeated forms type I

Table 2 Distribution of archetypal and rearranged form of JCV TCR in CSF collected from PML patients studied before and after the introduction of HAART treatment and in urine from Italian healthy individuals

		HAART PML		
TCR Organization	*Pre-HAART PML*	*Long survivors*	*Short survivors*	*Healthy Italian population*
Singular				
I	0	0	0	0
II	0	2 (33%)	0	20 (100%)
Repeat				
I	10 (55.5%)	0	0	0
II	8 (44.6%)	4 (67%)	6 (100%)	0

(IR) and 8 repeated forms type II (IIR), which are rearranged forms from the archetype regulatory region, have been amplified.

The results obtained among the PML patients treated with HAART are very interesting. In fact, for the first time in our experience, archetype (singular form type II, IIS) TCR organization was amplified from CSF of two PML patients who were classified as long survivor. All the other TCR detected in the CSF obtained from those groups of PML patients were defined as repeated forms type II. Twenty TCR regions were amplified in the urine collected from healthy controls and all of them had, as expected, a completely conserved archetype organization.

Discussion

After the introduction on a large scale in developed Western countries of the HAART treatment for HIV infection, a dramatic decrease in the incidence of many AIDS-related complications has been observed, though the incidence of some neurological disorders such as PML has been less affected (Clifford *et al*, 1999; De Luca *et al*, 2000). Moreover, among the PML patients treated with HAART, it has been observed that half of the cases show classical course of the disease, whereas the remaining patients are affected by a clinical variant of PML with a prolonged survival time. (Albrecht *et al*, 1998; Cinque *et al*, 1998; Miralles *et al*, 1998).

Many aspects of the changes observed, after the introduction of HAART, in the epidemiology and clinical manifestations of neurological complication affecting AIDS patients are unclear.

As regard to PML, there are no clear explanation for the observation of the milder form that affects the long-survivor patients. The reconstitution of the immune system is one of the main factor; however, it can be also hypothesized that viral factor might play a role. To verify this possibility, we have studied the distribution of JCV genotypes and the nucleotide sequence of TCR region in CSF samples collected from PML patients.

We focused our study on performing molecular analysis to determine the molecular characterization of the JCV DNA, which were amplified from PML not treated with HAART and from a group of short- and long-survivor HAART-treated PML patients.

The analysis of VP1 nucleotide sequence indicates that the distribution of JCV genotypes in PML patients is quite different between the pre-HAART and HAART era. In fact, even if the type 1 is the most frequent in both the studied groups, the frequency of type 2 and type 4 was clearly different. In the patients studied in the pre-HAART era, JCV type 2 was so overrepresented in comparison to the healthy controls that its association with a higher risk of PML was suggested (Ferrante *et al*, 2001; Agostini *et al*, 1998). The present study shows that in the HAART-treated patients, the distribution of JCV genotypes is more similar to that observed in the general population, especially regarding the presence of type 2, whose frequency is decreased in comparison to that observed in the pre-HAART era. Moreover, among the HAART-treated PML patients, we have observed three cases with a JCV type 4 in the CSF, a genotype found also in seven healthy controls, but not among the nontreated PML patients. All together, these data confirm that the introduction of the effective antiretroviral therapy in AIDS patients has determined a pattern of JCV genotype distribution more similar to that of the general population than in the past.

From our results there is no evidence in favor of a significant association between one of the JCV genotypes and PML clinical course.

The data obtained, studying the organization of the highly hypervariable TCR are, in our opinion, of particular relevance. Briefly, the type II singular (IIS) (Jensen and Major, 2001) is the conserved archetype form that is generally found in the urine of healthy individuals (Chang *et al*, 1999; reviewed in Jensen and Major, 1999); type I repeats (IR) and type II repeats (IIR) (archetype derived) are the rearranged forms, with tandem repeats and deletions that are distributed in different body compartments, but especially present in the brains and CSF of PML patients (Ault and Stoner, 1993). The nucleotide organization of TCR amplified in our study confirmed in part these previous observations; in fact we found among the PML/AIDS patients not treated with HAART the exclusive presence of rearranged forms, both type I and II. In comparison with those results, the data obtained in the PML patients under HAART were surprising, because, for the first time in our experience, we have detected in the CSF of two PML patients a completely conserved archetype TCR. This observation is even more interesting taking into account that these two cases were two long-survivor PML patients and represents the first evidence in favor of the possibility that a better clinical outcome of the disease could be related to the presence of an archetype TCR.

On the whole, the data obtained indicate that after HAART introduction, the genomic features of JCV strains involved in PML are slightly changed, and that in general, JCV strains detected in those patients are more related to the virus circulating in the general healthy population. Further studies including larger number of patients are needed to fully clarify the interaction between HAART treatment, JCV genomic features, and the clinical course of PML.

Methods

Subjects

The main features of PML patients studied in the pre-HAART era have been previously described (Ferrante *et al*, 2001). In the present study, CSF samples were collected from 17 AIDS patients treated with HAART

and followed at the Clinic of Infectious Disease of the San Raffaele Hospital. Nine of those patients were considered long survivors because they had a survival time longer than 6 months, whereas the remaining eight cases died within 6 months from the onset of PML and were considered short survivors.

As control, we have also studied the distribution of JCV genotypes and TCR organization of JCV strains, amplified in the urine collected from 23 healthy subjects living in the northern Italy. All the samples analyzed in the present study were previously defined as positive for JCV LT region DNA.

PCR for VP1 and transcriptional control regions
Molecular characterization of a short fragment of major capsid protein (VP1) gene was performed on the collected samples that proved previously to be positive for large T antigen JCV DNA. In order to characterize JCV genotypes isolates, a PCR designed to amplify a 215-bp fragment of the VP1 gene was adopted. The amplification was carried out in a total volume of 100 μl using 25 pmol of each primer, JLP-15B at positions 1710 to 1734 and JLP-16 at positions 1924 to 1902 (Agostini *et al*, 1997), and 2 U of TaqGold in a 48-cycles protocol with annealing step performed at 63°C. A nested polymerase chain reaction (n-PCR) was used to amplify a 353-bp fragment belonging to JCV TCR. The first round of PCR was carried out in a total volume of 50 μl with 2 U of Taq polymerase (Roche Diagnostics) and 20 pmol of the primers JRE1 (nucleotides 4989–5009) and LP2 (nucleotides 518–537), in a 30-cycles protocol with annealing temperature at 59°C. Ten microliters of the outer-PCR product were added to the inner PCR reaction mixture contained 20 pmol of each inner primers, RFORB (nucleotides 5082–5104) and RREV (nucleotides 291–310) (nucleotide numbering is based on prototype Mad-1 from Frisque *et al*, 1984). The program included 30 cycles with annealing at 63°C.

The PCR products were detected by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Direct nucleotide sequencing
PCR were performed using one standard and one biotinylated primer and the PCR-amplified products were used as template for direct sequencing. Sequences reactions were performed using the AutoLoad Solid Phase Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and the ALFexpress DNA sequencer, as previously described (Ferrante *et al*, 2001). The genotyping and sequence homology search were performed using BLAST (National Center for Bioinformatics, Bethesda, MD, USA) (NCBI) according to Agostini *et al* (1996) and to Jensen and Major (2001).

References

Agostini HT, Ryschkewitsch CF, Mory R, Singer EJ, Stoner GL (1997). JC virus (JCV) genotypes in brain tissue from patients with progressive multifocal leukoencephalopathy and in urine from controls without PML: increased frequency of JCV type 2 in PML. *J Infect Dis* **176:** 1–8.

Agostini HT, Ryschkewitsch CF, Singer EJ, Baumhefner RW, Stoner GL (1998). JCV type 2B is found more frequently in brain tissue of progressive multifocal leukoencephalopathy patients than in the urine from controls. *J Hum Virol* **1:** 200–206.

Agostini HT, Ryschkewitsch CF, Stoner GL (1996). Genotype profile of human polyomavirus JC excreted in urine of immunocompromised individuals. *J Clin Microbiol* **34:** 159–164.

Albrecht H, Hoffmann C, Degen O, Stoehr A, Plettenberg A, Mertenskotter T, Eggers C, Stellbrink HJ (1998). Highly active antiretroviral therapy significantly improves the prognosis of patients with HIV-associated progressive multifocal leukoencephalopathy. *AIDS* **12:** 1149–1154.

Ault GS, Stoner GL (1993). Human poliomavirus JC promoter/enhancer rearrangements pattern from progressive multifocal leukoencephalophaty brain are unique derivates of a single archetypal structure. *J Gen Virol* **74:** 1499–1507.

Berger JR, Kaszovitz B, Post MJ, Dickinson G (1987). Progressive multifocal leukoencephalopathy associated with human immunodeficiency virus infection. *Ann Intern Med* **107:** 78–87.

Berger JR, Major EO (1999). Progressive multifocal leukoencephalopathy. *Semin Neurol* **19:** 193–200.

Brooks BR, Walker DL (1984). Progressive multifocal leukoencephalopathy. *Neurol Clin* **2:** 299–313.

Chang D, Sugimoto, Wang M, Tsai RT, Yogo Y (1999). JC virus genotypes in a Taiwan aboriginal tribe (Bunun): implications for its population history. *Arch Virol* **144:** 1081–1090.

Chesters PM, Heritage J, McCance DJ (1983). Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infect Dis* **147:** 676–684.

Cinque P, Casari S, Bertelli D (1998). Progressive multifocal leukoencephalopathy, HIV, and highly active antiretroviral therapy. *N Engl J Med* **339:** 848–849.

Cinque P, Koralnik IG, Clifford DB (2003). The evolving face of HIV-related progressive multifocal leukoencephlaopathy: defining a consensus terminology. *J NeuroVirol* **9(s1):** 88–92.

Clifford DB, Yiannoutsos C, Glicksman M, Simpson DM, Singer EJ, Piliero PJ, Marra CM, Francis GS, Mcarthur JC, Tyler KL, Tselis AC, Hyslop NE (1999). HAART improves prognosis in HIV-associated progressive multifocal leukoencephalopathy. *Neurology* **52:** 623–625.

De Luca A, Giancola ML, Ammassari A, Grisetti S, Paglia MG, Gentile M, Congolani A, Murri R, Liuzzi G, D'arminio Monforte A, Antinori A (2000). The effect of potent antiretroviral therapy and JC virus load in cerebrospinal fluid on clinical outcome of patients with AIDS-associated progressive multifocal leukoencephalopathy. *J Infect Dis* **182:** 1077–1083.

Ferrante P, Mediati M, Caldarelli-Stefano R, Losciale L, Mancuso R, Cagni AE, Maserati R (2001). Increased frequency of JC virus type 1 and 2 in Italian progressive multifocal leukoencephalopathy patients. *NeuroVirology* **7:** 35–42.

Frisque RJ, Bream GL, Cannella MT (1984). Human polyomavirus JC virus genome. *J Virol* **51:** 458–469.

Giudici B, Vaz B, Bossolasco S, Casari S, Brambilla AM, Luke W, Lazzarin A, Weber T, Cinque P (2000). Highly active antiretroviral therapy and progressive multifocal leukoencephalopathy: effects on cerebrospinal fluid markers of JC virus replication and immune response. *Clin Infect Dis* **30:** 95–99.

Jensen PN, Major EO (1999). Viral variant nucleotide sequences help expose leukocytic positioning in the JC virus pathway to the CNS. *J Leukoc Biol* **65:** 428–438.

Jensen PN, Major EO (2001). A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region. *J NeuroVirol* **7:** 280–287.

Miralles P, Berenguer J, Garcia de Viedman D, Padilla B, Cosin J, Lopez-Bernaldo de Quiros JC, Munoz L, Bouza E (1998). Treatment of AIDS-associated progressive multifocal leukoencephalopathy with highly active antiretroviral therapy. *AIDS* **12:** 2467–2472.

Miralles P, Berenguer J, Lacruz C, Cosin J, Lopez C, Padilla B, Munoz L, Garcia de Viedma D (2001). Inflammatory reactions in progressive multifocal leukoencephalopathy after highly active antiretroviral therapy. *AIDS* **15:** 1900–1902.