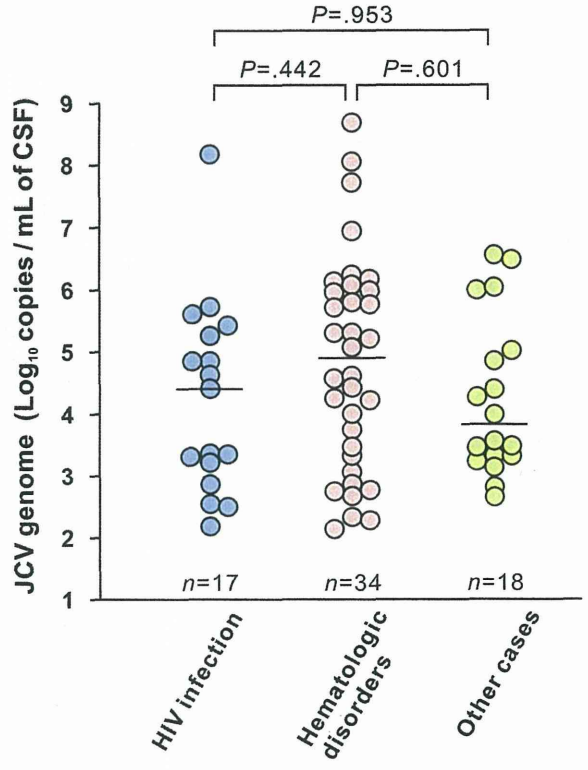

Figure 3 The JCV DNA levels in the CSF specimens from PML patients. The CSF viral loads for patients with HIV infection, hematologic disorders, or other diseases are shown (left to right). The open circles indicate the copy number of JCV DNA in each sample, and the horizontal lines represent the medians. In several cases, JCV DNA measurement was performed for different specimens from the same patient during the follow-up period.

without need for these treatments. These data indicate that PML occurred in patients with hematologic disorders not only after HSCT but also after treatment with chemotherapeutic or immunosuppressive drugs.

Discussion

The present study clarified the characteristics of PML cases in Japan based on clinical data obtained through the laboratory testing for JCV DNA in CSF specimens. Mass screening of PML patients has not been feasible in Japan due to the lack of a suitable database for PML. The current strategy deals with a relatively small number of patients but has a distinct advantage in collecting precise real-time information for patients as well as specimens. The testing was constantly requested by the physicians via websites, despite the fact that there were at least 4 commercial laboratories providing similar assays during the study period according to our own survey. Thus, this internet-based approach is thought to be useful for sampling data for rare infectious diseases. In addition, as this diagnostic support system was conducted regardless of patient age, gender, underlying disease or medical history, precise information could be obtained not only from PML patients but also from CSF-JCV-negative individuals with similar conditions. These data are considered to be valuable for the examination of the overall background to PML in Japan.

A large number of PML patients had HIV infection / acquired immunodeficiency syndrome (AIDS) or hematologic disorders. Recent database analyses and other clinical studies in the USA have suggested that approximately 79–82% of PML patients are positive for HIV and 7.7–13% have hematological malignancies [24,26,31]. In contrast, the proportion of HIV-related PML cases in Japan was approximately 33%, which is much lower than that in the USA. The difference in the ratios of HIV-related PML between these two countries must be interpreted based on the epidemiological status of HIV infection. According to the latest data from the Joint United Nations Programme on HIV/AIDS, World Health Organization [32], the prevalence of HIV

Table 2 Hematopoietic stem cell transplantation (HSCT) in patients positive or negative for CSF JCV

Underlying disease	Proportion (%) of JCV-positive patients with HSCT (*n*=27)		Proportion (%) of JCV-negative patients with HSCT (*n*=68)	
Acute myeloid leukemia	2/2	(100)	9/10	(90.0)
Acute lymphoblastic leukemia	1/1	(100)	4/6	(66.7)
Chronic lymphocytic leukemia	0/1	(0)	0/1	(0)
Adult T-cell leukemia	0/1	(0)	0/1	(0)
Hodgkin's lymphoma	1/1	(100)	0/1	(0)
Non-Hodgkin's lymphoma	1/6	(16.7)	5/21	(23.8)
Aplastic anemia	2/2	(100)	0/2	(0)
Primary immunodeficiency syndrome	0/2	(0)	1/6	(16.7)
Multiple myeloma	1/2	(50.0)	0/1	(0)
Primary macroglobulinemia	0/1	(0)	0/0	NA
Total	8/19	(42.1)	19/49	(38.8)

NA, not applicable.

Table 3 Characteristics of PML patients with a history of hematopoietic stem cell transplantation

Patient	Age	Sex	Underlying disease	Type of transplant	Interval (Days) [a]
1	50	M	Acute myeloid leukemia	Allogeneic bone marrow	279
2	44	M	Acute lymphoblastic leukemia	Allogeneic bone marrow	442
3	42	M	Aplastic anemia	Allogeneic bone marrow	614
4	58	M	Aplastic anemia	Allogeneic bone marrow	493
5	43	M	Acute myeloid leukemia	Umbilical cord blood	450
6	16	M	Hodgkin's lymphoma	Autologous bone marrow	285
7	61	M	Non-Hodgkin's lymphoma	Autologous peripheral blood	775
8	52	M	Multiple myeloma	Autologous peripheral blood	833

M, male.
[a] Days between transplantation and the initial testing for JCV DNA in CSF specimens.

infection among adults in the USA (0.6%) is at least 6-fold higher than that in Japan (< 0.1%). Thus, it is reasonable to suppose that the relatively low proportion of HIV-related PML in Japan is associated the low prevalence of HIV infection. As a large proportion of HIV-infected individuals in Japan are male [32], it is also reasonable that the sex ratio of HIV-related PML showed a predominance of males.

A notable finding of the present study is that hematologic disorders are a main risk factor for PML in Japan. Five of 19 patients in this group had received allogeneic HSCT, suggesting that this type of transplantation is an important risk factor of PML. In the other 14 PML cases, 11 individuals (patients 6–16) were administrated with chemotherapeutic and / or immunosuppressive agents for the treatment of hematologic malignancies. Thus, it is likely that these therapies are associated with the high incidence of PML cases in this category. The present study also demonstrates that the majority of PML patients with hematologic disorders are males. In contrast, the percentages of male patients with hematologic malignancies were similar to or slightly higher than those of females (leukemia, 59.1%; lymphoma, 52.9%; MM, 52.1%) according to the most recent statistics from the National Database for Cancer Incidence in Japan [33]. The reason for the male predominance among PML patients with hematologic disorders remains unknown. Further studies are needed on larger populations of PML patients to clarify the mechanism and significance of this sexual dimorphism. However, these data are thought to be beneficial for patients having similar underlying diseases.

In 50 subjects with autoimmune disorders, 3 SLE patients were diagnosed as having PML. These patients had been treated with immunosuppressive agents, such as tacrolimus, mesalazine, mycophenolate mofetil, prednisolone, and / or cyclophosphamide, but not with therapeutic antibodies. No PML cases were observed among individuals with other types of autoimmune disorders. In Japan, natalizumab and efalizumab are not

Table 4 Chemotherapy or immunosuppressive treatment in PML patients without hematopoietic stem cell transplantation

Patient	Age	Sex	Underlying disease	Chemotherapeutic or immunosuppressive agents
9	78	M	Non-Hodgkin's lymphoma	CPA, THP, VDS, PSL, R
10	66	M	Non-Hodgkin's lymphoma	CPA, DXR, VCR, PSL, R
11	72	F	Non-Hodgkin's lymphoma	CPA, DXR, VCR, PSL, THP, R
12	64	F	Non-Hodgkin's lymphoma	CPA, DXR, VCR, PSL, ETP
13	77	M	Adult T-cell leukemia	CPA, DXR, VCR, PSL
14	64	M	Multiple myeloma	DEX, DXR, VCR, Bzb
15	67	M	Chronic lymphocytic leukemia	CPA
16	71	M	Primary macroglobulinemia	R
17	62	M	Non-Hodgkin's lymphoma	NA
18	22	M	Primary immunodeficiency syndrome	NA
19	24	M	Primary immunodeficiency syndrome	NA

M, male; *F*, female; *CPA*, cyclophosphamide; *THP*, pirarubicin; *VDS*, vindesine; *PSL*, prednisolone; *R*, rituximab; *DXR*, doxorubicin; *VCR*, vincristine; *ETP*, etoposide; *DEX*, dexamethasone; *Bzb*, bortezomib; *NA*, not administered.

currently approved for use, and rituximab is not licensed for the treatment of autoimmune disorders. Therefore, increased awareness may be needed about the potential for PML in accordance with the wide spread use of therapeutic monoclonal antibodies in this country. It was also shown that the occurrence of PML is uncommon in individuals receiving solid-organ transplantation. Among the total study population, only 10 subjects underwent kidney, liver, or heart transplantation, and PML developed in one liver-transplanted patient. As this patient had suffered from common variable immunodeficiency, the association between the transplantation and PML remains unclear. This situation can be explained by the limited number of patients, who underwent organ transplantation, especially from brain dead donors [34]. However, it is predicted that the risk of PML will increase in accordance with the revision of the transplantation law in 2010, which extends the availability of transplantation therapy [35].

Conclusions

The results of this study suggest that the internet-assisted laboratory surveillance system might be a useful strategy for elucidating the characteristics of PML on a national level. The present database provides important background information for the diagnosis and treatment of patients with risk factors for PML in Japan.

Additional files

Additional file 1: Figure S1. Schematic diagram of the standard DNA and primer / probe sets for PCR testing. Yellow and grey lines represent the sequences of the JCV genome and pBR322 vector within the standard DNA (pJC1-4->pJCV), respectively. The numbers in the circle correspond to the nucleotide positions within the JCV genome. Three primer / probe sets detect the JCV T and VP1 genes and the boundary sequence of the JCV genome and pBR322 (green, red, and blue, respectively).

Additional file 2: Figure S2. Examples of real-time PCR amplifications. Three real-time PCR assays were designed to detect the JCV T (A) and VP1 (B) sequences and the contamination of samples with standard DNA (C). The reactions were performed in the absence or presence of standard DNA (2.0×10^8 to 0.8 copies per reaction). Relative fluorescence is plotted against cycle number. These PCR assays were capable of detecting at least 4 copies of JCV DNA per reaction under the same conditions. The data are representative of three independent experiments.

Abbreviations

PML: Progressive multifocal leukoencephalopathy; JCV: JC virus; HIV: Human immunodeficiency virus; SLE: Systemic lupus erythematosus; CSF: Cerebrospinal fluid; NIID: National institute of infectious diseases; MRI: Magnetic resonance imaging; VP1: Viral protein 1; cART: Combination antiretroviral therapy; CD4: Cluster of differentiation 4; AA: Aplastic anemia; MM: Multiple myeloma; HSCT: Hematopoietic stem cell transplantation; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; HL: Hodgkin's lymphoma; NHL: Non-Hodgkin's lymphoma; AIDS: Acquired immunodeficiency syndrome.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN conceived of the study, carried out real-time PCR testing, and created the database of patients. KN and MS analyzed the clinical data and drafted the manuscript. HM and MY supervised the PML surveillance program in Japan. SK and YM participated in the clinical study of PML cases. TS performed the statistical analyses. TT supported the internet-assisted support system for JCV testing. HM, MY, SK, YM, TS, TT, CKL, and IK participated in the study design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Suppressive Effect of PARP-1 Inhibitor on JC Virus Replication In Vitro

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The incidence of progressive multifocal leukoencephalopathy (PML) has increased due to the AIDS pandemic, hematological malignancies, and immunosuppressive therapies. Recently, the number of cases of monoclonal antibody-associated PML has increased in patients treated with immunomodulatory drugs such as natalizumab. However, no common consensus regarding PML therapy has been reached in clinical studies. In order to examine the suppression of JC virus (JCV) replication by 3-aminobenzamide (3-AB), a representative PARP-1 inhibitor, a DNA replication assay was carried out using the neuroblastoma cell line IMR-32 and IMR-adapted JCV. The suppression of JCV propagation by 3-AB was also examined using JCI cells, which are a carrier culture producing continuously high JCV titers. The results indicated that PARP-1 inhibitors, such as 3-aminobenzamide (3-AB), suppress JCV replication and propagation significantly in vitro, as judged by DNA replication assay, hemagglutination, and real-time PCR analysis. It has been also shown that 3-AB reduced PARP-1 activity in IMR-32 cells. According to the results of the MTT assay, the enzyme activity of 3-AB-treated cells was slightly lower than that of DMSO-treated cells. However, the significant suppression of JCV propagation is not related to the slight decrease in cell growth. To our knowledge, this is the first report that PARP-1 inhibitor suppresses the replication of JCV significantly in neuroblastoma cell lines via the reduction of PARP-1 activity. Thus, PARP-1 inhibitors also may be a novel therapeutic drug for PML. ***J. Med. Virol. 85:132–137, 2013.***

KEY WORDS: 3-aminobenzamide; JCI cells; IMR-32 cells; PARP-1

INTRODUCTION

JC virus (JCV) is a causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) in immunocompromised patients [Major et al., 1992]. PML occurs predominantly in immunocompromised patients, such as those with the acquired immunodeficiency syndrome (AIDS) and hematological malignancies, or those receiving immunosuppressive therapies [Berger and Major, 1999]. Several reports have suggested that highly active antiretroviral therapy (HAART) is effective in reducing mortality among patients with AIDS-related PML [Clifford, 1999; Guidici et al., 2000]. However, previous preclinical reports have failed to demonstrate adequately the efficacy of treatment with antiviral and antineoplastic drugs such as cytarabine and cidofovir in non-AIDS-related

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PML patients [Hall et al., 1998; Marra et al., 2000]. On the other hand, several studies have demonstrated that JCV uses $5HT_{2A}R$, which is a serotonin receptor, to bind to the cell surface [Elphick et al., 2004; Schaumburg et al., 2008]. Antibodies directed against $5HT_{2A}R$ as well as serotonin antagonists were found to inhibit JCV propagation in cell culture [Elphick et al., 2004; Nukuzuma et al., 2009]. However, risperidone, a serotonin antagonist, did not inhibit JCV attachment, internalization or replication in primary human fetal glial (PHFG) cells [Chapagain et al., 2008]. Thus, the efficacy of serotonin antagonists as therapeutic drugs for PML remains controversial. Recently, mefloquine was shown to inhibit JCV replication in cells infected previously with JCV [Brickelmaier et al., 2009].

Poly(ADP-ribose) polymerase 1 (PARP-1) is an abundant nuclear enzyme that catalyzes the formation of extensive branched polymers of poly (ADP-ribose) by using NAD^+ as a substrate [Jeggo, 1998]. Previous studies have indicated that PARP-1 is required for the efficient replication of HIV-1 and herpes simplex virus in infected cells [Kameoka et al., 2004; Li et al., 2012]. It has also been shown that the PARP-1 inhibitor, 3-aminobenzamide (3-AB), reduces SV40 propagation consistently [Gordon-Shaag et al., 2003].

In this study, the suppressive effect of 3-AB on JCV replication and propagation in a JCV-permissive cell line (IMR-32) [Tumiowicz et al., 1970] and cells infected persistently with JCV (JCI) [Nukuzuma et al., 1995] was assessed on the basis of DNA replication assay, hemagglutination (HA) assay, and real-time PCR analysis. In addition, the relationship among JCV propagation, PARP-1 activity and cell growth in the presence of this compound was examined to clarify the suppressive mechanism of 3-AB on JCV replication and propagation.

MATERIALS AND METHODS

Cell Lines and Compounds

The human neuroblastoma cell line IMR-32 [Tumiowicz et al., 1970] was obtained from DS-Pharma Biomedical (Osaka, Japan). JCI cells was obtained by supporting the continuous production of a high JCV titer, as described previously [Nukuzuma et al., 1995]. IMR-32 cells and JCI cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. 3-Aminobenzamide (3-AB) was obtained from Sigma–Aldrich (St Louis, MO) and dissolved in DMSO (Wako Pure Chemical Industries, Osaka, Japan).

Plasmids

Recombinant DNAs containing the full-length genomes of JCV clones M1-IMRa, M1-IMRb, and M1-IMRc were constructed from an IMR-32-adapted JCV population, with M1-IMRb showing the highest HA activity in IMR-32 cells among the three constructed clones, as described previously [Yogo et al., 1993]. The recombinant DNA containing M1-IMRb was kindly provided by Dr. Yogo (Tokyo University).

Quantitation of Cell Proliferation

Cell proliferation was determined by measuring mitochondrial succinate dehydrogenase activity using an MTT assay as described previously [Nukuzuma et al., 2012]. IMR-32 cells were plated into 96-well microtiter plates at a concentration of 7.1×10^4 cells/well in 100 μl medium and incubated in the absence or presence of 3-AB at a final concentration of 20 mM for 5 days at 37°C in a CO_2 incubator. After incubation for 5 days, cell proliferation was assessed using a Cell Proliferation Kit I (MTT; Roche, Indianapolis, IN) according to the manufacturers' instructions.

DNA Replication Assay

A DNA replication assay was carried out as described previously [Nukuzuma et al., 2012]. IMR-32 cells were plated into poly-L-lysine-coated 35-mm dishes in 2 ml of DMEM containing with 10% FBS until they reached 70–80% confluency. For transfection, 1.0 μg viral DNA, excised from recombinant plasmid, was introduced into the cells using the FuGENE 6 transfection reagent (Roche), and the cells were incubated in culture medium in the absence (DMSO) or presence (3-AB) of 3-AB at a final concentration of 20 mM or DMSO. At 48 and 72 hr after 3-AB treatment, the cells were harvested, and low-molecular weight DNA was extracted from cells according to the Hirt procedure [Hirt, 1967]. DNA was recovered by ethanol precipitation and dissolved in a small volume of TE buffer. The extracted DNA (1 μg) was digested with *Dpn*I and *Bam*HI. The fragments, separated on a 1.0% agarose gel, were transferred onto a nylon membrane (Roche) and then hybridized to digoxigenin-labeled M1-IMRb DNA at 65°C in an incubator using a DIG DNA Labeling Kit (Roche). The inclusion of a digoxigenin-labeled DNA Molecular Weight Marker II in an electrophoretic run served as the size marker on the gel. JCV DNA was detected using a DIG Luminescent Detection Kit (Roche), exposed to film overnight, and the exposed X-ray films were quantified using Image J (National Institutes of Health, Bethesda, MA, http://rsbweb.nih.gov/nih-image/). The relative band intensities were normalized against the intensities of the input DNA and background.

Treatment With the 3-AB Compound for JCI Cells

JCI cells were incubated in 2 ml of DMEM containing 10% FBS in absence (DMSO) or presence (3-AB) of the 3-AB compound at a final concentration of 20 mM. Four days after 3-AB treatment, the cells were harvested, passaged at a split ratio of 1:3, and

further incubated in fresh culture medium containing the 3-AB compound. The cells were passaged for 18 days according to this protocol.

Hemagglutination (HA) Assay

The HA assay was carried out essentially as described previously [Nukuzuma et al., 2009]. Briefly, JCI cells that had been untreated or treated with 3-AB in a 35-mm dish, were resuspended in 250 μl of 1 mM Tris–HCl (pH 7.5) containing 0.2% BSA and then frozen and thawed three times. The cell lysates were treated with 50 μg/ml of neuraminidase (Type V; Sigma–Aldrich) at 37°C overnight, incubated at 56°C for 30 min, and centrifuged at 1,500 rpm for 10 min at 4°C. The resultant supernatant was serially diluted with 50 μl PBS containing 0.2% BSA and assayed for HA using human type O erythrocytes obtained from the Hyogo Red Cross Blood Center.

Real-Time PCR Analysis

Real-time PCR to quantify JCV DNA copies was carried out essentially as described previously [Nukuzuma et al., 2009]. Briefly, viral DNA was extracted from four HA samples on days 12 and 18 using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The real-time PCR amplification was carried out using an ABI PRISM 7900HI sequence detection system (Applied Biosystems, Foster City, CA). The ratios of the amounts of JCV DNA were calculated with reference to the values for DMSO-treated cells.

Assay for PARP-1 Activity

The enzymatic activity of PARP-1 was estimated by measuring the level of poly ADP-ribose (PAR) by ELISA using an HT Colorimetric PARP/Apoptosis Assay 96 Tests kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. It was first examined whether JCV infection increases PARP-1 activity. PARP-1 activity was measured in uninfected (IMR-32 cells) and JCV-infected IMR-32 cells (JCI cells). The cells in 96-well microtiter plates were resuspended in 200 μl of Cell Extraction Buffer. Next, it was examined whether 3-AB suppresses PARP-1 activity in IMR-32 cells. IMR-32 cells were plated into 96-well microtiter plates at a concentration of 5.0×10^4 cells/well in 200 μl of medium and incubated in the absence or presence of 3-AB at a final concentration of 20 mM for 72 hr. The cell lysates were incubated in 200 μl of Cell Extraction Buffer on ice, and then 25 μl (1.25 μg protein) of each lysate were transferred to the wells of a histone-coated microtiter plate. Next, an anti-PAR monoclonal antibody, goat anti-mouse IgG-HRP conjugate, and HRP substrate were used to generate a colorimetric signal, and the reaction was then stopped by adding 0.2 M HCl solution. The absorbance of the colorimetric signal was measured at a wavelength of 450 nm using a BIO-RAD Model 3550 Microplate Reader (Bio-Rad, Hercules, CA). The measured absorbance correlates with PARP-1 activity.

Statistical Analysis

The significance of intergroup differences was determined statistically using a statistical program (SPSS 18 for Windows; SPSS, Chicago, IL) and Student's *t*-test.

RESULTS

3-AB Does Not Decrease IMR-32 Cell Proliferation

The doubling time of cells was in agreement with the proliferation of the cells as assessed by the MTT assay. As shown in Table I, the enzyme activity of IMR-32 cells treated with 3-AB at a concentration of 20 mM was slightly lower than that of the untreated IMR-32 cells, although this difference was not statistically significant. These results indicate that 3-AB at a concentration of 20 mM does not induce any cytotoxic effect on IMR-32 cells.

3-AB Suppresses JCV Replication in IMR-32 Cells

The human neuroblastoma cell line IMR-32 cells can support the production of JCV efficiently. Three JCV variants (M1-IMRa, M1-IMRb, and M1-IMRc) have alterations to the regulatory DNA sequences within the viral genomes associated with the adaptation in IMR-32 cells [Yogo et al., 1993]. JCV clone M1-IMRb was used for the DNA replication assay in this study because it showed the highest HA activity in IMR-32 cells [Yogo et al., 1993]. M1-IMRb DNA was introduced into cells and then transferred to a medium with absence (DMSO) or without (3-AB) the 3-AB compound at a final concentration of 20 mM. Treated IMR-32 cells were harvested at 48 and 72 hr post-transfection. The low-molecular weight DNA extracted from IMR-32 cells transfected with M1-IMRb was then treated with restriction endonuclease *Dpn*I and *Bam*HI. *Dpn*I cleaves methyl-adenosine residues and multicuts the input plasmid DNA containing methylated adenosine, but not DNA newly replicated in IMR-32 cells. The digested DNA fragments separated by electrophoresis were transferred onto a nylon membrane and hybridized with digoxigenin-labeled JCV DNA. JCV-replicated DNA was clearly detected and 3-AB treatment suppressed JCV replication at

TABLE I. Growth Rate of 3-AB-Treated IMR-32 Cells Compared With Untreated IMR-32 Cells*

Cells	Absorbance[a]
Untreated IMR-32	0.917 ± 0.023
3-AB-treated IMR-32	0.888 ± 0.026

[a]Mean ± SD of absorbance is shown for five samples.
*IMR-32 cells (7.1×10^4) were plated in DMEM containing 10% FBS in the absence (untreated IMR-32) or presence (3-AB-treated IMR-32) of 3-AB at a final concentration of 20 mM. After 5 days, cell absorbance was measured using the MTT method.

both 48 and 72 hr in the treated IMR-32 cells (3-AB) when compared to that in untreated IMR-32 (DMSO) (Fig. 1A). The columns of the histogram were derived from analyzing the intensities of the replicated DNAs (*Dpn*I-resistant bands) from three independent experiments using Image J (Fig. 1B). The relative intensities were calculated with reference to the values for the DMSO-treated control at 48 hr posttransfection. It has been shown that the relative intensities in the 3-AB-treated IMR-32 cells were lower (by 61.0% at 48 hr and 59.0% at 72 hr, respectively) than in non-treated IMR-32 cells (Fig. 1B). The difference at 72 hr was statistically significant ($P < 0.05$). These results indicate that 3-AB suppresses significantly JCV replication in IMR-32 cells.

3-AB Suppresses JCV Propagation in JCI Cells

To examine further the suppression of JCV propagation by 3-AB, JCI cells were used for a virus propagation experiment. JCI cells are persistently infected with JCV and have no need for virus inoculation or transfection with viral DNA. The cells were harvested on days 12 and 18 after treatment with 3-AB, and the virus titer was determined by HA assay (Fig. 2). In the 3-AB-untreated cells, the HA titers of the JCI cell extracts were 320 ± 128 HA units on day 12, increasing to 1,024 ± 724 HA units on day 18. On the other hand, there was no significant increase in the HA titers of the 3-AB-treated cells: 192 ± 74 HA units on day 12 and 224 ± 192 HA units on day 18. Under the assay conditions, 3-AB at a concentration of 20 mM did not induce any cytotoxic effects in JCI cells (data not shown). These results indicate that 3-AB suppresses JCV propagation in JCI cells.

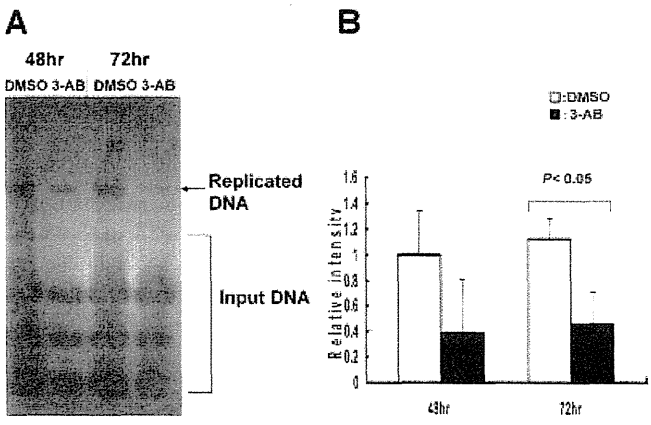

Fig. 1. Suppression of JCV replication in IMR-32 cells by 3-AB. **A**: IMR-32 cells were transfected with 1.0 μg each of DNA from M1-IMRb clone and incubated in culture medium in the absence (DMSO) or presence (3-AB) of 3-AB at a final concentration of 20 mM. Low-molecular weight DNA was extracted from cells at 48 and 72 hr posttransfection and digested with *Dpn*I and *Bam*HI. Digested DNA fragments were separated on a 1.0% agarose gel, transferred onto a nylon membrane, hybridized to digoxigenin-labeled M1-IMRb DNA, and exposed to film overnight. Position of bands representing replicated (5.1 kb, *Dpn*I-resistant) and input (various fragments, *Dpn*I-sensitive) DNA are shown. **B**: The relative intensities of *Dpn*I-resistant bands were determined as described in the Materials and Methods Section. The columns of the histogram were derived from analyzing the intensities of the replicated DNAs (*Dpn*I-resistant bands) from three independent experiments using Image J. The relative band intensities were normalized against the intensities of the input DNA and background. The relative intensities were calculated with reference to the values for DMSO-treated control at 48 hr posttransfection. $P < 0.05$ (Student's *t*-test).

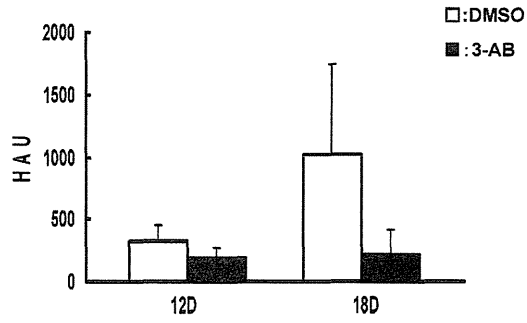

Fig. 2. Suppression of JCV propagation in JCI cells by 3-AB. JCI cells were incubated in 2 ml of complete growth medium in the absence (DMSO) or presence (3-AB) of 3-AB at final concentrations of 20 mM for 12 and 18 days (D), and quadruplicate cultures were subjected to HA assay. HA activity was determined with human type O erythrocytes on samples from four cultures. Data are shown as the mean ± standard deviation of the means.

3-AB Suppresses JCV DNA Replication in JCI Cells

To confirm that 3-AB suppresses JCV DNA replication in JCI cells, total DNA was extracted from each HA sample on days 12 and 18. The amount of JCV DNA copies was determined using real-time PCR. In preliminary experiments, it was been shown that the detectable level of the real-time PCR system used in this study was above 100 copies per reaction. In the 3-AB treated cells, the amount of JCV DNA was decreased to 18.5% on day 12 and 25.0% on day 18, respectively, when compared to untreated cells. The differences on days 12 and 18 were both statistically significant ($P < 0.01$).These results indicate that 3-AB suppresses the replication of JCV DNA significantly in JCI cells (Fig. 3).

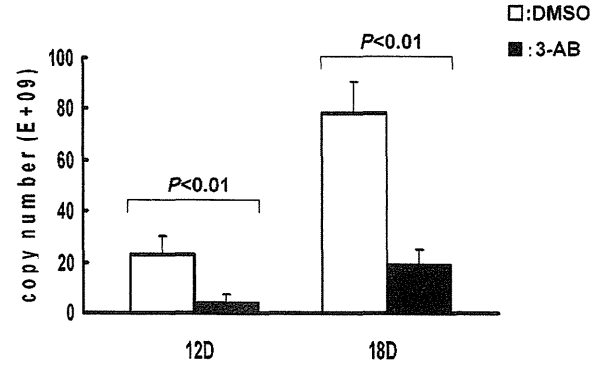

Fig. 3. Suppression of JCV DNA replication in JCI cells by 3-AB. Total DNA was extracted from JCI cells that had been maintained in the absence (DMSO) or presence (3-AB) of 3-AB at final concentrations of 20 mM for 12 and 18 days (D). The real-time PCR was carried out to quantify JCV DNA copies. Data are shown as the mean ± standard deviation of the means ($n = 4$ per group). $P < 0.01$ (Student's *t*-test).

TABLE II. PARP Activity of JCI Cells Compared With IMR-32 Cells*

Cells	Absorbance[a]
IMR-32	2.263 ± 0.206
JCI	1.963 ± 0.186

[a]Mean ± SD of absorbance is shown for four samples.
*IMR-32 cells and JCI cells were plated into microtiter plates using DMEM containing 10% FBS. At confluency, the absorbance of PARP activity was measured using a microplate reader.

3-AB Suppresses PARP-1 Activity in IMR-32 Cells

PARP-1 activity was quantified using a HT Colorimetric PARP/Apoptosis Assay in IMR-32 cells, as described in the Materials and Methods Section. A HT Colorimetric PARP/Apoptosis Assay is an ELISA kit which semi-quantitatively detects PAR, synthesized by the enzymatic activity of PARP-1, deposited onto immobilized histone proteins in a 96-well microplate. Thus, the measured absorbance correlates with PARP-1 activity. PARP-1 was clearly detected, and PARP-1 activity was measured in uninfected (IMR-32 cells) and JCV-infected IMR-32 cells (JCI cells). PARP-1 activity in JCI cells was similar to that in IMR-32 cells (Table II). This result indicated that JCV infection did not increase PARP-1 activity.

It was examined whether 3-AB suppresses PARP-1 activity in IMR-32 cells. PARP-1 activity in 3-AB treated cells was lower (reduction of 35.8%) than that in non-treated cells. These results indicate that 3-AB suppresses PARP-1 activity in IMR-32 cells (Table III).

DISCUSSION

The incidence of PML has increased due to the AIDS pandemic as well as increases in hematological malignancies and the use of immunosuppressive therapies [Berger and Major, 1999]. Recently, the number of cases of monoclonal antibody-associated PML has increased in patients treated with immunomodulatory drugs such as natalizumab, rituximab, and efalizumab [Carson et al., 2009]. HAART was confirmed to possess adequate efficacy for the treatment of AIDS-related PML [Clifford, 1999; Giudici et al., 2000]. However, the drugs are not effective for non-AIDS-related PML [Hall et al., 1998; Marra et al., 2002]. Recently, it was shown that mefloquine inhibited JCV DNA replication in vitro [Bricklmaier et al., 2009]. Thus, mefloquine has attracted much interest as a potential therapeutic drug for non-AIDS-related PML.

TABLE III. PARP Activity of 3-AB-Treated IMR-32 Cells Compared With Untreated IMR-32 Cells*

Cells	Absorbance[a]
Untreated IMR-32	1.564 ± 0.783
3-AB-treated IMR-32	1.007 ± 0.579

[a]Mean ± SD of absorbance is shown for four samples.
*IMR-32 cells (5.0×10^4) were plated in DMEM containing 10% FBS in the absence (untreated IMR-32) or presence (3-AB-treated IMR-32) of 3-AB at a final concentration of 20 mM. After 72 hr, the absorbance of PARP activity was measured using a microplate reader.

From another viewpoint, it is necessary to develop a novel therapy that targets host factors required for efficient JCV replication to avoid the appearance of drug-resistant mutants. Previous studies have indicated that PARP-1 plays important roles in the efficient replication of both HIV-1 and HSV in the infected cells [Kameoka et al., 2004; Li et al., 2012]. On the other hand, a previous study has indicated that a PARP-1 inhibitor, 3-AB, reduced SV40 propagation [Gordan-Shaag et al., 2003].

The results from the DNA replication assay in this study indicated that 3-AB suppresses JCV replication significantly at 48 and 72 hr posttransfection in IMR-32 cells. A previous study demonstrated that the maximum suppression of SV40 propagation by 3-AB occurs at a concentration of 20 mM [Gordan-Shaag et al., 2003]. As JCV and SV40 belong to the same genus, the current finding is thought to be consistent with the earlier investigation.

The present study has also demonstrated that 3-AB inhibits JCV propagation in JCI cells, which continuously produce high JCV titers in a carrier culture. Using an HA assay and real-time PCR analysis, we demonstrated that 3-AB exerts a significant suppressive effect on JCV propagation in JCI cells. The suppressive effect of 3-AB on JCV replication in IMR-32 cells was assessed on the basis of DNA replication assay. JCV DNA replication was inhibited in a short time (48 and 72 hr). On the other hand, the suppressive effect of 3-AB on JCV propagation in JCI cells was examined using HA assay and real-time PCR analysis. Since JCI cells produces high titers of JCV continuously, a rather long time (days 12 and 18) was required for suppressing HA titers and the amount of JCV DNA. The real-time PCR analysis is more sensitive than HA assay. Thus, the results from DNA replication assay, HA assay and real-time PCR assay were not inconsistent. As the enzyme activity of 3-AB-treated cells was reduced only slightly, as assessed by MTT assay, it is unlikely that JCV propagation in the presence of 3-AB is reduced as a consequence of decreased cell growth. Rather, it is thought that the replication and propagation of JCV in IMR-32 cells were directly diminished by 3-AB, which reduced PARP-1 activity. It was previously shown that a small-interfering RNA (siRNA) directed against PARP-1 markedly suppresses HIV-1 replication in human cell lines [Kameoka et al., 2004]. It was also demonstrated that although 3-AB does not suppress SV40 replication or capsid protein production, PARP-1 enhances virus release to the medium by inducing necrosis of the infected cells [Gordan-Shaag et al., 2003].

The data in this study suggest that the mechanism underlying the suppressive effect of 3-AB on JCV replication was different from that on SV40. To our knowledge, this is the first report that PARP-1 inhibitor suppresses the replication of JCV significantly in

neuroblastoma cell lines by the reduction of PARP-1 activity. The PARP-1 inhibitor may suppress JCV propagation and serve as both a novel drug candidate and a lead compound in the development of more potent inhibitors for the treatment of PML.

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ORIGINAL ARTICLE

Sequential changes in the non-coding control region sequences of JC polyomaviruses from the cerebrospinal fluid of patients with progressive multifocal leukoencephalopathy

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Abstract Progressive multifocal leukoencephalopathy (PML) is caused by JC polyomavirus (JCV) infection in the brain. JCV isolates from PML patients have variable mutations in the non-coding control region (NCCR) of the genome. This study was conducted to examine sequential changes in NCCR patterns of JCV isolates obtained from the cerebrospinal fluid (CSF) of PML patients. CSF specimens were collected from PML patients at different time points, the NCCR sequences were determined, and their compositions were assessed by computer-based analysis. In patients showing a marked increase in JCV load, the most frequent NCCR sequences in the follow-up specimens were different from those in the initial samples. In contrast, the dominant NCCRs in the CSF remained unaltered during the follow-up of individuals in whom the viral load decreased after therapeutic intervention. These data demonstrate that the majority of JCV variants emerge with the progression of PML and that these changes are suppressed when the viral load is decreased.

Introduction

JC polyomavirus (JCV) is a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* [1]. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system in humans [2–5]. PML develops in HIV-positive patients as well as in those who are immunodeficient due to hematological malignancies, chemotherapy, transplantation, lymphocyte depletion or the treatment of autoimmune disorders with immunosuppressive agents [2, 5, 6]. In addition, PML has recently been

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diagnosed in patients receiving immunomodulatory therapies with monoclonal antibodies, such as natalizumab, rituximab, and efalizumab [2, 4, 5, 7, 8]. Currently, no specific treatment has been established for PML. Restoration of the immune system, either by combination antiretroviral therapy (cART) for patients with acquired immunodeficiency syndrome (AIDS)-related PML or by moderating the immunosuppressive therapies for non-AIDS-related PML patients, is the only treatment option for the management of PML, although several experimental treatments are being investigated [2].

Clinical isolates of JCV can be classified into two groups, primarily on the basis of sequence divergence in the non-coding control region (NCCR; also referred to as the regulatory region or transcription control region) of the viral genome [5, 9–11]. This region includes the origin of replication as well as sequences that control transcription of viral early and late genes [12, 13]. Nonpathogenic JCV strains isolated from the urine of healthy individuals contain a consistent NCCR sequence known as the archetype [14]. In contrast, JCV isolates recovered from the brain or cerebrospinal fluid (CSF) of PML patients are characterized by multiple rearrangements within the NCCR [11, 15]. The changes in the NCCR sequences are thought to be related to the activation of virus replication during disease progression [11, 15, 16]. These mutated sequences are thought to be derived from the archetype NCCR via deletions and/or duplications [17, 18], leading to the alteration of promoter activity [3, 19, 20]. Accumulating evidence suggests that the rearranged NCCRs of JCV variants recovered from PML patients exhibit highly variable and patient-specific sequences [15, 19, 21–33]. It has also been suggested that NCCRs contain increased variations in rearrangements correlated with immunosuppression [15]. Recent *in vitro* studies have demonstrated that the replicative efficacy of JCV variants with rearranged NCCRs is higher than that of archetype viruses [16, 19, 34]. Cellular factors are likely to contribute to the replication capacity of JCV [1, 35]. However, the precise mutational mechanism of the NCCR in the PML brain remains to be elucidated.

Measurement of the JCV DNA level in the CSF is a reliable and less-invasive marker for the diagnosis of PML [7]. In addition, CSF testing for JCV DNA is repeated during the follow-up period to monitor the progression of the disease. It has been reported that the copy numbers of JCV DNA in CSF increase in accordance with PML progression [36]. It has also been suggested that CSF viral loads are diminished in PML patients receiving cART or being administered mefloquine, a potential drug candidate for the treatment of PML [36–40]. It is hypothesized that JCV variants with rearranged NCCRs are released into the CSF. The question thus arises as to whether the patient-specific NCCR pattern is maintained in the CSF during the course of PML progression.

In the present study, the relationship between sequential changes in CSF JCV loads and rearrangements of NCCR sequences was analyzed. For this purpose, NCCR patterns were examined using different sets of CSF specimens collected from PML patients in whom JCV DNA levels increased or decreased during the follow-up term.

Materials and methods

CSF specimens

The study protocol was approved by the Ethical Committee for Biomedical Science in the National Institute of Infectious Diseases (application number 338). CSF specimens were collected by lumbar puncture from 11 patients after informed consent from patients or their family members was obtained, and they were transferred from the respective hospitals to the Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, for the routine clinical testing of JCV DNA as a part of medical practice. Anonymised clinical information was collected through standardized questionnaires. The subjects were diagnosed as having PML on the basis of neurological symptoms, magnetic resonance imaging patterns, and the detection of JCV DNA in the initial CSF testing. These patients were immunocompromised because of severe underling diseases or immunosuppressive therapies, such as hematopoietic stem cell transplantation, chemotherapy, and the administration of immunosuppressive agents. The study population comprised 8 males and 3 females. The mean age of subjects was 58.4 years (median 62.0 years, range 40–78 years, SD = 14.5). The subjects were categorized into three groups on the basis of the underlying diseases and therapeutic interventions. The first group, Group I, consisted of four non-AIDS-related PML patients in whom the amounts of JCV DNA in the CSF increased during the follow-up period. The individuals in this group did not receive mefloquine. Four patients in the second group, Group II, were diagnosed as having non-AIDS-related PML and showed a reduction in CSF JCV loads after the oral administration of mefloquine hydrochloride tablets (Mephaquine Hisamitsu; Hisamitsu Pharmaceutical Co., Inc., Saga, Japan). The mefloquine-based therapy was experimentally performed with informed consent from the patients or their family members and approval from the ethics committee of each institution. The third group, Group III, consisted of three AIDS-related PML patients who showed a reduction in JCV DNA levels in the CSF during cART.

DNA extraction and real-time PCR

Total DNAs were extracted from CSF specimens using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA), and the JCV DNA copy number in each sample was determined using a real-time PCR assay as described previously [41].

Cloning of NCCRs

The NCCRs were amplified by nested PCR using a high-fidelity DNA polymerase with proofreading activity (Blend Taq Plus; Toyobo, Tokyo, Japan) as reported earlier [29, 41]. To equalize the amplification efficiency of the nested PCR, each DNA extract was diluted so that the amount of JCV DNA was 20 copies per reaction. Amplified DNA fragments were purified using a MinElute PCR Purification Kit (QIAGEN) and ligated to the TA cloning vector pGEM-T Easy Vector (Promega, Madison, WI) according to the protocols supplied by the manufacturers. Competent *Escherichia coli* cells (ECOS Competent *E. coli* DH5α; Nippon Gene, Tokyo, Japan) were transformed with the ligation mixture and plated on an LB agar medium containing ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Promega) as specified by the suppliers. White colonies were randomly selected for each clinical sample and were grown in a liquid LB medium containing ampicillin. Plasmid DNA was extracted from the bacterial pellets using a QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and then subjected to sequence analysis of the NCCR inserts (20 clones/patient, 10 clones/time point). This amplification and cloning method could detect not only the major but also at least 10 % of the minor NCCRs in a mixed population of JCV strains (data not shown).

Sequence analysis

Sequencing reactions were performed in a DNA Engine Dyad thermal cycler (Bio-Rad Laboratories, Hercules, CA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the protocols supplied by the manufacturers. Sequencing of each NCCR clone from both sides of the insert was performed using universal primers (5′- TAA TAC GAC TCA CTA TAG GG-3′ and 5′- ATT TAG GTG ACA CTA TAG -3′) in an ABI 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences of the NCCR clones were aligned to that of the archetype CY strain recovered from a healthy Japanese individual (GenBank: AB038249.1) [32] using CLC DNA Workbench 6.1 software (CLC bio, Aarhus, Denmark). When the NCCR sequence of any clone was different from that of the archetype JCV due to insertions and/or deletions, the mutated NCCR sequence of the patient-derived JCV was subdivided into fragments *in silico*, and the partial sequences were aligned to the archetype NCCR. The fragmentation and alignment of the NCCR clones were repeated until each fragment overlapped with the

Table 1 Characteristics of non-AIDS-related PML patients showing an increase or decrease in CSF viral load

			JCV testing (copies/mL CSF)			
Group[a]	Patient	Underlying disease[b]	Initial	Follow-up[c]	Days[d]	Ratio[e]
I	1	Liver cirrhosis (HCV carrier)	1.74×10^3	7.11×10^4	26	41
I	2	Primary macroglobulinemia	1.64×10^5	5.23×10^7	58	319
I	3	Chronic lymphocytic leukemia	2.98×10^3	9.82×10^5	49	330
I	4	Acute lymphoblastic leukemia	5.64×10^3	8.79×10^6	48	1559
II	5	Hepatocellular carcinoma	1.18×10^6	1.64×10^4	52	0.0139
II	6	Systemic lupus erythematosus Sarcoidosis	3.05×10^6	2.51×10^4	39	0.0082
II	7	Non-Hodgkin's lymphoma	1.36×10^6	1.16×10^3	182	0.0009
II	8[f]	Acute myeloid leukemia	9.11×10^5	7.43×10^2	68	0.0008

[a] Non-AIDS-related PML patients were categorized into Groups I and II on the basis of changes in CSF viral load

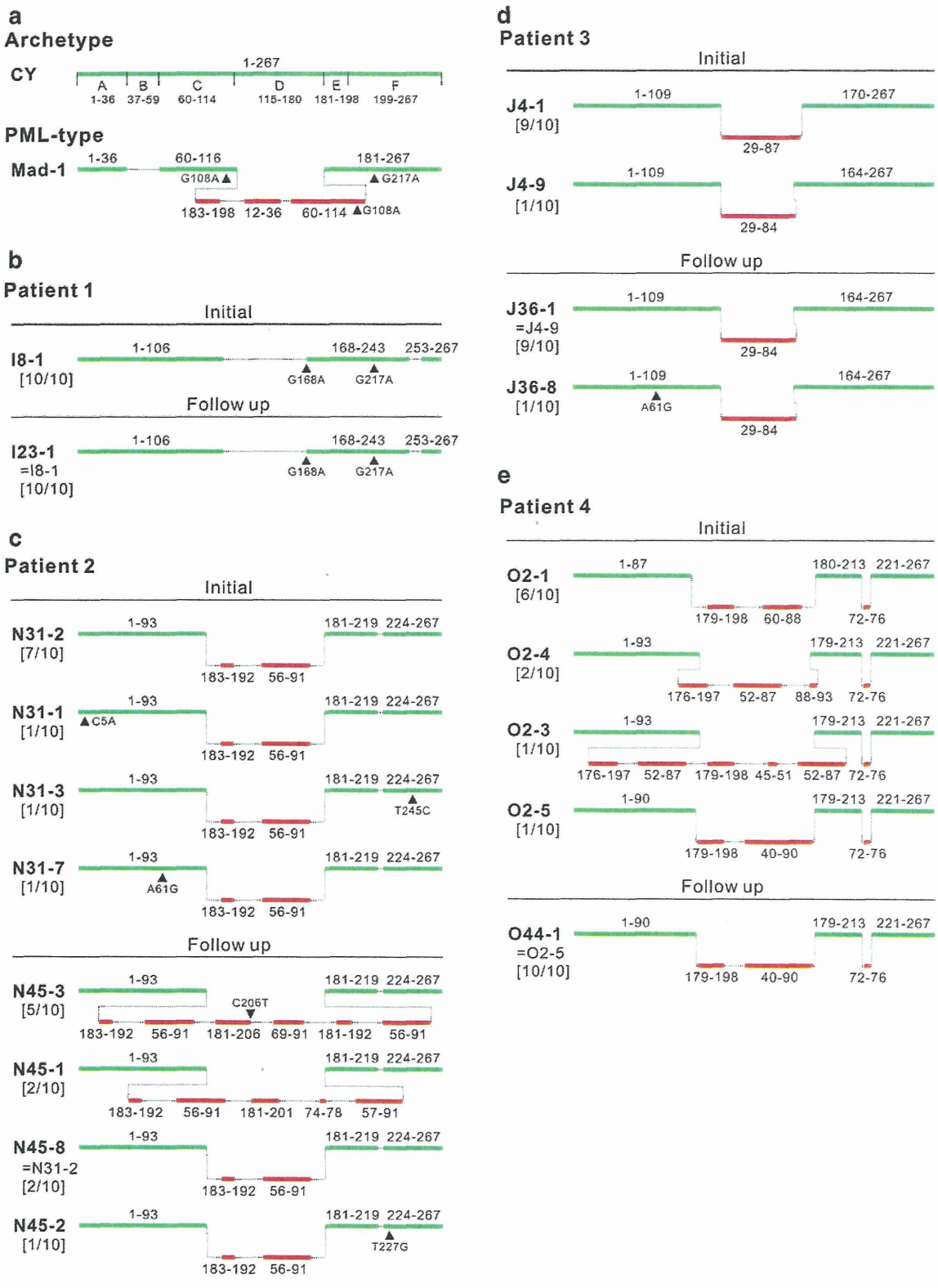
[b] HCV, hepatitis C virus

[c] Patients in Group II were administered mefloquine after initial JCV testing

[d] Days between the initial and follow-up measurements of JCV DNA levels

[e] Amount of JCV DNA in follow-up sample divided by that in the initial sample

[f] Clinical features including CSF viral loads were reported previously by the authors as a patient report (ref. 39)



archetype NCCR. Single-nucleotide substitutions and/or deletions within the NCCR were also identified. The origin-identified fragments were then joined together to reconstitute the full-length NCCR sequence.

Nucleotide sequence accession numbers

NCCR sequences were submitted to the DNA Data Bank of Japan (DDBJ) and were assigned accession numbers

◀ **Fig. 1** NCCR patterns in the CSF from PML patients showing an increased viral load (Group I). The NCCR sequences were compared to that of the archetype CY strain, and their compositions were illustrated on the basis of *in silico* analysis. (**a**) NCCRs of the archetype (CY) and PML-type (Mad-1) strains. The horizontal green lines indicate the DNA fragments identical to the archetype NCCR (5' and 3' nucleotide positions 1-267 within the JCV genome). The letters A to F represent the regions of the archetype NCCR mentioned in the main text. The dotted lines represent the deleted sequences. The red lines indicate the duplicated sequences inserted into the deleted region. The nucleotide numbers corresponding to the archetype NCCR are shown above or below the solid lines. Single-base differences are represented by closed triangles (e.g., "G108A" indicates that G at nucleotide position 108 in the archetype NCCR is substituted by A). (**b–e**) NCCRs of JCV variants detected in the CSF from patients 1 to 4. The NCCR patterns in the initial (upper side) and follow-up (lower side) specimens are shown. The name of each NCCR pattern (e.g., I8-1) is indicated on the left side of the green lines. As several NCCR clones in each specimen had identical sequences, frequencies of each NCCR pattern in the respective specimens are shown in square brackets. Identical NCCR sequences between the initial and follow-up samples are represented by equal symbols

(GenBank/EMBL/DDBJ: AB699037–AB699089). A list of the accession numbers as allocated to the individual CSFs of individual patients is shown in Supplementary Material, Table S1.

Results

Nucleotide sequences and the JCV NCCR patterns in CSF specimens from PML patients showing increased viral loads (Group I)

The first series of analyses was conducted to examine the NCCR patterns of JCV detected in the CSF from PML patients in Group I (Table 1). For patients 1 to 4, the immunosuppressive therapies were tapered off or discontinued after the detection of JCV DNA in the initial CSF testing, but no mefloquine was administered. The CSF specimens for the follow-up JCV testing were collected between 26 and 58 days after the initial examination. The CSF viral loads in patients 1 to 4 increased by 41-, 319-, 330- and 1559-fold, respectively, during the follow-up term. The NCCR patterns of the archetype (CY strain), PML-type (Mad-1 strain), and patient-derived viruses are shown in Fig. 1. In the archetype CY JCV, the 267 bases of the NCCR are commonly divided into regions A, B, C, D, E, and F [17]. In the rearranged NCCR of the Mad-1 strain (GenBank: J02226) [42], nucleotides 37-59 and 117-180, corresponding to regions B and D, respectively, were deleted, and nucleotides 183-198, 12-36, and 60-114 were duplicated (Fig. 1a). Two single-nucleotide differences were found in regions C and F of Mad-1 NCCR compared to the CY strain sequence. The NCCR sequences of 80 clones from the PML patients (20 clones/patient, 10 clones/time point) were determined. In each specimen, several NCCR clones had identical sequences, and 11 and 8 NCCR patterns were identified in the initial and follow-up specimens, respectively (Fig. 1b-e). All the NCCR sequences of these clones had deletions and/or duplications, primarily within region D, and were apparently different from the archetype NCCR (Fig. 1a). Although the numbers and sequence compositions of these NCCRs appeared to differ among patients, it was noticed that similar NCCR sequences were identified in both the initial and follow-up specimens from the respective individuals (Fig. 1b-e). Thus, the variations and frequencies of each NCCR pattern were compared. The NCCR sequences of clones I8-1, N31-2, J4-9 and O2-5 in the initial CSF specimens were identical to those of clones I23-1, N45-8, J36-1 and O44-1, respectively, in the follow-up samples (Fig. 1b-e). In the initial and follow-up specimens from patient 1, who showed a 41-fold increase in viral DNA load, the NCCR patterns of all clones were the same as the sequence of clone I8-1 (Fig. 1b). In patients 2 to 4, in whom viral DNA levels were elevated by more than 300-fold during the follow-up period, the most frequent NCCR patterns in the initial CSF specimens were N31-2, J4-1 and O2-1, respectively. However, these NCCR patterns were in the minority (N45-8 in patient 2) or were not identified (J4-1 and O2-1 in patients 3 and 4, respectively) in the follow-up specimens (Fig. 1c-e). In addition, no major NCCR patterns in the follow-up specimens were identified (N45-3 in patient 2) or were detected at low frequency (J36-1/J4-9 and O44-1/O2-5 in patients 3 and 4, respectively) in the initial testing samples. These data suggest that the dominant NCCR patterns changed in the CSF from PML patients in accordance with the increase in viral load during the follow-up period.

Nucleotide sequences and JCV NCCR patterns in CSF from PML patients in whom viral DNA loads decreased (Group II)

The next analyses were conducted to assess the NCCR patterns in CSF specimens from PML patients in whom CSF viral loads decreased during the follow-up period (Group II). PML patients 5 to 8, who were all administered mefloquine, showed a reduction in DNA levels at between 39 and 182 days after the initial testing (Table 1). The JCV DNA copy numbers in the follow-up specimens from these patients were reduced by approximately 99 % compared to those in the initial samples. The sequence compositions of 80 NCCR clones (20 clones/patient, 10 clones/time point) in the CSF specimens from these patients are shown in Fig. 2 (patients 5-7) and Fig. 3 (patient 8). Several NCCR clones in each specimen had identical sequences, and thus

16 and 13 rearranged NCCR patterns were detected in the initial and follow-up samples, respectively. To elucidate the variations in JCV NCCRs in the CSF from patients 5 to 8, the frequencies of each NCCR pattern were examined. The NCCR sequence compositions of clones M55-1, K40-1, Y41-1, T37-1 and T37-6 completely matched those of clones M56-1, K26-1, Y17-1, T7-1 and T7-8, respectively. The NCCR patterns M55-1, K40-1, Y41-1 and T37-1 were most frequently detected in the initial CSF specimens from patients 5 to 8, respectively. These NCCR patterns were also dominant in the follow-up specimens from patients 5 to 8 (M56-1/M55-1, K26-1/K40-1, Y17-1/Y41-1 and T7-1/T37-1, respectively) (Fig. 2 and Fig. 3). These data suggest that the dominant NCCR patterns remained virtually unaltered in the CSF samples from PML patients when viral loads are reduced after mefloquine administration.

NCCR patterns in the CSF from AIDS-related PML patients in whom viral DNA loads decreased after cART (Group III)

As the dominant JCV variants in the CSF remained dominant during the follow-up in the mefloquine-treated PML patients, a final set of analyses was performed to elucidate whether this phenomenon could also be observed in the absence of mefloquine treatment. Three different sets of CSF specimens were obtained from AIDS-related PML patients in whom JCV loads decreased during cART. The

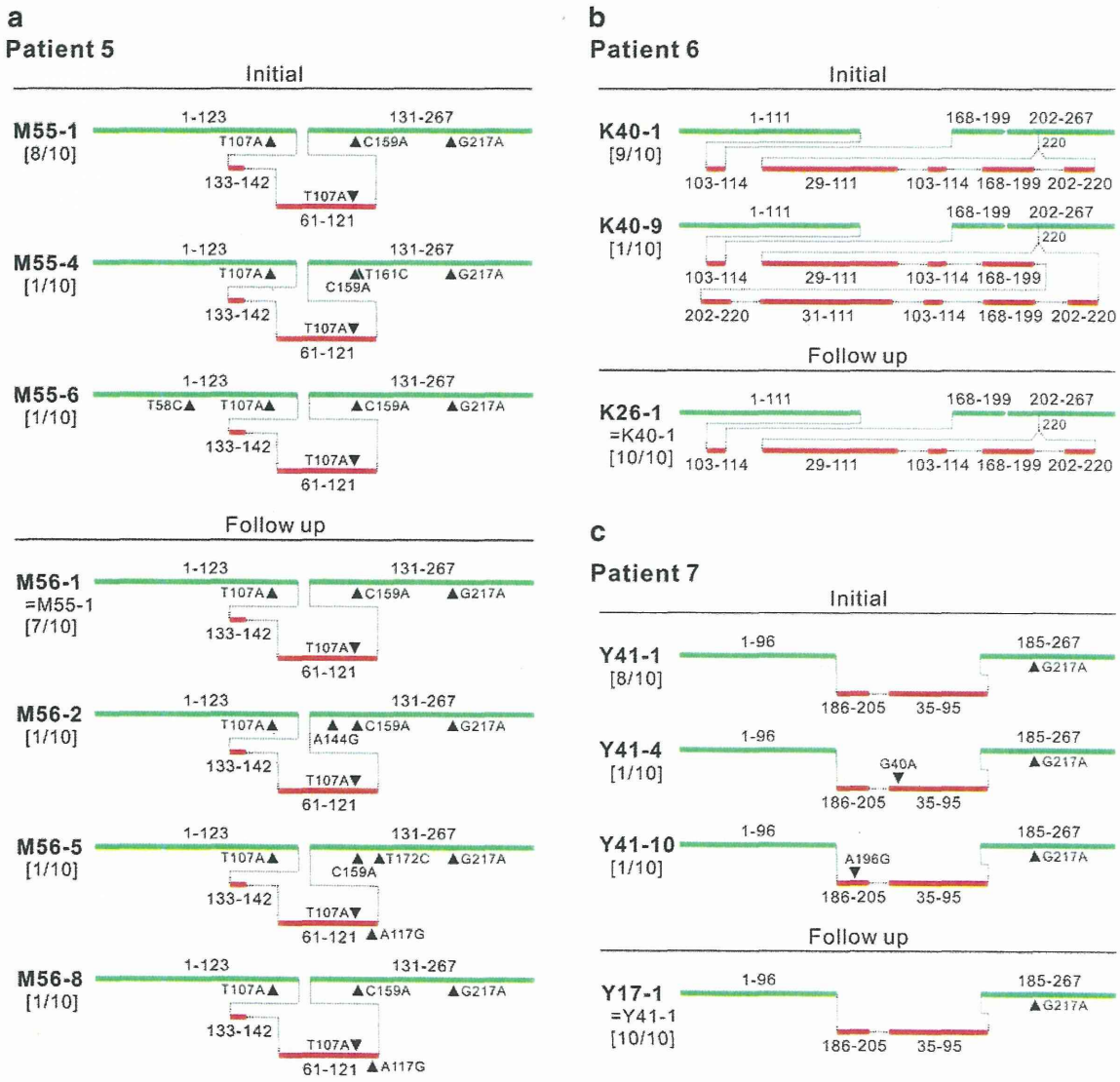


Fig. 2 NCCR patterns in the CSF from PML patients showing a decreased viral load (patients 5-7). The NCCR patterns in the CSF from patients 5 to 7 in Group II were compared to that of the archetype CY strain (**a**–**c**, respectively). The method of schematic representation is the same as that in Fig. 1

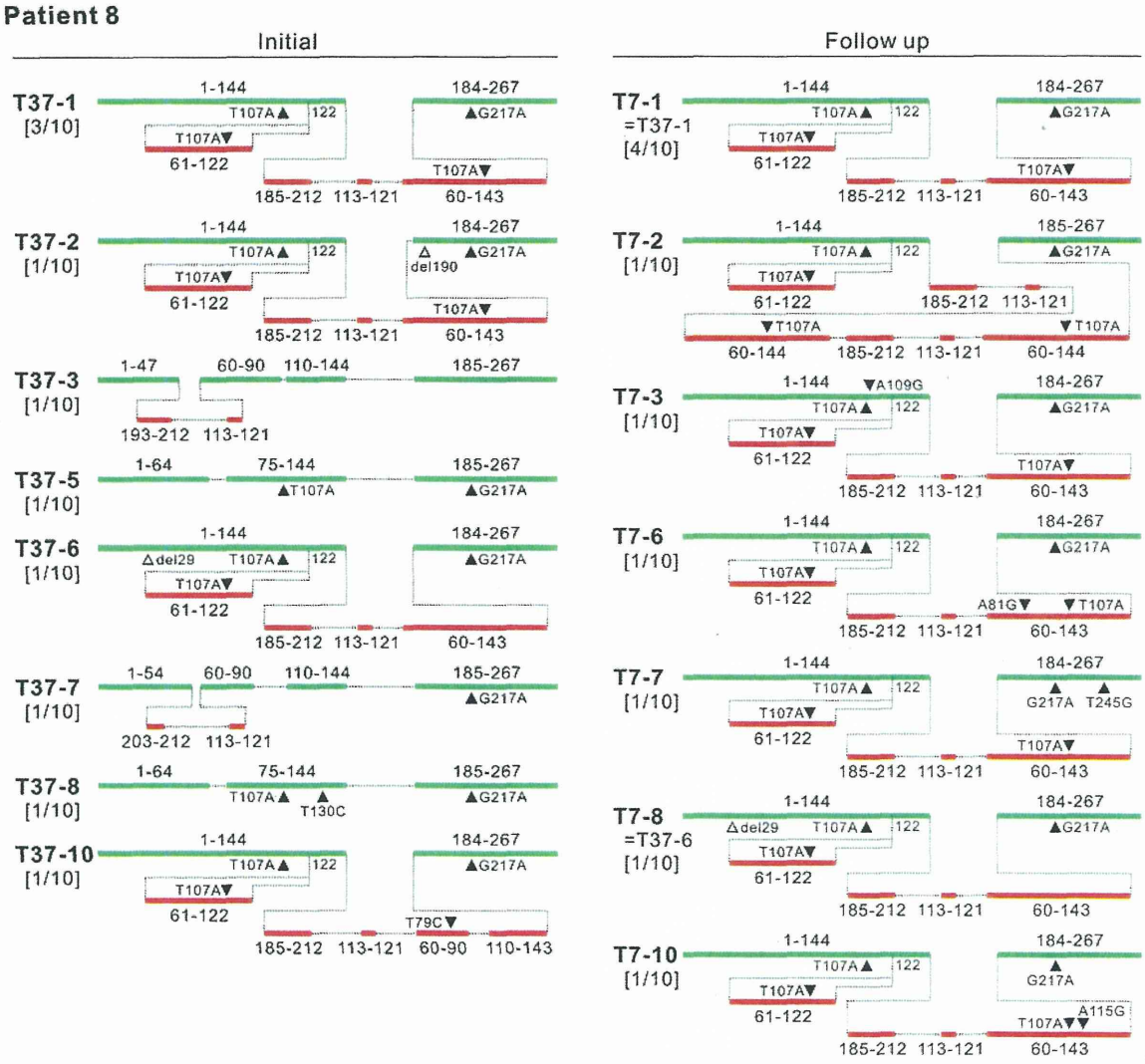


Fig. 3 NCCR patterns in the CSF from PML patients showing a decreased viral load (continued). The NCCR patterns in the CSF from patient 8 in Group II were compared to that of the archetype CY strain. The method of schematic representation is the same as that in Fig. 1. In addition, single-base deletions are represented by open triangles (e.g., "del190" in NCCR pattern T37-2 indicates that nucleotide 190 in the archetype NCCR is deleted)

changes in the quantities of JCV genome in the CSF samples obtained for the initial and follow-up examinations are shown in Fig. 4a. Patients 9 and 10 received cART after PML diagnosis, and the JCV DNA levels decreased to undetectable levels at 85 and 69 days after the initial testing, respectively. Thus, alterations in JCV populations during the follow-up could not be analyzed in the Group III patients other than patient 11. Patient 11 received cART before PML diagnosis, and the CSF viral load in the follow-up specimen decreased by approximately 99 % at 41 days after the initial testing. The arrangement orders of 20 NCCR clones derived from the CSF from patient 11 are shown in Fig. 4b. Three types of NCCR sequences were identified in the initial testing sample, with S18-2 the most frequently detected. In the follow-up CSF specimen, two NCCR patterns were identified, with S3-1 dominant. The nucleotide sequence of S3-1 in the follow-up specimen was identical to that of S18-2 in the initial sample. These data suggest that the dominant JCV variant in the CSF remained unchanged.

Discussion

Although numerous studies have demonstrated that rearranged NCCRs can be detected in CSF specimens from PML patients [15, 19, 21–33, 43–49], it remains unclear whether NCCR patterns are altered during the progression of PML. The value of this study is in revealing the sequential patterns of NCCR sequences in PML patients

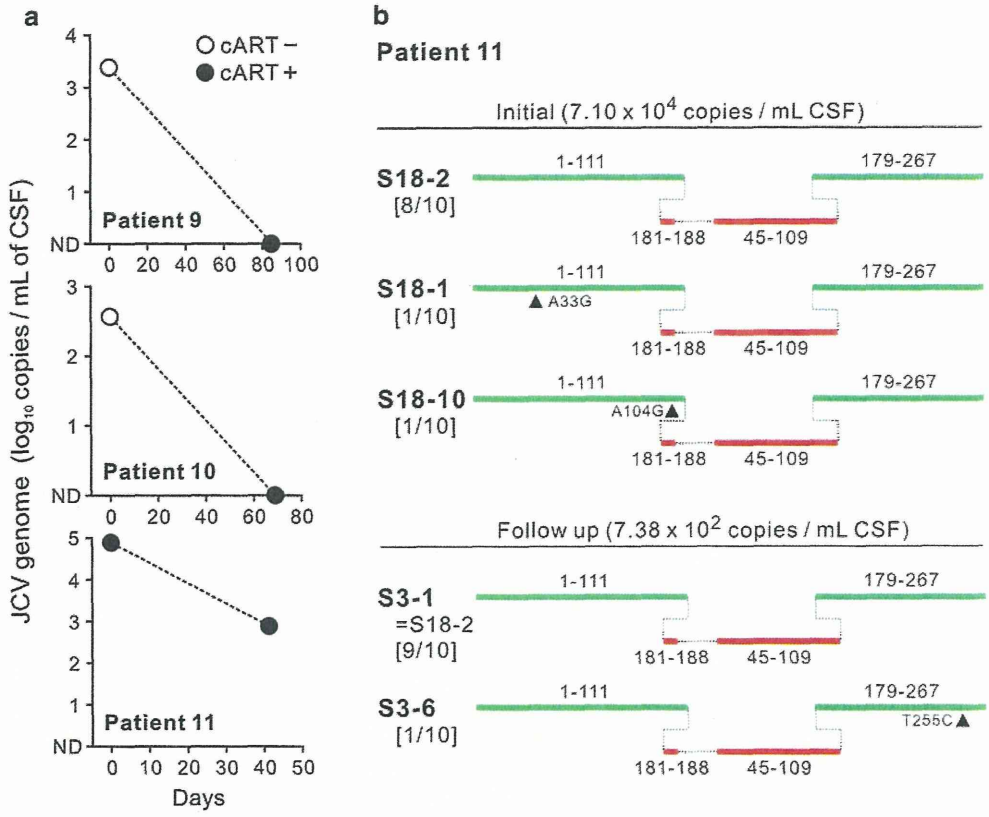


Fig. 4 The CSF JCV loads and NCCR patterns in AIDS-related PML patients who received cART (Group III). (**a**) The JCV DNA levels in the CSF during the course of the follow-up period. The CSF specimens were collected from patients 9 to 11 for the PML diagnosis (day 0) or for follow-up after the initial testing. The open and closed circles indicate the specimens obtained before and after cART, respectively. ND indicates "not detected". (**b**) Diagrammatic representation of NCCR sequences detected in the CSF specimens from patient 11. The NCCRs are shown as described for Fig. 1. In addition, the copy numbers of JCV genomes in the CSF specimens are shown in parentheses

using CSF specimens obtained at different time points. The present data demonstrate that the majority of JCV variants emerge with the progression of PML and that these changes are suppressed when the viral load is decreased. Studies on the mutational mechanism of JCV NCCRs in the human brain have been hampered by the lack of suitable animal models that mimic JCV infection in the brain of PML patients. Although JCV testing is occasionally performed on biopsied or autopsied brain tissues for the diagnosis of PML, it is difficult to monitor the continuous changes in NCCR patterns using these samples. The current investigation focused on the NCCR patterns of JCV variants in CSF specimens collected for the diagnosis and management of PML. JCV DNA levels in CSF are reported to decrease in AIDS-related PML patients receiving cART [38]. In agreement with the reported results, we found that the JCV DNA levels decreased in AIDS-related PML patients (Group III). It has been reported that the JCV promoter is transactivated by the Tat protein of HIV-1 [50, 51]. It is likely that although PML was induced by HIV infection, JCV replication was inhibited due to the early initiation of cART during the follow-up period. Thus, the analyses were primarily performed on samples collected from patients with non-AIDS-related PML.

Although the relationship between the complexity of rearranged NCCRs and the pathogenesis of PML is unclear [27], Gosert *et al.* demonstrated that NCCR rearrangements, particularly deletions in region D, commonly increase JCV early gene expression [19]. In the present study, relatively high levels of JCV DNA (9.11×10^5–5.23×10^7 copies/mL) were detected in the follow-up CSF specimens from patients 2 to 4 and in the initial samples from patients 5 to 8 (Table 1). It is apparent that JCV actively propagated in the brains of these patients over the course of disease progression. Highly complicated NCCR patterns were dominant in the follow-up specimens from patient 2 (N45-3 in Fig. 1c) and the initial samples from patients 6 and 8 (K40-1 in Fig. 2b and T37-1 in Fig. 3 respectively). However, NCCR patterns in the majority JCV populations in the follow-up specimens from patient 3 (J36-1 in Fig. 1d) and in the initial samples from patient 7 (Y41-1 in Fig. 2c) were simple in composition. Thus, the complexity of the dominant NCCR pattern is thought not to be associated with viral DNA levels in the CSF. These data provide an important background to the analysis of NCCR patterns for PML diagnosis. Although the exact mechanisms of the genomic rearrangement of NCCR sequences have yet to be fully delineated, Tan *et al.* have suggested that the rearranged NCCRs could be detected in the brain and peripheral organs of immunosuppressed individuals without PML [15]. They also indicated that the active replication of JCV under immunosuppression is correlated

with increased variations in NCCR sequences [15]. Based on previous findings, it can be postulated that in patients 2, 6, and 8, JCV actively replicated and underwent frequent NCCR mutations prior to the development of PML, probably due to severe immunosuppression. JCV variants with complicated NCCR patterns might have subsequently led to PML.

The most frequent NCCR patterns in the initial CSF samples were different from those in the follow-up CSF samples in the three patients in Group I in whom the viral loads increased by more than 300-fold. In one patient in this group, the viral DNA load in the CSF increased by only 41-fold, and the NCCR pattern remained unchanged during the follow-up period. These data indicate that the dominant JCV variants with distinct NCCRs in the CSF readily change in accordance with the marked increases in viral DNA levels. It was suggested that the detected NCCR patterns changed within 3 days in one PML patient [19], probably due to highly dynamic virus replication, as reported for the closely related BK virus [52]. This viral evolution in the CSF might come from the same or different replication foci in the brain, as discussed in an earlier report [19]. It has been shown previously that naturally occurring JCV variants with rearranged NCCRs from PML patients confer increased early gene expression and higher replication rates compared to those of JCV with archetype NCCR and thereby increase cytopathology [19, 53]. In the present study, the rearranged NCCR sequences were readily detected in the initial test samples, implying that JCV variants had increased replicative efficacy at the onset of PML. It will be important to determine whether the change in the dominant NCCR patterns during the follow-up period is associated with an exacerbation of PML. It is likely that the sequential rearrangement of the NCCR during the follow-up period augments the replicative efficacy of JCV during disease progression.

It is of interest to speculate whether the JCV variants with rearranged NCCRs emerge from preexisting viruses that are suppressed by the immune system or from *de novo* generation during replication. Based on the NCCR patterns in patients 2 to 4 (Fig. 1), it appears that the most frequent NCCR variants in the follow-up CSF specimens are produced from among the populations of the dominant NCCR viruses in the first samples via one of the following possible modes. In patient 2, N45-3 is thought to be created by the tandem insertion of nucleotides 181-206, 69-91, 181-192 and 56-91 into the duplicated region (next to nucleotides 183-192 and 56-91) of N31-2, and a single point mutation (C to T at nucleotide position 206) might occur during this process (Fig. 1c). In patient 3, J36-1 can be generated by a three-base deletion within the duplicated region (nucleotides 29-87) and by a six-base insertion (nucleotide 164-169) in J4-1 (Fig. 1d). In patient 4, O44-1 is considered to be a derivative of O2-1 generated by the replacement of nucleotides 60-88 with 40-90 (Fig. 1e). Therefore, it is reasonable to suppose that JCV variants detected in the follow-up periods are generated *de novo* by simple mutational events during viral replication or spread within the brain.

A notable finding of the present study is that the dominant NCCR patterns remained unchanged in the CSF specimens of non-AIDS-related PML patients when viral loads were decreased after mefloquine administration (Group II). Mefloquine is widely used for the prophylaxis and treatment of malaria and is known to cross the blood-brain barrier [54, 55]. It has been reported that mefloquine inhibits JCV replication *in vitro* [37]. However, the molecular mechanisms underlying the effect of mefloquine on JCV replication remain unknown, and the efficacy of mefloquine treatment for PML has not been validated through a randomized controlled trial and remains controversial. Several recent studies on Japanese PML patients receiving mefloquine have demonstrated a reduction in CSF JCV loads and/or the arrest of disease progression as judged by clinical signs and imaging features [39, 56–58]. However, the data obtained in the present study must be interpreted with caution because the immunosuppressive therapies were tapered off or discontinued in most cases. It is possible that the immune restoration, not mefloquine administration, led directly to the reduction in viral load in some PML cases. In addition, the stabilization of the dominant JCV population was observed not only after administration of mefloquine but also after cART, suggesting that this phenomenon seems not to be specific to mefloquine-treated patients. Therefore, it is considered that the alteration in the dominant JCV population is inhibited as a consequence of the decrease in viral replication efficiency within the brain. It was also found that several NCCR sequences were identified at low frequencies in the follow-up CSF specimens from patients in Group II and that most of these patterns were not identified in the initial test samples. It can be speculated that although the high-frequency variants were controlled, the low-frequency viruses emerged, probably due to incomplete inhibition of JCV replication. Although JCV propagation in the main lesion might be attenuated, the virus might evade the immune system and/or the effect of mefloquine and undergo NCCR mutations in a small, localized area within the brain. As patients' sera were not obtained from the hospitals, anti-JCV antibody titers could not be measured. It is important to examine the immune responses of patients in whom the viral load decreased during the follow-up period.

In summary, the data obtained in the current study demonstrate that the majority of JCV variants change in accordance with the marked increase in CSF viral load

during PML progression and that these changes are suppressed when the viral load is decreased after therapeutic intervention. NCCR typing, as well as determination of viral load, may assist in the evaluation of the effect of treatment on PML during the follow-up period.

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Conflict of interest The authors declare that they have no conflict of interest.

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