Sequencing and Analysis of JC Virus DNA From Natalizumab-Treated PML Patients

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Background. Progressive multifocal leukoencephalopathy (PML) in natalizumab-treated MS patients is linked to JC virus (JCV) infection. JCV sequence variation and rearrangements influence viral pathogenicity and tropism. To better understand PML development, we analyzed viral DNA sequences in blood, CSF and/or urine of natalizumab-treated PML patients.

Methods. Using biofluid samples from 17 natalizumab-treated PML patients, we sequenced multiple isolates of the JCV noncoding control region (NCCR), VP1 capsid coding region, and the entire 5 kb viral genome.

Results. Analysis of JCV from multiple biofluids revealed that individuals were infected with a single genotype. Across our patient cohort, multiple PML-associated NCCR rearrangements and VP1 mutations were present in CSF and blood, but absent from urine-derived virus. NCCR rearrangements occurred in CSF of 100% of our cohort. VP1 mutations were observed in blood or CSF in 81% of patients. Sequencing of complete JCV genomes demonstrated that NCCR rearrangements could occur without VP1 mutations, but VP1 mutations were not observed without NCCR rearrangement.

Conclusions. These data confirm that JCV in natalizumab-PML patients is similar to that observed in other PML patient groups, multiple genotypes are associated with PML, individual patients appear to be infected with a single genotype, and PML-associated mutations arise in patients during PML development.

The emergence of progressive multifocal leukoencephalopathy (PML) in multiple sclerosis (MS) patients treated with natalizumab has motivated a search for biological factors contributing to the risk of this serious brain infection. PML is caused by the JC virus (JCV), which is estimated to be present in 50%–60% of the world's adult population [1–3]. In infected individuals, JCV can persist and replicate asymptomatically in the urinary tract and possibly other organs [4]. In immunocompetent individuals, the virus is rarely found outside of the urinary tract [5]. However, under conditions of severe immunosuppression or treatment with specific immunomodulating drugs, the virus may establish a lytic infection in oligodendrocytes, leading to PML [6, 7].

Although the percentage of people infected with JCV is high, PML is a rare disease. It is most widely seen in HIV/AIDS patients in whom the lifetime incidence ranges from 3% to 5% [8, 9], although these numbers have begun to decline with the use of combined antiretroviral therapy [10]. In the past decade, however, an increasing number of non–HIV/AIDS–related cases of PML have been reported. Many of these new cases occur in individuals on new and emerging immunomodulating therapies [11]. In 2005, 3 cases of PML were reported in patients undergoing natalizumab therapy for MS or Crohn's disease [12–14]. PML has also been linked to other immunomodulating therapies, including efalizumab, mycophenolate mofetil, and rituximab [15]. Since 2005, 108 additional cases of PML associated with natalizumab therapy have been reported. Natalizumab is a humanized monoclonal antibody that limits the entry of immune cells to the central nervous system (CNS) by binding to the alpha-4 chain of integrin molecules and

Received 7 December 2010; accepted 23 February 2011.

Potential conflict of interest: All authors are employees of Biogen Idec, Inc.

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The Journal of Infectious Diseases 2011;204:237–44

0022-1899 (print)/1537-6613 (online)/2011/2042-0012$14.00

DOI: 10.1093/infdis/jir256

blocking adhesion of peripheral mononuclear blood cells to endothelial cells of the blood-brain barrier [16]. Initially, natalizumab-associated PML was reported in patients on combination therapy, but all subsequent cases have occurred in patients on monotherapy [17]. While the presence of JCV has been established in all confirmed cases of natalizumab-associated PML, the DNA sequences of the viruses have not been reported.

JC virus is a member of the genus *Polyomavirus*, which includes JCV, BK virus, WU virus, KI virus, Merkel cell polyomavirus, Simian Virus 40, and mouse polyomavirus [18]. The genome is a double-stranded, circular DNA molecule of roughly 5100 bases. The genome encodes 6 proteins and can be divided into 3 segments: early genes, late genes, and a noncoding control region (NCCR; also known as the transcription control region, or TCR) [19]. The proteins encoded by the early region genes (small t-antigen and large T-antigen) are involved in viral replication and transcription of the late region genes. The late genes encode the capsid proteins VP1, VP2, and VP3, as well as the regulatory protein agnoprotein. The NCCR includes the origin of replication, as well as sequences that control transcription of both early and late genes. Within individual hosts, JCV persists in at least 2 forms: a latent, nonpathogenic form and a virulent neurotropic form. The neurotropic form contains a rearranged NCCR and is typically found in the cerebrospinal fluid (CSF), brain, or blood of PML patients. The nonpathogenic form is most frequently detected in urine, and its NCCR is not rearranged [20]. NCCR rearrangements involve deletions and duplications of specific sequence elements (reviewed by Yogo and colleagues [21]) and are thought to play a role in the pathogenesis of the virus by altering its cellular tropism. More recently, observations of point mutations in the VP1 capsid protein have also been shown to be associated with PML [22–25]. Currently there is no consensus on whether specific JCV genotypes are preferentially associated with PML [26–32], and existing literature does not address the question of whether the pathogenic form is acquired as a new infection or if it arises from alterations of latent virus.

To better understand the emergence of the pathogenic form of JCV in natalizumab-treated patients, we conducted a cross-sectional study, incorporating samples obtained at or near the time of PML diagnosis. We isolated and sequenced JCV from available plasma, urine, and CSF samples of 17 PML patients. Using a combination of locus-specific and whole viral genome analysis, we obtained data on JCV genotype, NCCR rearrangement, and VP1 capsid protein sequence variation. To analyze the JC virus associated with natalizumab-treated PML patients, we sequenced viral isolates from urine, blood, and CSF of individual patients wherever all 3 were available. Our analysis of multiple sequences, often from more than 1 anatomical location in individual patients, provides new insights into JCV sequence diversity, features of NCCR rearrangements, and the relationship between NCCR rearrangement and VP1 point mutations.

METHODS

Samples

CSF, plasma, serum, and urine samples were obtained from natalizumab-treated MS patients suspected of having or confirmed to have PML. All samples were kept frozen and thawed on ice prior to use for extraction of viral DNA.

Viral DNA Extraction

JCV DNA was extracted from biofluids using commercial kits according to the manufacturers published protocols. For extraction of viral DNA from urine (3.5 mL) samples, the QIAamp Viral RNA Mini Kit was used (Cat 52904; Qiagen, Inc.). For all other samples (CSF, plasma) we used the QIAamp MinElute Virus Spin Kit (Cat 57704; Qiagen, Inc.).

Polymerase Chain Reaction (PCR) Amplification

For amplification of the NCCR and VP1 coding sequences from the viral DNA, we used the Herculase II Fusion Enzyme system (Cat 600677; Agilent Technologies, Inc.). For NCCR amplification we used the following primers: 5′ GATTCCTCCCTATTCAGCACTTTG 3′and 5′ TCCACTCCAGGTTTTACTAA 3′. The entire VP1 coding region was amplified with the following primers: 5′ CCTCAATGGATGTTGCCTTT 3′ and 5′ AAAACCAAAGACCCCTC 3′.

Cloning and Sequencing

PCR amplification products were cloned using the TOPO TA Cloning Kit for sequencing (Cat 45-0030; Qiagen, Inc.). Prior to ligation of the PCR products into the TOPO vector, 3′ terminal adenines were added to the insert by incubation with Taq polymerase and additional deoxyribonucleotide triphosphates (dNTPs) for 15 minutes at 72°C. Ligation products were transformed and plated according to manufacturer's specifications. When possible, up to 48 individual colonies for each cloned product were screened and sequenced by Applied Biosystems 3730XL DNA Analyzer with BigDye Terminator version 3.1 chemistry.

Viral Sequence Accession Numbers

All viral sequences have been deposited into GenBank and assigned the following accession numbers: JF424834 – JF426135.

JCV Whole Genome Amplification

Whole genome amplification of JCV was accomplished in 3 steps using a modification of the protocol from Agostini HT, Stoner, GL [33]. In the first step, template DNA was amplified by multiple displacement amplification (MDA) using the REPLI-g kit (Cat 150043; Qiagen, Inc.). In the second step, the MDA product was linearized by digestion with a single cutting enzyme, EcoR1, which resulted in fragments of whole genome length. In the final step, the fragments were used as a template for full-length genome amplification using Phusion Hot Start

High-Fidelity DNA Polymerase (NEB Cat F-540S; Finnzymes). We used the following primers (the EcoR1 sites are in bold):5′ GTTTCTTA**GAATTC**CACTACCCAATCTAAATGAGGAT 3′ and 5′ GTTTCTTTG**GAATTC**TGGCCACACTGTAACAAG 3′.

PCR products of appropriate length were confirmed by gel electrophoresis, cloned, and sequenced.

Data Analysis

The determination of JCV genotype was based on known polymorphisms in the VP1 capsid coding sequence [34]. Genotypes were defined by alignment and degree of identity to reported genotypes at 12 amino acid positions. When the VP1 sequences were not perfectly matched to any of the genotypes, we mapped them to the nearest genotype and appended a "v" to indicate they were variants of a standard JCV genotype. To confirm strain designations we performed phylogenetic analysis on our VP1 sequences and found that all isolates from the same genotype clustered together (data not shown). Outside of the genotype defining sites, any variations in VP1 were compared with a VP1 variability table [23], which identified amino acid positions that were hypervariable in virus derived from PML patients compared with non-PML patients.

For analysis of the NCCR, the sequences were aligned and mapped to the archetype JCV NCCR [35]. The 267 bases of the archetype are commonly divided into segments to describe recurrent patterns of deletion and duplication [27, 32, 36]. The 7 regions are ORI, A, B, C, D, E, and F sequentially from 5′ to 3′. We used the combinations of letters to describe different NCCR patterns. In our notation, a capital letter in the NCCR pattern represents a perfect match to the corresponding region of the archetype sequence, while a lowercase letter indicates a region with deletion, and an asterisk denotes a point mutation.

For whole genome analysis, we used the MargFreq (software) Program to score and annotate amino acid positions that varied across the groups of genomic sequences. In total, 396 individual JCV whole genome sequences were retrieved from Genbank (25 from brain, 371 from urine), translated into individual viral protein sequences, and compared with 152 individual viral genome sequences from Tysabri PML patients.

RESULTS

Comparative analysis of JCV within and among natalizumab-treated PML patients showed that patterns of DNA sequence variation are similar to what has been observed in AIDS and other PML patients. CSF, blood, and urine samples from natalizumab-treated PML patients were collected at or near the time of diagnosis. To maximize the use of samples, we performed 3 independent amplifications on each viral DNA sample: NCCR, VP1, and whole genome. At least 1 of these reactions succeeded for samples from 17 natalizumab-treated PML patients. Whole JCV genomes were successfully amplified from 6 PML patients. Independent amplifications of the VP1 region (without whole genome amplification) were completed for 16 patients, and for 13 patients we obtained both VP1 and NCCR sequence data (Table 1). The 6 patients from which the JCV whole genome was sequenced also have independently derived VP1 and NCCR sequences that matched the sequences found on the whole genomes. All sequence data were obtained from an average of 16 isolates of cloned PCR products. We controlled for cross-contamination by including a "buffer-alone" sample each time we performed the viral DNA extraction and PCR amplification protocols. Analysis of the NCCR sequence data revealed that every viral DNA sample that was rearranged had a unique NCCR sequence. This served as an internal control for our NCCR amplification reactions and confirmed that none of our NCCR sequences matched the Mad-1 laboratory strain. While the most frequently observed genotype was type 1B, found in 7 patients, we did not observe a relationship between JCV genotype and PML development (Table 1).

NCCR Sequences

As shown in Table 1, we obtained NCCR sequences from 25 PML samples (14 patients). Five samples were from urine, 11 from CSF, and 9 from blood. For most samples, the PCR products were cloned and as many as 47 isolates sequenced. We sequenced a total of 579 NCCR sequences. Consistent with other studies [20, 22], we found that all NCCR sequences from urine samples matched the archetype sequence. In contrast, all sequences from PML-associated CSF or blood were rearranged, containing deletions, tandem repeats, or both relative to the archetype reference sequence. In certain patients, we observed multiple patterns of NCCR rearrangement in a given compartment (ie, CSF or blood) (Table 1; Figure 1A–C). In some of these patients, the more common variant in each body compartment was identical. Analysis of how these rearrangements impacted transcription factor binding sites within the JCV NCCR revealed that the NF-1 binding site was the most frequently duplicated, with other sites such as AP-1 and SP-1 being less affected (see Supplemental Material). While the NCCR rearrangements were distinct from one patient to another, the most frequent events involved deletions of all or part of segment D. Deletion of all or part of segment D occurred in all but 1 rearranged virus from the CSF or blood of all the PML patients we studied (Figure 2).

VP1 Sequences

We and others have shown that mutations near the sialic acid binding pocket of the viral VP1 capsid protein may be associated with PML development [22, 23]. We sequenced the entire VP1 coding region of JCV DNA isolated from blood, CSF, and/or urine of 16 patients. Our findings are summarized in Table 1. Eighty-one percent of patients (13/16) had VP1 point mutations. For patients with urine, as well as blood or CSF sequences, the mutations were not detected in virus from urine. Nearly 50%

Table 1. Patient Samples, Strain Type, and Mutational Status of JC Viruses From 17 Natalizumab-Treated PML Subjects

		VP1			NCCR		
ID	Matrix	Genotype	Mutation	No. of Clones	TCR	Pattern	No. of Clones
PML-001	CSF	2B	WT	48	R	ORI_A_b_c_c_e_f	44
PML-002	CSF	1B	D66H	41	–	–	–
PML-004	PLASMA	1BV	WT	26	–	–	–
	URINE	1BV	WT	50	A	ORI_A_B_C_D_E_F	39
PML-005	CSF	1B	D66G	48	–	–	–
PML-006	CSF[a]	1AV	WT	3	R	ORI_A_B_c_d_e_b_c_d_E_f	32
			L55F	25		ORI_A_B_c_d_E_f	1
			N265S	7			
			L55F, N265S	4			
	SERUM	1AV	S267F	24	R	ORI_A_B_c_d_e_b_c_d_E_f	38
						ORI_A_B_c_d_E_f	1
PML-007	CSF[a]	1B	S269F	6	R	ORI_A_B_c_c_E_F	20
PML-009	CSF[a]	1A	S267L	5	R	ORI_A_B_C_d_E_f_c_d_E_f	18
						ORI_A_B_C_d_e_c_d_e_f	3
						ORI_A_B_C_d_E_f_c_d_E_f_c_d_E_f_c_d_E_f	1
	PLASMA	1A	S267L	23	R	ORI_a_b_c_c_F	23
	URINE[a]	1A	WT	2	A	ORI_A_B_C_D_E_F	22
PML-012	CSF	1B	L55F	17	R	ORI_A_C*_E_a_C*_E_f	21
	PLASMA	–	–	–	R	ORI_A_C*_E_a_C*_E_f	20
	URINE[a]	1B	WT	22	A	ORI_A_B_C_D_E_f	13
PML-013	PLASMA[a]	1AV	L55F	21	R	ORI_A_B_c_f_c_E_f	30
	URINE[a]	1AV	WT	23	A	ORI_A_B_C*_D_E_f	17
PML-019	CSF[a]	2B	S61P	46	R	ORI_A_B_c_d_e_f_b_c_d_e_F	23
	SERUM	2B	S61P, S61T	19	R	ORI_A_B_c_d_e_f_c_d_e_f	9
						ORI_A_B_c_d_e_f	1
	URINE	2B	WT	1	–	–	–
PML-021	CSF[b]	1A	S269F	1	R	ORI_A_B_c_d_E_F	21
	PLASMA[b]	1A	S269F	1	–	–	–
PML-022	CSF	1A	WT	46	R	ORI_A_B_C_E_C_E_f	21
PML-025	CSF[b]	2AV	S269F	1	R	ORI_A_B*_C_D_E_f_f	22
	SERUM	2AV	S269F	1	R	ORI_A_B*_C_D_E_f_f	24
	PLASMA	2AV	S269F	1	R	ORI_A_B*_C_D_E_f_f	24
	URINE	2AV	WT	1	A	ORI_A_B_C*_D_E_F	1
PML-030	CSF[b]	1B	S267F, Q271H	1	R	ORI_A_B_c_E_f_b_c_E_F	25
PML-032	CSF	–	–	–	R	ORI_A_B_c_b_c_E_F	23
	PLASMA	–	–	–	R	ORI_A_B_c_b_c_E_c_E_f	22
						ORI_A_B_c_b_c_E_f	1
PML-033	SERUM	1BV	L55F, Q271K	23	R	ORI_A_B_c_e_b_c_E_F	7
						ORI_A_B_c_e_c_E_F	6
						ORI_A_B*_C_e_c_E_F	5
						ORI_A_B_c_e	1
142–101	PLASMA	2B	L55F	5	–	–	–
	Total			542			579

NOTE. Summary of viral sequence data. VP1 coding region and transcriptional control regions were PCR amplified, cloned, and sequenced. The number of clones sequenced for each amplification is indicated. All sequences were compared with reference genotype NC_001699 (Genbank). Wild-type (WT), rearranged (R), archetype (A), no data (-). The NCCR patterns are described using letters to designate NCCR segments (Frisque and Yogo), and are described further in the Methods section. Capital letters indicate that a segment is identical to the reference archetype. Lowercase letters indicate that a segment has an insertion or deletion. Asterisks (*) indicate a point mutation or single nucleotide polymorphism relative to the reference archetype.

[a] Sequenced JCV whole genome from this sample.

[b] Sample not cloned (sequenced bulk PCR product). CSF, cerebrospinal fluid; NCCR, noncoding control region; PCR, polymerase chain reaction; PML, progressive multifocal leukoencephalopathy; TCR, transcription control region.

of the mutations observed were at 2 positions (L55 and S269), residues known to be important for sialic acid binding by JCV [22, 23, 38–41]. The other VP1 mutations observed, though less frequent, were in positions where mutations associated with PML have been reported elsewhere [22].

JCV Whole Genome Analysis

Eight samples from 6 patients were suitable for whole genome amplification and sequencing (Table 2). For each sample we amplified and cloned the genome, followed by sequencing between 2 and 45 individual isolates. We looked for evidence of

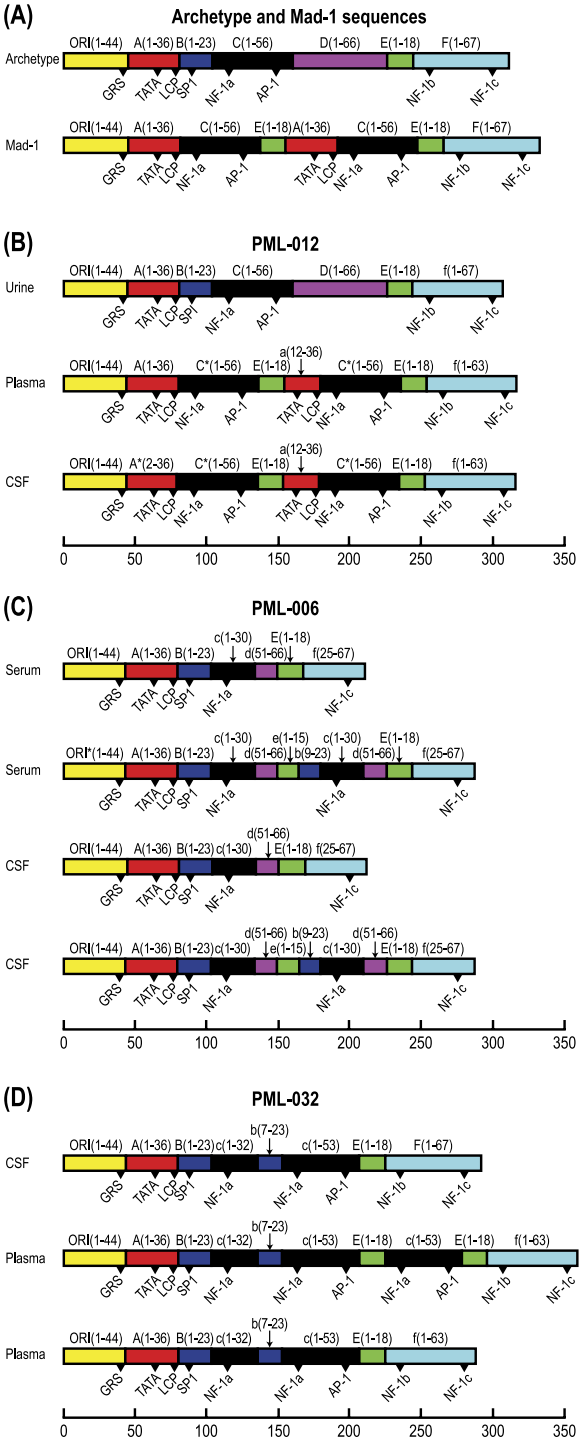

Figure 1. Example of NCCRs from different biofluids of 3 natalizumab-treated PML patients. *A*, Archetype and Mad-1 NCCRs (shown for comparison); *B*, PML-012; *C*, PML-006; and *D*, PML-032. Urine-derived sequences always matched archetype, while sequences from CSF and blood were often identical to each other or contained related patterns of rearrangement. Letter designations and numbering for NCCR segments are described in Table 1 and in Methods section. CSF, cerebrospinal fluid; NCCR, noncoding control region; PML, progressive multifocal leukoencephalopathy.

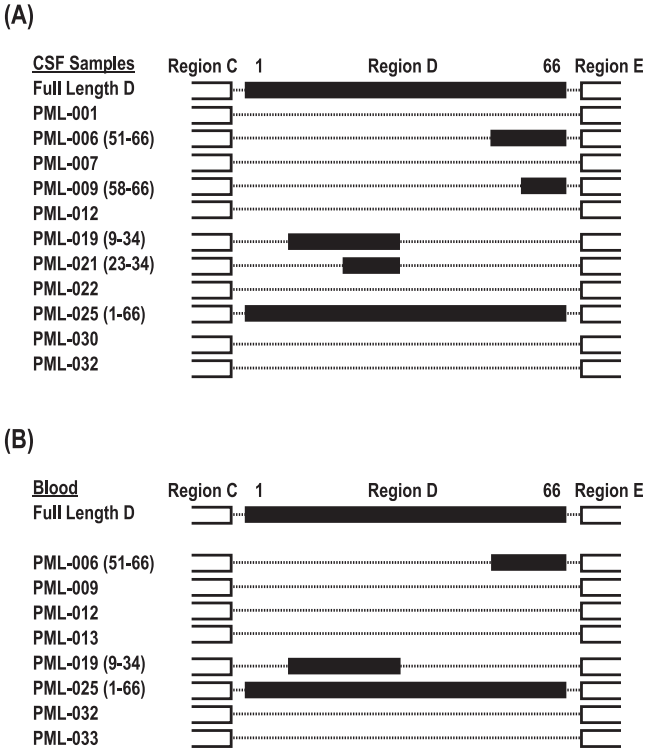

Figure 2. The NCCR from CSF or blood of PML patients frequently contained deletion of all or part of region D. *A*, Schematic of region D from CSF samples. *B*, schematic of region D from blood samples. Numbering according to Yogo and colleagues and Frisque and colleagues [20, 37]. CSF, cerebrospinal fluid; NCCR, noncoding control region; PML, progressive multifocal leukoencephalopathy.

comutations between the NCCR and VP1 coding regions and for mutations in the 5 other protein coding genes (agnoprotein, VP2, VP3, large T- and small t-antigens) that might suggest the involvement of these viral proteins in the development of PML. We compared the JCV genomic sequences of natalizumab-treated PML patients to publicly available JCV genomes. JCV genomes derived from CSF samples were the most heterogeneous, while urine-derived genomic sequences were relatively homogeneous. In every case, the VP1 and NCCR sequences from whole genomes matched their counterparts from the targeted sequencing (Tables 1 and 2). We found 2 novel variants (T125A, C560S) in the large T coding region among the genomes we sequenced (Table 3; online only). All other coding variants observed in the natalizumab-treated PML patient genomes were at sites of known genotype-specific heterogeneity.

DISCUSSION

This is the first detailed study of JCV sequence variation in natalizumab-associated PML. In 17 natalizumab patients from the United States and Europe, we observed 6 different JCV genotypes or genotype variants. The most frequently observed genotype was

Table 2. NCCR and VP1 Mutation Patterns Observed in Full-Length JC Virus Sequences From 6 Natalizumab-Treated PML Patients

ID	Matrix	Genotype	NCCR	VP1	No. of Clones
PML-006	CSF	1AV	R	L55F	18
				N265S	9
				S269Y	1
				WT	10
PML-007	CSF	1B	R	S269F	7
PML-009	CSF	1A	R	S267L	20
	Urine	1A	A	WT	17
PML-012	Urine	1B	A	WT	2
PML-013	Plasma	1AV	R	L55F	45
	Urine	1AV	A	WT	6
PML-019	CSF	2B	R	P51S	8
				S61P	9
	Total				152

NOTE. Summary of JCV whole genome sequence data. Wild-type (WT), rearranged (R), archetype (A). For each of these samples, we also obtained results from PCR and cloning of NCCR and VP1 independently, and those data are represented in Table 1. For all genomic clones a single species of TCR was seen in each sample matching the pattern described in Table 1. CSF, cerebrospinal fluid; NCCR, noncoding control region; PCR, polymerase chain reaction; PML, progressive multifocal leukoencephalopathy.

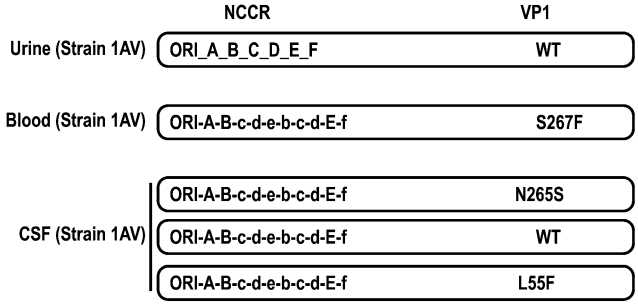

Figure 3. PML-associated mutations develop within individual hosts. Comparison of VP1 and NCCR sequences from whole genomes of a natalizumab-treated PML patient (PML-006). Analysis of virus from different biofluids showed that this patient was infected with a single viral genotype and that PML-associated mutations were present in virus found outside of the urine. While multiple VP1 capsid mutations were observed, all mutated viral species contained identical NCCR rearrangements. NCCR, noncoding control region; PML, progressive multifocal leukoencephalopathy; WT, wild type.

type 1B in 30% of our subjects. This is a relatively common genotype in the United States and Europe [28, 42]. We also observed genotypes 1A, 1AV, 1BV, 2AV, and 2B. Although this is a limited sample set, it does not appear that particular geographic genotypes are associated with PML in our patient cohort.

JCV in natalizumab-treated PML patients appears to be similar to that in AIDS and other PML patients, with the most variable regions of the viral genome being the NCCR and VP1 coding sequences. Rearrangements of the NCCR were observed in 100% of the virus isolated from CSF or blood (Table 1). Mutations in the VP1 capsid protein were found in virus isolated from the CSF or blood of 13 (81%) of 16 patients analyzed and were not found in urine. In 5 patients we had matched urine and nonurine samples in which the VP1 mutation was present in the blood and/or CSF but not present in the urine. Each of these patients had a single genotype of virus, suggesting within-patient changes in this gene.

While both NCCR rearrangements and VP1 point mutations were prevalent in JCV from the CSF and/or blood of natalizumab-treated PML patients, the NCCR changes were more common and appeared to precede VP1 mutations. This was suggested by whole genome data that showed a variety of VP1 mutations in the background of a constant NCCR pattern (Table 2, Figure 3). Also, 2 of the 3 PML patients without VP1 mutations had NCCR rearrangements (the other subject's NCCR failed to amplify).

The NCCR sits between the viral early gene large T and the late gene agnoprotein and is 267 base pairs in the archetype JCV sequence. The 267 bases are commonly divided up into 6 unequal fragments, A–F, facilitating the description of recurrent patterns of deletion and duplication [27, 32, 36]. Analysis of the NCCR regions of our PML cohort revealed a variety of rearranged patterns, with each patient having a unique pattern of deletions and duplications (Table 1). Segment D was the most commonly affected segment, being partially or completely deleted in CSF or blood of 12 (92%) of 13 patients (Figure 2). Segment B was also frequently deleted. These observations are consistent with NCCR rearrangements seen in AIDS patients with PML [43].

In cases where more than 1 rearranged NCCR pattern was present in a sample, we often observed a short form of the NCCR, with deletion but no duplication, and a long form with both deletion and duplication (Figure 1*C*, 1*D*). This observation suggests that deletions may occur prior to duplication in the process of NCCR rearrangement. In patients from whom we obtained NCCR sequences from both CSF and blood, most contained similar or identical NCCR rearrangement patterns across those biofluids within the individual patients. While this suggests that the rearranged virus spread from one compartment to the other, our data do not reveal exactly where in the host NCCR rearrangements occur. They may occur outside of the CNS and travel through the blood, or they may occur first in the CNS with subsequent release into the blood. Indeed, it also remains possible that viral mutations may occur transiently in the urinary tract (or bone marrow) [15] prior to release to other organ systems. Longitudinal studies combined with deep sequencing of viral populations may help reveal the sequence of mutational events leading to changes in tissue tropism and behavior of JCV.

Our analysis of complete JCV genomic sequences from 6 natalizumab-treated patients revealed several important features

of PML-associated mutations. As noted above, we observed NCCR rearrangement without VP1 mutation, but never the opposite. Additionally, we did not observe any pattern of NCCR rearrangement associated with specific VP1 capsid mutations or JCV genotypes. This suggests that NCCR rearrangement and VP1 mutation may be independent events that impact distinct elements of JCV cellular tropism. However, since VP1 mutations have not been observed in JCV with archetype NCCR, VP1 mutations may only arise in JCV with the pathogenic forms of NCCR. With 2 exceptions the sequence variations observed in protein-coding genes other than VP1 were previously described genotype-associated polymorphisms. In 1 patient (PML-013), the virus contained a novel T125A mutation in the gene encoding the large T-antigen protein. In a second patient (PML-019), we observed a C560S mutation, also in large T-antigen (Table 3; online only). Both of these mutations occurred in more than 1 clonal isolate, and neither was found in a search of published JCV T-antigen sequences (Genbank). The importance of these sequence variants is unknown at this time.

PML-associated mutations were notably absent from virus in the urine of natalizumab-treated PML patients. The pattern observed for PML patients is essentially the same as that observed for AIDS and other PML patients: Only archetype virus is detected in urine, and, post-PML diagnosis, rearranged or mutated virus is observed in the bloodstream and CSF, but not in urine. This observation suggests that if NCCR rearrangement ever occurs in the urinary tract, it is rare and is not subject to preferential selection over archetypal virus replication. This is in sharp contrast to a recent description of virus with rearranged NCCR in the urine of a non-PML MS patient treated with natalizumab [44]; the reasons for this discrepancy are not understood at the moment. However, in the unusual case where rearranged virus was reported in urine, it was not associated with PML.

While the immunological perturbations leading to PML in natalizumab-treated MS patients and in AIDS patients appear to be different, the patterns of DNA sequence variation that we observed are strikingly similar. We have shown that in natalizumab-treated PML patients, VP1 mutations and the previously reported NCCR rearrangements are the most common viral alterations associated with PML. Our data are consistent with the model of a common asymptomatic infection with archetype JC virus, frequently found in urine, combined with a rare occurrence of changes in the viral genome that can alter cellular tropism and potentially lead to CNS infection and PML. Since our data are derived from samples that were collected at or near the time of PML diagnosis, it remains to be determined if any of the mutations we observe appear prior to the clinical manifestations of PML. Our findings broaden the information available on JC virus and PML and may be useful in the context of earlier diagnosis of PML or in predicting who is at greater risk for developing PML. Additional research into tissue distribution of JCV in healthy patients, and patients with immune diseases such as HIV or MS, as well as longitudinal studies of patients on therapies associated with PML, will undoubtedly reveal even more about JCV and PML. Combined with these translational approaches, improved methods for the detection of low levels of viral DNA may help stratify PML risk and guide therapeutic strategies.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

Funding

This study was funded by Biogen Idec Inc. Funding to pay the Open Access publication charges for this paper was provided by Biogen Idec Inc.

Acknowledgments

We would like to acknowledge our collaborators and colleagues for their material and technical contributions. We are particularly thankful to Bill Aschenbach, Chao Sun, Helen McLaughlin, Norm Allaire, Sarah Ratsch, and Suzanne Szak for their efforts in support of this publication.

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Reid, CE; Li, H; Sur, G; Carmillo, P; Bushnell, S; Tizard, R; McAuliffe, M; Tonkin, C; Simon, K; Goelz, S; Cinque, P; Gorelik, L; Carulli, JP

Title:
Sequencing and Analysis of JC Virus DNA From Natalizumab-Treated PML Patients

Date:
2011-07-15

Citation:
Reid, C. E., Li, H., Sur, G., Carmillo, P., Bushnell, S., Tizard, R., McAuliffe, M., Tonkin, C., Simon, K., Goelz, S., Cinque, P., Gorelik, L. & Carulli, J. P. (2011). Sequencing and Analysis of JC Virus DNA From Natalizumab-Treated PML Patients. JOURNAL OF INFECTIOUS DISEASES, 204 (2), pp.237-244. https://doi.org/10.1093/infdis/jir256.

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