

Case Report

Genetic Analysis of JC Virus and BK Virus From a Patient With Progressive Multifocal Leukoencephalopathy With Hyper IgM Syndrome

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A case of acute progressive multifocal leukoencephalopathy (PML) with hyper IgM syndrome 1 is reported. Viral DNA and VP1 protein of JC virus (JCV) and BK virus (BKV) were detected by immunohistochemistry, in situ hybridization, semi-nested polymerase chain (PCR) and PCR-restriction enzyme analysis. JCV DNA and VP1 protein were found in the nuclei of oligodendrocytes. The non-coding control region (NCCR) and VP1 region of the JCV genome were sequenced; this revealed a novel rearrangement pattern of the NCCR in the brain tissue. The VP1 regions of brain and urine JCV were identical and of genotype type 2A. The BKV in the urine sample was genotype I. No BKV genome was found in the brain. The novel genomic rearrangement of the JCV NCCR in the brain tissue may have altered JCV pathogenesis to induce PML; the impaired immunity from hyper IgM syndrome 1 may have enabled the rearrangement. The JCV NCCR rearrangement in the brain may have originated from the archetypal form in the urine through deletion and duplication. **J. Med. Virol.** 76:398–405, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: JCV; BKV; NCCR; VP1; nucleotide sequence; hyper IgM syndrome

(PML) as a result of targeted viral lysis of oligodendrocytes [Jensen and Major, 2001]. The brains of patients with PML invariably contain JCV with sequence variations in the non-coding control region (NCCR); these variants may enable the switch from latent to lytic infection. It is accepted generally that these JCV variants are generated from archetypal strains through sequence rearrangement (deletion, duplication, and insertion). Rearrangement is thought to occur during the persistent infection [Ault and Stoner, 1993; Vaz et al., 2000; Jensen and Major, 2001; Pietropaolo et al., 2003].

Hyper IgM syndrome 1 is a rare, X-linked, recessive immunodeficiency disease characterized by failure of immunoglobulin isotype switching from IgM to IgG, IgA, and IgE, leading to low serum IgG, IgA, and IgE, and normal to increased IgM serum levels [Notarangelo et al., 1992]. The disease is caused by mutations of the gene encoding CD40 ligand (CD40L), a cell-surface ligand expressed by CD4⁺ T cells in an activation-mediated regulated fashion [Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Kroccek et al., 1994]. The functional effect of these CD40L mutations is to limit the interaction of the CD4⁺ T cells with the CD40 glycoprotein on the surface of B cells, monocytes, and dendritic cells. The interaction of CD40 and CD40L induces activated monocytes/dendritic cells to secrete IL-12, and binding of IL-12 to the activated T cells induces their differentiation into IFN- γ -producing T cells, which are critical for protection against intracellular pathogens [Grewal et al., 1995]. Thus, individuals

INTRODUCTION

JC virus (JCV) and BK virus (BKV), members of the family Papovaviridae, genus *Polyomavirus*, have a very high prevalence of infection worldwide but very low pathogenicity. Primary infection by JCV or BKV is usually asymptomatic, and the viruses can remain in a latent state in the kidney. Immunodeficiencies that occur in conjunction with leukemia, HIV infection, and organ transplantation can contribute to JCV activation, leading to progressive multifocal leukoencephalopathy

Grant sponsor: Japan China Medical Association Fund.

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Accepted 17 March 2005

DOI 10.1002/jmv.20377

Published online in Wiley InterScience
(www.interscience.wiley.com)

with hyper IgM syndrome 1 suffer from abnormal cellular immune responses and are susceptible to bacterial infection [Notarangelo et al., 1992; Durandy and Honjo, 2001].

In this article, the genetic characteristics of JCV and BKV from an individual with acute PML with the hyper-IgM syndrome 1 were examined.

CLINICAL DATA

A 37-year-old male was admitted to hospital because of blurring of vision. Four months before admission, he had experienced left field loss in writing, and 1 month later, he could not catch a ball coming from the left side. Examination showed a left homonymous hemianopia. A cranial magnetic resonance imaging (MRI) study showed a T2-weighted hyperintensity area in bilateral posterior lobes involving subcortical white matter and sparing the cortex, with no mass effect (Fig. 1). In his past history, he had pneumonia at 12 years of age, and his level of IgA was found to be low. Agranulocytosis was found at 23 years, which resolved spontaneously. Later, chronic otitis media and chronic paranasal sinusitis recurred. In family history, his younger brother died of pneumonia in childhood. His two children were healthy. Laboratory tests showed that, serum IgA, IgG, and IgM were less than 5 mg/dl (normal range 110–410), 539 mg/dl (870–1700), and 488 mg/dl (35–220), respectively. Thus hyper-IgM with low IgA and IgG were found. Flow cytometry demonstrated a deficiency of CD40L, which was consistent with hyper IgM syndrome 1. In brain biopsy from the right posterior lobe, massive demyelination with many lipophages, reactive astrocytes, and perivascular lymphoid infiltration was found. Degeneration was more severe in the white matter than in the cortex. In the periphery of the affected lesions, many oligodendrocytes with intranuclear inclusions and a few large astrocytes with hyperchromatic nuclei were found.

PML associated with the hyper-IgM syndrome 1 was suspected. To test further this hypothesis, immunohistochemistry with antibody against JCV VP1 protein and in situ hybridization with BKV whole genome probe, which has cross-reactivity with JCV genome were undertaken. Positive results by both assays confirmed the diagnosis of PML. After the diagnosis was made, intravenous IgG was administered, but the affected area expanded to the whole brain and the patient died 6 months after diagnosis.

MATERIALS AND METHODS

Immunohistochemistry and In Situ Hybridization

Formalin-fixed, paraffin-embedded brain tissue was available for analysis, and formalin-fixed autopsy brain tissues without known neurological disease were used as negative control. An antibody against JCV VP1 protein (DAKO, Kyoto, Japan), which has no cross-reactivity with BKV [Aoki et al., 1996], was used for immunohistochemistry. Biotinylated whole BKV genome hybridization probe, which has cross-reactivity with JCV genome, and in situ hybridization kit were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). Immunohistochemistry and in situ hybridization were carried out according to the manufacturer's instructions. Cytochemical detection was carried out with horseradish peroxidase and diaminobenzidine tetrahydrochloride as chromogen.

DNA Purification

DNA was extracted from serum, urine, and brain tissue by digestion with protease K. DNA was purified on silica columns (Qiagen GmbH, Germany) by elution with double-distilled water, according to the manufacturer's protocol.

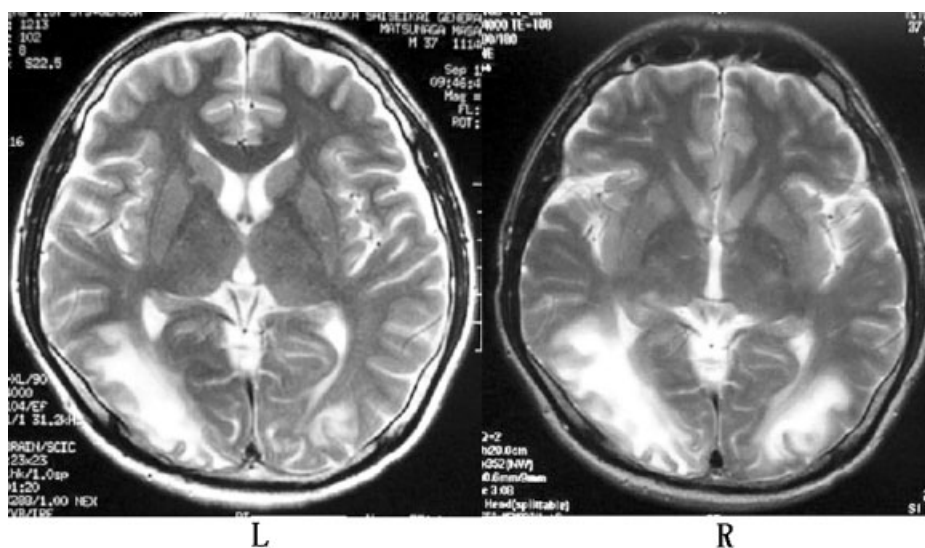


Fig. 1. (L) A cranial magnetic resonance imaging (MRI) study showed a T2-weighted hyperintensity area in bilateral posterior lobes involving subcortical white matter and sparing the cortex, with no mass effect. (R) MRI after 45 days shows the enlargement of the hyperintensity area.

BKV and JCV DNA Detection by Nested PCR

To detect JCV and BKV DNA, a semi-nested PCR assay was designed to amplify the highly conserved large T coding region; primers and conditions were described in detail in previous studies [Arthur et al., 1989; Nickeleit et al., 1999]. This assay was used for all samples including serum, urine, and brain. β -globin genes were amplified as a positive control DNA.

JCV VP1 and NCCR Amplification

For nested PCR, a 215-bp fragment of the viral structural late protein VP1 gene was chosen, which includes the genotyping sites that distinguish the eight JCV genotypes and the different subtypes. The primers were JLP-15 (5'-ACAGTGTGGCCAGAATTCCACTACC-3', 1710–1734) and JLP-16 (5'-TAAAGCCTCCCCCAACAGAAA-3', 1924–1902); nucleotide numbering is based on the prototype JCV Mad-1 from Frisque et al. [1984]. Nested PCR was used to amplify a 353-bp fragment belonging to JCV NCCR. The first pair primers were JRE1 (5'-CCTCCCTATTTCAGCACTTTGT-3', 4989–5009) and LP2 (5'-TACGTGACAGCTGGCGAAGAA-3', 518–537), and the inner primers were RFOR (5'-GCCTCCACGCCCTTACTACT-3', 5085–5104) and RREV (5'-CAGAAGCCTTACGTGACAGC-3', 310–329). The PCR conditions were as described by Pagani et al. [2003].

Nucleotide Sequence

The purified JCV VP1 and NCCR PCR products were sequenced using the primers JLP-15, JLP-16, RFOR, and RREV. The cycle sequencing reaction was performed using the ABI PRISM BigDye^R Terminator v3.1 cycle sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Fluorescence-based nucleotide sequence analysis was obtained on an ABI PRISM 3100 DNA analyzer instrument.

BKV VP1 Amplification and PCR-Restriction Enzyme Analysis

PCR primers 327-1 (5'-CAAGTGCCAAAACACTACTAAT-3', 1630 to 1649) and 327-2 (5'-TGTCATGAAGGTTAAGCATGC-3', 1937 to 1956) were chosen to anneal with invariant regions flanking the subtype-specific region in BKV VP1 region (Nucleotide numbering is based on BKV Dun from Seif et al. [1979].) A 327-bp fragment was used to distinguish the four subtypes of BKV by PCR-RE. The PCR product (15 μ l) was digested with two units of endonuclease (XmnI or AvaII) in a total of 20 μ l of the supplied buffer at 37°C for 1 hr. The reaction mixture was electrophoresed on a 3% agarose gel. The BKV subtype was determined according to the digestion bands as described previously [Li et al., 1995].

RESULTS

Immunohistochemistry and In Situ Hybridization in the Brain With PML

Immunohistochemistry demonstrated that the JCV VP1 protein was expressed in the patient's brain (Fig. 2L). The brain tissues were hybridized with whole BKV DNA probe, which also hybridized with the JCV genome (about 70% homology with BKV genome). Combined with the positive immunohistochemical results of the JCV VP1 protein, the in situ hybridization results (Fig. 2R) confirmed the presence of the JCV genome in the brain. With respect to localization, the JCV VP1 protein and JCV genome were found in the nuclei of some oligodendrocytes in the demyelinated area (Fig. 2).

Identification of BKV and JCV Large T Antigen

A 146-bp fragment encoding large T antigen indicative of the JCV genome was amplified from both brain and urine (Fig. 3A,L). A 149-bp fragment encoding large T antigen indicative of the BKV genome could be

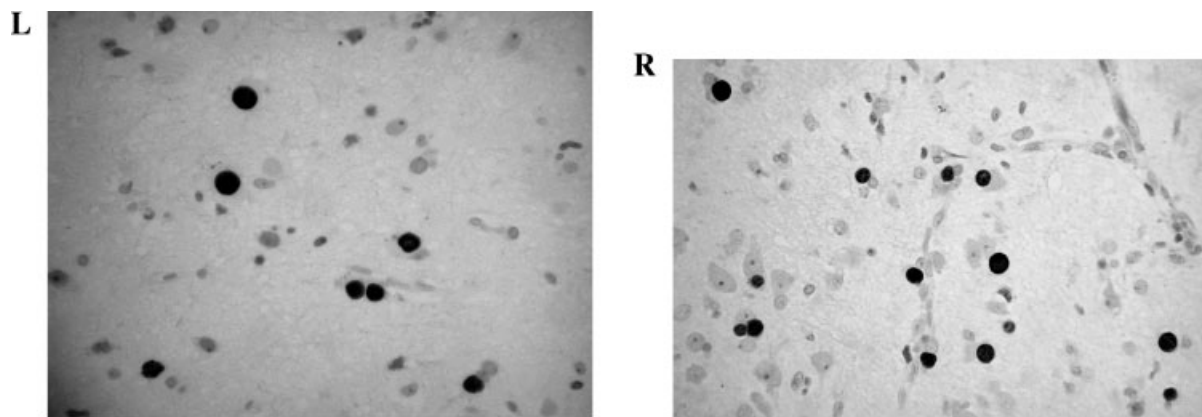


Fig. 2. Detection JCV VP1 protein (L) and JCV DNA (R) in the brain tissue by immunohistochemistry and in situ hybridization (ISH), respectively. Some oligodendrocytes in the demyelination area show nuclear positivity (brown) in both immunohistochemistry and ISH. Immunohistochemistry: Biotin-labeled anti-JCV VP1 protein antibody, ISH: Biotin-labeled DNA probe, hematoxylin counterstain, original magnification $\times 200$.

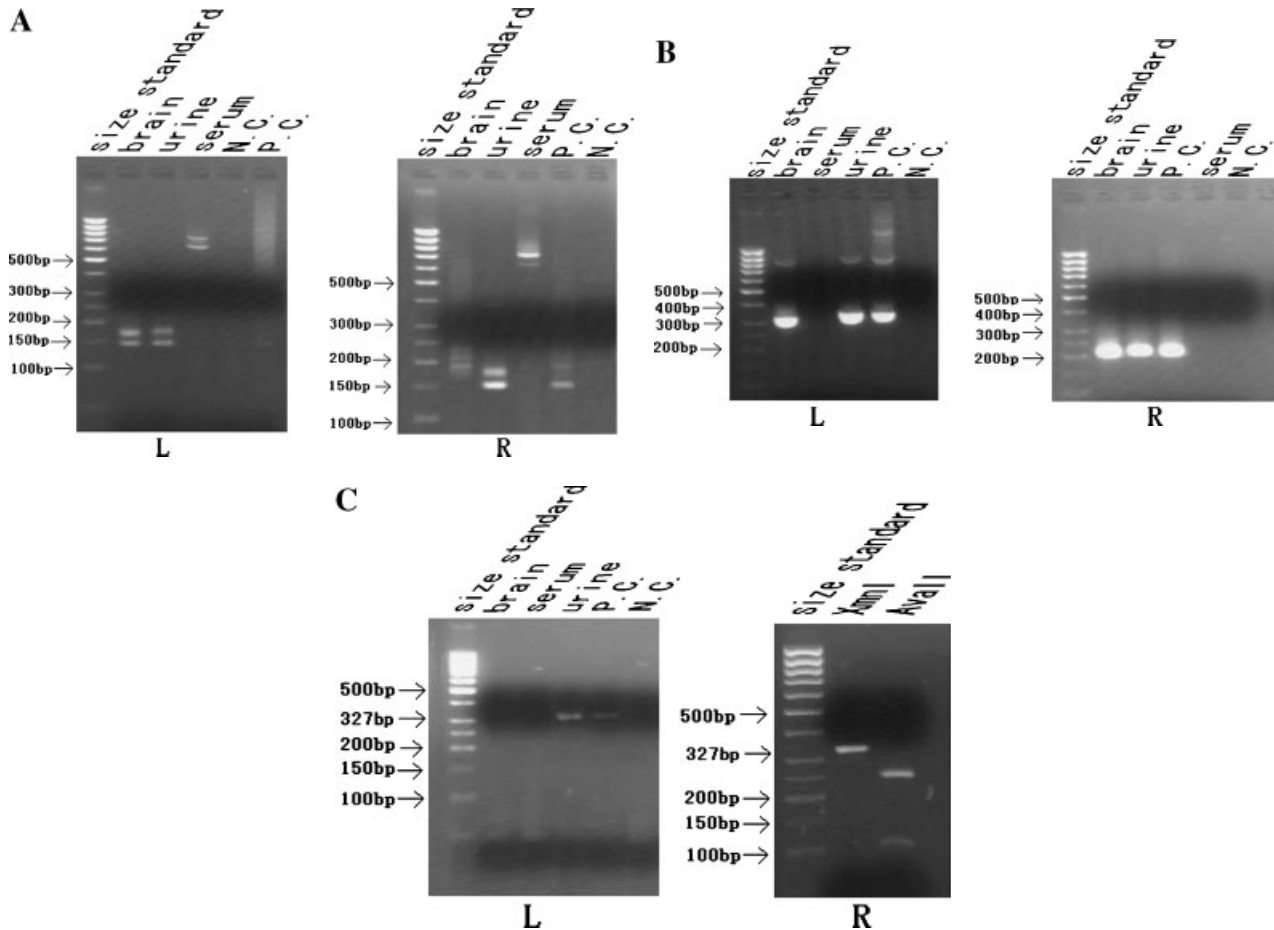


Fig. 3. **A:** Nested-PCR detection of JCV and BKV large T antigen genomes in urine, brain and serum samples. Left image (L): On the agarose gel, 173 bp amplicon for JCV in the first PCR and 146 bp amplicon of JCV in the second nested PCR were amplified in the brain and urine DNA. Right image (R): 176 bp amplicon for BKV in the first PCR and 149 bp amplicon of BKV in the second nested PCR were amplified in urine DNA. With excess target, the semi-nested PCR technique gives rise to a double band. **Lane 1:** Size standards, **lane 2:** brain, **lane 3:** urine, **lane 4:** serum, **lane 5:** negative control (N.C.) on the left image and positive control (P.C.) on the right image, **lane 6:** P.C. on the left image and N.C. on the right image. **B:** Detection of PCR-amplified JCV VP1 region and NCCR genomes in agarose gels. On the left image (L), approximately 350 bp amplicons of JCV NCCR were amplified in brain and urine, but not amplified in serum. On the right

image (R), 215 bp amplicons of JCV VP1 region were amplified in brain and urine, but not amplified in serum. **Lane 1:** size standards, **lane 2:** brain, **lane 3:** serum on the left image and urine on the right image, **lane 4:** urine on the left image and positive control (P.C.) on the right image, **lane 5:** P.C. on the left image and serum on the right image, **lane 6:** negative control (N.C.). **C:** Detection of 327 bp fragments of BKV VP1 region and restriction patterns for the VP1 region digested with restriction endonuclease XmnI and AvaII. On the left image (L): 327 bp fragments of BKV VP1 region is amplified in urine, but not amplified in brain and serum. **Lane 1:** size standard, **lane 2:** brain, **lane 3:** serum, **lane 4:** urine, **lane 5:** positive control (P.C.), **lane 6:** negative control (N.C.). On the right image (R): 327 bp fragment of BKV DNA generated by PCR remains uncut by XmnI (lane 2) and is cut with AvaII to produce 237 and 90 bp of two fragments (lane 3). Lane 1: size standard.

amplified only from urine (Fig. 3A,R). In serum, however, neither of these large T antigen DNAs of JCV and BKV was amplified (Fig. 3A).

Sequence Analysis Identifying Rearrangement of JCV NCCR in the Brain

An approximately 350 bp fragment from the JCV NCCR was amplified from the urine and the brain (Fig. 3B,L). Nucleotide sequences of these fragments were analyzed. The sequence of the entire NCCR from urine was almost identical to that of isolate "MY" of Yogo et al. [1990] with one point mutation (the 95th base was C instead of G). Figure 4 shows the sequence of the hypervariable parts of NCCR, from the origin of DNA replication to the late protein coding sequences. These hypervariable parts of NCCR from brain showed se-

quence rearrangements; as shown in Figure 4, Boxes A and F were retained intact, but Box D was deleted. The NCCR from brain also had two repeats of a 61-bp sequence inserted between Box B and Box F. This sequence consisted of the last seven nucleotides of Box B (termed B'), the first 32 nucleotides of Box C (termed C'), the three nucleotides "TAA," the last 15 nucleotides of Box E (termed E'), and the first four nucleotides of Box F (termed F'). The hypervariable region in JCV NCCR from brain (239 bp) was shorter than that of archetype (267 bp).

Identification of JCV Genotypes

The PCR products of JCV VP1 region amplified from the urine and the brain samples are shown in Figure 3B and R. Both these sequences were identical to that

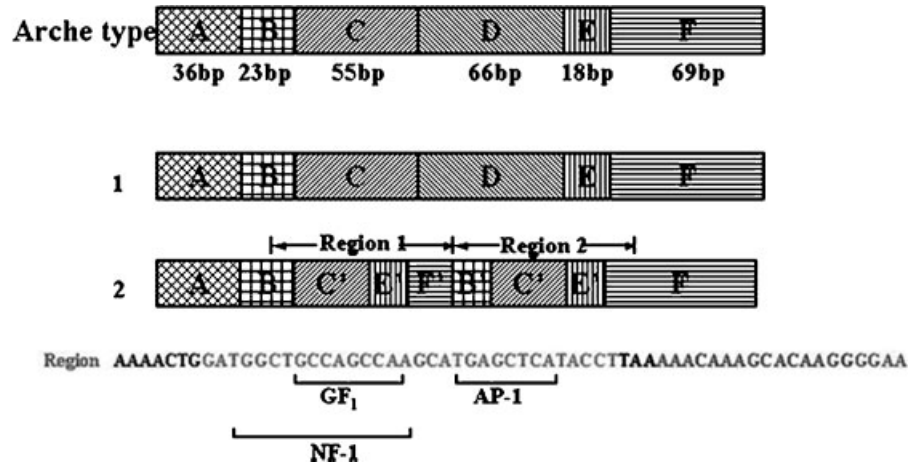


Fig. 4. Comparison of archetypal NCCR to sequences isolated from urine (**lane 1**) and brain tissue (**lane 2**) of this case. Region 1 and 2 has identical nucleotide sequences which repeat twice. Nuclear factor-1 (NF-1) shows the NF1 protein bind sites. Glial factor-1 (GF₁) represents GF₁ proteins bind sites. Activator Protein-1 (AP-1) represents AP-1 bind sites. Boxes are depicted maintaining the proportion of nucleotide sequence length. On the bottom line, the repeated sequences are listed.

of isolate Tky-1 [Kato et al., 1994]. The VP1 region identifies the JCV as type 2A (Table I) [Stoner et al., 2000].

Identification of BKV Genotype

A 327-bp fragment of BKV VP1 region was amplified in the urine, but brain was negative for this DNA (Fig. 3C). The 327-bp fragment remained uncut by XmnI and was digested with AvaII to produce two fragments, of 237 and 90 bp (Fig. 3C,R). This pattern is typical of BKV type I. Therefore, the genotype of the urine BKV was determined to be type I.

DISCUSSION

Rearrangement of the JCV NCCR

The cellular host range of JCV could depend upon a number of factors. One such factor is the NCCR, the definitive modulator of viral activity. The JCV NCCR is an approximately 300-bp sequence positioned between the early and late protein-coding sequences in the viral DNA genome and containing promoter and/or enhancer elements [Yogo et al., 1990; Pietropaolo et al., 2003]. The JCV NCCR contains hypervariable sequence between the origin of DNA replication and the late protein coding sequences. In what is called the archetypal sequence, this hypervariable region is subdivided into six distinct regions, designated Box A, B, C, D, E, and F, each containing binding sites for cellular factors involved in viral transcription [Ault and Stoner, 1993; Vaz et al., 2000; Pietropaolo et al., 2003]. Sequence modification in this hypervariable NCCR affects markedly the levels of viral transcription and replication and in this way may govern both JCV cellular tropism [Martin et al., 1985; Ault, 1997; Vaz et al., 2000; Jensen and Major, 2001; Pietropaolo et al., 2003] and the switch

between lytic and latent infection [Jensen and Major, 2001; Pietropaolo et al., 2003].

In the NCCR in JCV from brain of the patient, Boxes A, B, and F were highly conserved, but Box D was deleted. The NCCR had partial repetition of Boxes B, C, E, and F, designated as B', C', E', and F'. The NCCR thus showed the pattern A, B, C', E', F', B' C', E', F (Fig. 4). This pattern in a PML-associated strain seems to fit with the previous suggestion that suggest Box D, to some extent, represses viral activity, while Boxes A, C, and E promote viral activity [Ault, 1997; Jensen and Major, 2001]. As for the preservation and partial repetition of Box B in this case, and its preservation in CSF of HIV-negative subjects with PML [Pietropaolo et al., 2003], a possible explanation is that Box B is necessary for JCV to complete its replication cycle in the absence of viruses other than JCV supplying trans-activators of JCV transcription and replication. Some studies have indicated direct intercommunication between HIV-1 and JCV through the HIV-encoded regulatory protein Tat, and HIV-1 Tat can increase JCV gene transcription [Pietropaolo et al., 2003].

Cytotoxic T lymphocytes (CTL) play a crucial role in the control of many viral infections in humans. Immunosurveillance by CTL is an important factor in preventing the onset of severe diseases in patients chronically infected by DNA viruses such as Epstein-Barr virus, cytomegalovirus, or herpes simplex virus [Du Pasquier et al., 2003]. Therefore, cell-mediated immunity may also play a role in controlling JCV reactivation and spread. A series of experiments demonstrated a strong association between the early presence of JCV-specific CTL in peripheral blood and the containment of PML [Du Pasquier et al., 2004], and indicate that JCV-specific CTL may be of a central importance in controlling the spread of JCV in immunosuppressed individuals [Koralnik et al., 2001, 2002; Du Pasquier et al., 2003]. CD8⁺ CTL recognize intracellularly synthesized viral proteins

TABLE I. Sites in VP1 Region for JCV Genotype Assignment

	Nucleotide										Position			
	1753	1756	1771	1786	1790	1804	1805	1818	1837	1843	1850	1869	1870	
Distributed region														
Type2A														
Urine of this case	A	C	A	G/T ^a	T	T	A/C ^b	C	T	T	A	G	A	
Brain of this case	A	C	A	G	T	T	C	C	T	T	A	G	A	
	A	C	A	G	T	T	C	C	T	T	A	G	A	

N.E. Asia, Northeast of Asia; N.A., Native American. T is a minor nucleotide at position 1786 in type 2A strain.

^aSites represent sequences unless a split site is indicated.

^b1805 is C in the Tokyo-1 strain and in some Guarani variants.

that have been degraded into peptides through the endogenous pathway and presented on MHC class I molecules at the surface of virus infected cells [Koralnik, 2002].

A general impairment of the Th1-type T-helper cell function of cell-mediated immunity has been found in PML [Weber et al., 2001]. For the patient with defective helper T cell function resulting from hyper IgM syndrome 1, the impaired cellular immune response to JCV may have led to PML. In addition, because of the impaired CD40L expression on the activated helper T cells, the patient's B cells were unable to proliferate and to undergo class switch recombination *in vivo*. The patient's low levels of IgG, IgA and IgE may have resulted in too few immunoglobulins specific to JCV to prevent proliferation of JCV. So the primary T cell defect and the secondary B cell abnormality of hyper IgM syndrome 1 may both have facilitated the modification of archetypal JCV genome in the urinary tract.

Origin of JCV in the Brain

The JCV VP1 sequences were identical in the urine and the brain, implying that the urine and the brain strains had the same origin. However, the site of initial infection in the body and the route of transmission to the brain have not been determined. Archetypal JCV is thought to be the form of virus that circulates in the asymptomatic population. The archetype could be the source of the rearranged forms in the brain, produced via sequence deletions and duplications that give variants that are more active and with a new tissue tropism [Yogo et al., 1990; Ault and Stoner, 1993; Ciappi et al., 1999]. This conclusion is supported by the observation that the urine and brain strains had the same origin, and that the JCV NCCR from the patient's brain is highly variable, in contrast to the archetypal virus from urine.

For the route of transmission, it has been hypothesized that lymphocytes have a role in spreading JCV to the brain [Ault and Stoner, 1993; Ciappi et al., 1999]. The lymphotropism of JCV is now well documented [Ciappi et al., 1999]. JCV with either rearranged or archetypal NCCR infects lymphocytes in peripheral sites of the initial infection, and in the lymphocytes, JCV may replicate and undergo rearrangement of the NCCR [Ciappi et al., 1999], Lymphocytes carry either the archetypal or the rearranged form of JCV to the brain, where it may persist in latent form and may be reactivated under immunosuppressive conditions [Ault and Stoner, 1993; Ciappi et al., 1999]. In the patient described here, although serum was negative for JCV and peripheral blood leukocytes were not available, the possibility that peripheral blood leukocytes may have carried JCV from the urinary tract to the brain cannot be excluded.

Genotypes of JCV and BKV

Genotype analysis of JCV from the urine of both PML and non-PML patients has revealed at least eight main

types that differ in nucleotide sequence by about 1–3% and have evolved in specific geographical regions [Jobes et al., 1998; Stoner et al., 2000]. Based on the sequence of a 215-bp region near the 5' end of the VP1 gene [Agostini et al., 1997a; Stoner et al., 2000], JCV in the brain and urine of the patient both belonged to type 2A, even though the NCCR patterns were different. This observation supports the suggestion that genotype 2 may be associated with a higher risk of developing PML compared to other JCV genotypes [Agostini et al., 1997b], but it does not support another report that type 2B was more frequent in the PML brain than in the urine from non-PML controls [Agostini et al., 1998]. These findings suggest that unknown factors that differ among JCV genotypes influence the neurotropism and/or neurovirulence of JCV, or interact with the JCV NCCR and contribute to the NCCR rearrangements that lead to PML types in the context of immunodeficiency.

In addition to JCV infection, the patient also had an active BKV infection in the urinary tract, as shown by amplification of BKV DNA encoding VP1 from urine. The BKV was of genotype I, which is common among Japanese. But BKV genome was not found in the brain tissue, which implies that the BKV had no role in the patient's PML or in JCV reactivation.

In conclusion, this is the first PML case secondary to hyper-IgM syndrome 1, and the JCV in brain had a novel rearranged form of the NCCR. It appears that this NCCR variation may have originated from the basic archetype in the urine as a consequence of hyper-IgM syndrome 1, and this genetic change may have resulted in pathogenic changes that allowed the JCV to cause PML in the brain. The genotypes of JCV VP1 region were type 2A and identical in the brain and the urine samples. The PML patient was infected actively with BKV subtype I in the urinary tract.

ACKNOWLEDGMENTS

We thank Nagura Kiyoko and Ihara Megumi of the first Department of Pathology for excellent sequencing assistance.

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