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JC virus VP1 loop-specific polymorphisms are associated with favorable prognosis for progressive multifocal leukoencephalopathy

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JC virus (JCV) is a human polyomavirus that causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that mainly affects immunocompromised subjects. Since its discovery, PML has been considered a rapidly progressing fatal disease; however, amino acid substitutions in the capsid viral protein have recently been tentatively associated with changes in PML clinical course. In order to provide more insight to PML pathogenesis and identify potential prognostic markers, seven cerebrospinal fluid (CSF) samples and four brain autopsy samples were collected from patients afflicted with PML with different clinical courses (fast- and slow-progressing), and the JCV VP1 coding region was amplified, cloned, and sequenced. In addition, urine samples were collected and analyzed from nine patients with PML or other neurological diseases (ONDs) as a control group. Sequencing analysis of the genomic region encoding the VP1 outer loops revealed polymorphic residues restricted to four positions (74, 75, 117, and 128) in patients with slow PML progression, whereas no significant mutation was found in JCV isolated from urine. Collectively, these data show that JCV VP1 loop mutations are associated with a favorable prognosis for PML. It is therefore possible that slower progression of PML may be related to the emergence of a less virulent JCV strain with a lower replication rate. *Journal of NeuroVirology* (2009) **15,** 51–56.

Keywords: amino acid substitutions; disease progression; JC virus; PML

Introduction

JC virus (JCV) is the etiological agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) that affects individuals with reduced immune competence (Walker and Padgett, 1983).

Before the introduction of highly active antiretroviral therapy (HAART), PML was one of the most frequent complications seen in acquired immunodeficiency syndrome (AIDS) patients, with a prevalence of up to 8% (Berger *et al,* 1998). Moreover, it was recognized as a rapidly progressing fatal disease capable of inducing death in less than 6 months from the onset of neurological symptoms (Berger *et al,* 1987; Brooks and Walker, 1984). The introduction of HAART treatment in human immunodeficiency virus (HIV)-positive subjects reduced the frequency of opportunistic infections observed in AIDS patients, but showed no such effect in PML (Antinori *et al,* 2001). However, an important change in the clinical course was observed: nearly half of PML patients receiving HAART developed slower clinical progression, with a survival time of up to

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1 year, which is significantly longer than that observed in classical PML (Albrecht *et al,* 1998; Cinque *et al,* 1998; Clifford *et al,* 1999). Based on this observation, definitions of slow and fast PML (SP and FP PML) progressors, on the basis of the survival time (less or more than 6 months) from the onset of the disease, as well as active and inactive PML (Cinque *et al,* 2003), have become common, but the underlying reasons for increased survival remain unclear.

JCV belongs to the polyomaviridae family of DNA viruses and has a 5130–base pair (bp) circular closed genome that encodes early regulatory proteins (large and small T antigen) as well as late structural proteins (agnoprotein, viral proteins 1, 2, and 3 [VP1, VP2, VP3]), and carries an untranslated transcriptional control region (TCR) (Frisque *et al,* 1984).

The crystal structure of the JCV VP1 protein is not yet available, but based on the homology of JCV with simian virus 40 (SV40) and murine polyomavirus (mPyV) (Liddington *et al,* 1991), it is likely that the JCV VP1 polypeptide is divided into BC, DE, EF, GH, and HI outer domains.

Changes and rearrangements in the JCV genome have been frequently observed, including the two primary types isolated from body tissues and fluids: the archetype (CY) strain found in the urine of healthy subjects and PML patients, and the PML-type (Mad-1 and rearranged forms) isolated from brain tissue and cerebrospinal fluid (CSF) of PML patients (Yogo *et al,* 1990; Ault and Stoner, 1993). Other polymorphisms can occur in the late genomic region that result in modification of the outer loops of the major capsid protein (VP1 loops), which are involved in recognition of the cellular receptor. Recently, new VP1 loop mutations were detected in PML-type JCV isolates at specific amino acid residues within the BC, DE, and HI loops, which were associated with the progression of PML (Zheng *et al,* 2005a, 2005b).

In order to provide insight into PML pathogenesis and identify potential prognostic markers especially after introduction of HAART, CSF and brain autopsy samples collected from patients afflicted with both SP and FP varieties of PML as well as urine collected from PML patients and those with other neurological diseases (ONDs) as a control group were analyzed for the presence of specific VP1 loop mutations.

Results

Nucleotide substitutions were detected in the VP1 region of all the isolates from the CSF, brain tissues, and urine samples of the enrolled individuals. In particular, five clones for each PCR fragment and for each patient were sequenced and 53 different nucleotide substitutions (1/815 sequenced nucleotides), both synonymous and nonsynonymous, were found in the isolates from CSF and brain samples, as compared to JCV Mad-1 strain. In the urine samples, 61 different nucleotide mutations (1/579 sequenced nucleotides) were found, as compared to JCV CY strain. Only substitutions detected in at least three of five clones for each DNA fragment were considered and translated.

Using this approach, 31 different VP1 loop mutations were detected in the CSF and brain samples, whereas 35 (20 in the PML patients and 15 in the OND patients) were identified in the urine samples.

In the urine, amino acid substitutions were detected in four of the five outer loops (not in the HI loop), whereas in the CSF and brain, mutations were found in all five outer loops.

The frequency of amino acid substitutions ranged from one to seven in the CSF and brain, and from one to three in the urine. Polymorphic residues in JCV strains isolated from the CSF and brain were restricted to two (74 and 75) in the BC loop, one (128) in the DE loop, and one (117) in the Alpha-B beta sheet. These substitutions occurred in four to seven different patients, of whom all except one were afflicted with slow-progressing PML.

In the CSF samples, Ala was found at position 128 in four SP PML patients; this configuration is usually found in the JCV GSB, but not in the Mad-1 strain (Loeber and Dorries, 1988). The same substitution was found in three of nine urine samples. In addition, Ser was found at position 74 in the CSF of all slow-progressing PML patients, and also in four of nine urine samples from the controls.

The detected amino acid mutations and their frequencies in CSF, brain, and urine samples are shown in Tables 1 and 2.

Discussion

The precise structure of the JCV virion is not yet known, but it likely shares common features with SV40 and MPyV. The crystal structures of both of these viruses have revealed that VP1 is the major protein in the capsid surface structure (Liddington *et al,* 1991; Stehle *et al,* 1996). In SV40, the capsid has icosahedral symmetry and each VP1 molecule displays elaborate loop structures emanating outward from the beta-sheet framework (Liddington *et al,* 1991). The loops, which also contain α-helix structures, have been named BC, DE, EF, GH, and HI. BC, DE, and HI closely interact at the outward end of the β-sandwich. The BC loop can be divided into two smaller loops, BC1 and BC2. The long EF loop forms a small jelly-roll structure on the side of the β-sandwich.

The external loops have been suggested to provide the principal antigenic structures, receptor-binding sites, and domains responsible for hemagglutination (Chang *et al,* 1996).

Table 1 Frequencies of amino acid substitutions in the VP1 region of JCV isolates from cerebral samples of PML patients

Amino acid position*	Amino acid substitution	Affected regions of VP1	CSF/Brain frequency
55	Leu → Phe	BC	2/11
58	Phe →Cys	BC	1/11
59	Ser →Leu	BC	1/11
60	Lys →Stop	BC	1/11
74	Asn →Ser	BC	4/11
75	Arg →Lys	BC	7/11
101	Leu →Ile	CD	1/11
106	Val →Ala	CD	1/11
113	Leu →Val	Beta-D	1/11
115	Gln →Pro	Beta-D	1/11
117	Ser →Thr	Alpha-B	4/11
123	Ser →Cys	DE	1/11
127	Ala →Gly	DE	1/11
128	Thr →Ala	DE	4/11
134	Gly →Arg	DE	1/11
138	Phe →Leu	DE	1/11
143	Ile →His	DE	1/11
146	Ser →Leu	DE	1/11
147	Ala →Arg	DE	1/11
158	Leu →Val	EF	2/11
163	Thr →Ser	EF	1/11
164	Lys →Met	EF	2/11
167	Glu →Asp	EF	1/11
178	Gln →Phe	EF	1/11
186	Met →Ile	EF	1/11
236	Val →Ala	Beta-G1	2/11
267	Phe →Ser	HI	1/11
268	Gly →Trp	HI	1/11
269	Leu →Phe	HI	1/11
282	Gln →Ala	Beta-I	1/11
284	Arg →Gly	Beta-I	1/11

*Amino acid numbers are those of Mad-1 strain (Frisque *et al,* 1984). BC, DE, EF, and HI: outer loops; Beta-D, Alfa-B, Beta-G1, Beta-I: beta sheets (Chang *et al,* 1996).

Table 2 Frequencies of amino acid substitutions in the VP1 region of JCV isolates from urine of patients with PML or OND

Amino acid position*	Amino acid substitution	Affected regions of VP1	Urine frequency
42	Cys → Trp	Beta-B	1/9
52	Asp →Asn	Beta-B	1/9
53	Glu →Asp	Beta-B	1/9
65	Ser →Tyr	BC	1/9
66	Asp →Thr	BC	2/9
70	Ser →Gly	BC	1/9
74	Asp →Ser	BC	2/9
74	Asn →Ser	BC	2/9
75	Lys →Arg	BC	1/9
92	Asn →Ser	CD	1/9
93	Glu →Gln	CD	1/9
101	Len →Ile	CD	1/9
102	Met →Thr	CD	1/9
104	Glu →Gly	CD	1/9
109	Lys →Stop	Beta-D	1/9
114	Gly →Ile	Beta-D	1/9
117	Thr →Ser	Alpha-B	2/9
118	Len →Ser	Alpha-B	1/9
127	Ala →Pro	DE	1/9
128	Thr →Ala	DE	3/9
133	Glu →Ala	DE	1/9
134	Gly →Ala	DE	2/9
135	Arg →Asn	DE	1/9
158	Leu →Val	EF	2/9
162	Lys →Thr	EF	1/9
164	Lys →Thr	EF	2/9
165	Tyr →Val	EF	1/9
165	Tyr →Asp	EF	1/9
167	Asp →Glu	EF	1/9
185	Glu →Val	EF	1/9
198	Val →Len	EF	1/9
205	Pro →Leu	EF	1/9
208	Asn →Asp	EF	1/9
230	Thr →Ala	Beta-G1	1/9
235	Thr →Ala	Beta-G2	1/9

*Amino acid numbers are those of CY strain (Yogo *et al,* 1990). BC, DE, EF, and HI: outer loops; Beta-D, Alfa-B, Beta-G1/G2, Beta-I: beta sheets (Chang *et al,* 1996).

Previous studies (Zheng *et al,* 2005a,b) have shown that several mutations can occur within the VP1 outer loops of viral strains isolated from the CSF and brain, but not the urine, of PML patients.

The purpose of our study was to determine whether there is any correlation between VP1 loop mutations and the clinical course of the disease, thereby providing insights into PML diagnosis and prognosis.

Among all the detected nucleotide mutations, only substitutions detected in at least three of five clones for each DNA fragment were considered to be present in the original samples; mutations detected less frequently were probably artifacts generated during PCR (Eckert and Kunkel, 1990).

The data obtained show that all virions amplified from urine samples were identical to JCV CY, with the exception of some sporadic and random point mutations. In the urine, 35 different amino acid changes were found within VP1 loops, but their frequencies were very low and the distribution among the patients were not significant. Contrarily, mutation hot spots in JCV isolated from the CSF and brain were restricted to four positions in four to seven different patients, all but one of whom were affected with SP PML. Interestingly, none of these polymorphic residues was previously reported (Zheng *et al,* 2005a, 2005b).

Given their high frequency in SP PML patients, it is likely that these substitutions have biological significance. In particular, residue 75 is localized within the BC loop, which comprises the main part of the viral binding pocket, is involved in recognition of the cellular receptor, and is a potential sialic acid–binding site for JCV infection. It was also shown that Arg75Gly substitution at this position resulted in nonviable viruses *in vitro* (Gee *et al,* 2004). Similarly, it was recently demonstrated that a single amino acid mutation within the BKV BC loop could produce nonviable viruses and significantly reduce viral spread (Dugan *et al,* 2007).

In our samples, we found an Arg to Lys substitution at position 75 that is usually present in the CY strain. On the basis of results previously reported (Gee *et al,* 2004), we consider that this mutation

could decrease the ability of the virus to bind to cellular receptors, thereby weakening its virulence.

We also found a Ser to Thr substitution at position 117 in the CSF of SP PML patients that is usually found in the CY strain; thus, it seems that a form of JCV similar to the archetypal strain and perhaps characterized by decreased replication ability infects the CNS of patients with SP PML.

On the other hand, a JCV strain with Lys60Stop and Ser267Phe mutations was isolated from the CSF of one patient affected with FP PML. These substitutions were already reported by Zheng and colleagues (2005b) and were associated with a worsened outcome. It is possible that these mutations either increase the affinity of the virus for the cell receptor or prevent the neutralization of JCV by specific antibodies, thereby permitting evasion of the immune system and access to oligodendrocytes.

In summary, we conclude that VP1 loop mutations at specific residues could be associated with a more favorable clinical course for PML and that after the introduction of HAART, the JCV genomic organization has changed, although the biological significance of these changes still needs to be elucidated and the case study to be enlarged.

Methods

Eleven HIV-positive PML patients (seven males and four females, mean age: 47 years) were enrolled in the study and, on the basis of the survival time from the onset of the disease, they were classified as six fast progressors (FP; less than 6 months of survival time) and five slow progressors (SP; more than 6 months of survival time). CSF samples were collected from two FP PML living patients and from five SP PML living patients, whereas four brain autopsy samples were taken, between 20 and 56 h after death, from FP PML patients and were formalin fixed and paraffin embedded.

In addition, four HIV-positive SP PML and five HIV-positive OND patients were enrolled in the control group, matched for sex and age with the cases (six males and three females, mean age: 49 years) and urine samples were collected. Main demographic and pathological data of the patients and the controls are shown in Table 3.

All patients were recruited from the Infectious Disease Department of Policlinico S. Matteo in Pavia, the Neurological Department of Neurological Clinic Mondino in Pavia, IRCCS, and the Infectious Disease Department of Ospedale Sacco in Milano and gave written consent for enrolment in the study.

Viral DNA was isolated from CSF and urine samples using RNA Virus Spin Kit (Macherey Nagel, Germany) following the manufacturer's instruction.

DNA was obtained from brain tissues according to standard procedures. Briefly, two 10-μm sections were deparaffined and incubated in digestion buffer, containing proteinase K (20 mg/ml), at 52°C overnight and, after phenol-chloroform extraction and ethanol precipitation, the DNA was resuspended in water and the DNA amount was read with a spectrophotometer.

Table 3 Main demographic and pathological features of the enrolled patients and controls

Patient	Age	Sex	Sample type	Diagnosis
1	51	F	CSF	FP PML
2	55	M	CSF	FP PML
3	60	M	CSF	SP PML
4	45	F	CSF	SP PML
5	48	M	CSF	SP PML
6	42	M	CSF	SP PML
7	47	F	CSF	SP PML
8	29	F	Brain autopsy	FP PML
9	48	M	Brain autopsy	FP PML
10	31	M	Brain autopsy	FP PML
11	65	M	Brain autopsy	FP PML
Control	Age	Sex	Sample type	Diagnosis
1	42	M	Urine	SP PML
2	60	M	Urine	SP PML
3	32	M	Urine	SP PML
4	55	M	Urine	SP PML
5	63	F	Urine	Neuropathy
6	42	F	Urine	Neuropathy
7	30	F	Urine	Neuropathy
8	73	M	Urine	Neuropathy
9	40	M	Urine	Neuropathy

Note. FP, fast progressing; SP, slow progressing.

All the collected samples were previously assayed for the presence of JCV LT antigen DNA by means of real-time polymerase chain reaction (PCR) (Delbue *et al,* 2005).

Seminested PCR

Seminested PCR, modified from Zheng *et al* (2005), was used to amplify the VP1 region of JCV DNA from CSF and brain tissue samples. The target of amplification was a 797-bp region of the VP1 gene corresponding to the BC, DE, EF, GH, and HI loops. The primers used for the first step of nested PCR were VP1 1.1 (nucleotides [nt] 1569 to 1592) and VP1 4a (nt 2353 to 2330), whereas the second step was performed using VP1 1.1 and JLP16 (nt 1902 to 1924) for the first fragment and VP1 1.5 (nt 1877 to 1898) and VP1 4a for the second one. Seminested PCR generated two fragments: the first, delimited by VP1 1.1 and JLP16, was 356 bp in length, whereas the second one, delimited by VP1 1.5 and VP1 4a, was 477 bp in length. Both the seminested PCR mixtures contained 5 units/μl of Taq DNA Polymerase (Roche, USA), buffer 10 ×, dNTP 0.6 μM, Mg^{2+} 2.0 mM, and forward and reverse primers at 0.6 μM.

From 3 to 7 μl of sample DNA from CSF, urine, and brain tissues (final concentration of DNA for each reaction: from 6 to 14 ng/ μl), for the first step, and 3 μl of the first amplification for the second step were added to the PCR mixture to obtain a total

reaction volume of 50 µl. After activation at 94°C for 5 min, the first step of amplification was performed at 63°C for 30 cycles and seminested PCRs were performed at 66°C and 67°C for 30 cycles.

To avoid contamination, three isolated rooms were used: one for DNA extraction, one for PCR, and one for analysis of PCR products.

Cloning of amplified fragments

The amplified fragments were analyzed with 2% agarose gel electrophoresis. The band corresponding to the fragment was excised with a clean cutter and DNA was extracted using a gel extraction kit (Qiagen, Germany), following the manufacturer's protocol. The fragments were cloned using a TA Cloning kit (Invitrogen, USA): PCR fragments were ligated into pCR 2.1 vector with a ratio of 1:1 (vector:insert) and then the construct was transformed into competent *Escherichia coli* INVαF strain.

For each PCR fragment, DNA was isolated from ten recombinant clones carrying partial VP1 gene sequences using a Plasmid Purification Minikit (Qiagen, Germany) and recombinant clones were selected by digestion with a combination of HindIII and XbaI (Roche, USA).

Sequencing of amplified fragments

Clones testing positive after digestion (at least five for each cloned PCR fragment) were sequenced using T7 primers and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) as previously described (Delbue *et al,* 2005). The obtained genome sequences were translated using ExPASy software available online (http://www.expasy.org/Expasy Hunt/; ExPASy & Health On the Net Foundation) and a consensus sequence for each patient was built using Clustal W software (http://www.ebi.ac.uk/Tools/clustalw/ (Chenna *et al,* 2003). Consensus sequences from CSF and brain samples were aligned with the JCV Mad-1 prototype isolate, whereas sequences from urine specimens were aligned with the JCV CY isolate (Yogo *et al,* 1990; Ault and Stoner, 1993).

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