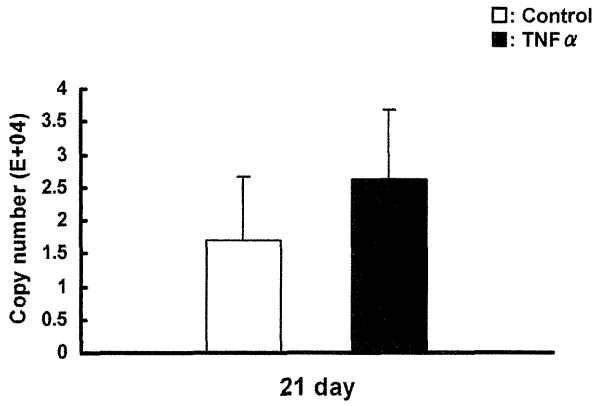


Fig. 4. DNase protection assay of JCV production in TNF-α-treated IMR-32 cells. The cells were transfected with the JCV genome and incubated for 21 days in the absence or presence of TNF-α. The extravirion JCV DNAs were eliminated with a potent DNase (Base-line zero DNase), and the amount of viral genome, which had not been digested by DNase in the particles, was measured by real-time PCR. The linear standard curve was generated from serial dilution with plasmid M1-IMRb. Data are shown as the mean ± standard deviation of the means.

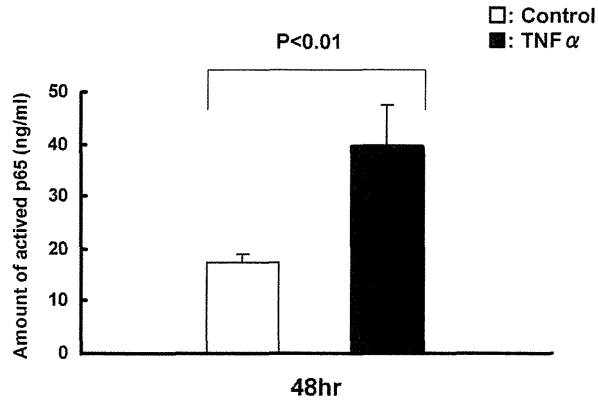


Fig. 5. Stimulation of the NF-κB pathway by TNF-α in IMR-32 cells. IMR-32 cells were plated into six-well plates at a concentration of $8.0 \times 10^5$ cells/well in 2 ml medium and incubated in the absence (Control) or presence (TNF-α) of TNF-α for 48 hr. The nuclear fraction was extracted from each of the four cultures. The amount of p65 was measured using ELISA. Data are shown as the mean ± standard deviation of the means. $^*P < 0.01$ (Student's *t*-test).

conditions used, at least $7 \times 10^9$ copies of the transfected JCV genome could be digested by DNase, while the replicated JCV particles remained intact (data not shown). The results of real-time PCR show that the amount of JCV DNA derived from virus particles in TNF-α-treated IMR-32 cells was 1.5-fold greater than that in control cells, although this difference was not statistically significant (Fig. 4).

### TNF-α Stimulates the NF-κB Pathway

Activation of the NF-κB pathway in TNF-α-treated IMR-32 cells was quantified by measuring the nuclear translocation of NF-κB/p65, as described in section Materials and Methods. The amount of p65 in TNF-α-treated cells was 2.3-fold greater than that in untreated cells, and this difference was statistically significant ($P < 0.01$) (Fig. 5). These results indicate that TNF-α stimulates the activation of the NF-κB pathway in IMR-32 cells.

## DISCUSSION

AIDS–PML has become prevalent and is found in approximately 4% of all AIDS patients [Major et al., 1992]. The high incidence of PML among individuals with AIDS suggests that PML is particularly associated with AIDS. HIV-1 induces the production of pro-inflammatory cytokines such as TNF-α in the CNS [Benveniste, 1994; Yeung et al., 1995; Kaul et al., 2005]. In this study, the effects of TNF-α on viral gene expression were examined in IMR-32 cells transfected with JCV DNA. The plasmid M1-IMRb contains the viral genome of the JCV mutant strain, which can productively replicate in IMR-32 cells. This mutant JCV was obtained from serial passaging of the JCV Mad-1 strain in IMR-32 cells. The viral genome of M1-IMRb has deletions of the TATA sequence distal from the origin of replication within the regulatory region, leading to a similar sequence to that of the Mad-4 strain. However, deletion endpoints, relative to Mad-1, were not identical between Mad-4 and M1-IMRb. The expression level of the JCV large T gene in IMR-32 cells was increased following TNF-α treatment. Since the NF-κB pathway in IMR-32 cells was activated in the presence of TNF-α, it can be speculated that TNF-α-mediated activation of the NF-κB pathway leads to the upregulation of JCV large T expression. The results of the DNA replication assay and real-time RT-PCR in the transfection experiment show that the replication of JCV genome DNA in IMR-32 cells was increased in the presence of TNF-α. Thus, the upregulation of large T expression is necessary for viral DNA replication.

In a previous study by other investigators, the effects of cytokines, TNF-α, IL-6, IL-1β, and TGF-β, on the transcription of JCV early and late genes were examined using reporter assays. Each reporter assay included phorbol 12-myristate 13-acetate (PMA) as a positive control. TNF-α stimulated the early promoter as strongly as PMA and TGF-β, IL-6 had a minimal effect, and the effect of IL-1β was intermediate. The expression of the late promoter of JCV in TNF-α-treated cells was statistically significantly higher than in cells treated with other cytokines [Wollebo et al., 2011]. It was suggested that TNF-α stimulates JCV transcription in both the early and late phases of infection in the human oligodendroglioma cell line [Wollebo et al., 2011]. These studies indicated a relationship between TNF-α and AIDS–PML pathogenesis. It has been noted that TNF-α stimulates the transcription of the JCV reporter construct containing a NF-κB binding site (κB element) [Wollebo et al.,

2011]. However, it has also been reported that TNF-α does not stimulate JCV transcription in human glial cells [Atwood et al., 1995] as the JCV reporter construct in the study did not contain a κB element.

NF-κB is a transcriptional factor that can be activated by TNF-α and, in turn, activates the expression of HIV-1 in T cells [Nabel and Baltimore, 1987; Fiers, 1991; West et al., 2001]. The κB element also regulates JCV promoter activity in CNS-derived cells [Ranganathan and Khalili, 1993]. When the amounts of p65 were increased in the presence of nuclear factor of activated T cells 4 (NFAT4), there was a synergistic enhancement of JCV early transcription [Wollebo et al., 2012]. NFAT4 has a role as a cell-signaling phosphatase in neurons and glia [Ho et al., 1994; Graef et al., 1999]. Further analyses, such as the gene expression of NFAT4, need to be conducted to better understand TNF-α mediated JCV replication in IMR-32 cells.

The regulation of JCV replication by TNF-α in AIDS-related PML is currently unknown. In fact, there have been no reports of TNF-α-stimulated JCV replication in culture cells to date because a previous report showed the effect of TNF-α on JCV early and late transcription in reporter assays [Wollebo et al., 2011].

While astrocytes are targets of HIV-1 infection, they are semi-permissive of JCV infection as they allow the expression on T antigen and late gene expression to a limited extent without virus production in vivo. Previous studies revealed that IMR-32 cells transfected with M1-IMRb could be transferred repeatedly accompanied by continuous JCV production. Since it is difficult to propagate JCV in astrocytes, IMR-32 cells are useful for studying the role of TNF-α in JCV production. In this study, JCV production in IMR-32 cells transfected with M1-IMRb was compared using real-time PCR analysis combined with DNase treatment. Extravirion DNAs derived from the transfected JCV genome were eliminated with a potent DNase, and the amount of JCV DNAs from the DNase-protected particles was determined. Further developments are needed to establish a JCV production system using astrocytic cell lines.

A previous report based on in situ hybridization analysis showed that TNF-α did not increase the multiplication of JCV in human fetal glial cells infected with JCV [Atwood et al., 1995]. The results of the DNA replication assay in this study were not identical with those of in situ hybridization analysis. This difference is not inconsistent as the DNA replication assay used in this study is able to distinguish between newly replicated JCV DNA and transfected DNA in IMR-32 cells. On the other hand, no significant increase in VP1 gene expression or virus multiplication was observed in real-time RT-PCR analysis and the DNase protection assay, respectively. This is in contrast to the effect of TNF-α on JCV replication through the upregulation of large T antigen expression in IMR-32 cells. These results suggest that TNF-α mainly induces the gene expression of the large T antigen, which is necessary for JCV replication in IMR-32 cells, leading to the stimulation of viral genome replication in the early stage of infection. Thus, TNF-α may induce JCV reactivation. Many important aspects of PML pathogenesis remain unclear, including the upregulation of viral transcription and replication. As TNF-α stimulated the expression of large T antigen and viral replication, this cytokine may contribute to JCV propagation in AIDS-related PML. This finding may contribute to understanding the pathogenesis of AIDS-related PML.

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ORIGINAL ARTICLE

# High-resolution melting analysis for mutation scanning in the non-coding control region of JC polyomavirus from patients with progressive multifocal leukoencephalopathy

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**Abstract** JC polyomavirus (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. JCV isolates from PML patients have hypervariable mutations in the noncoding control region (NCCR) of the viral genome. Although nucleotide sequencing analysis of NCCR mutation is useful for the confirmation of PML diagnosis and basic studies examining JCV variants, it is often labor-intensive, time-consuming, and expensive. This study was conducted to evaluate the feasibility of a high-resolution melting (HRM) analysis technique for the rapid and low-cost scanning of NCCR mutations. The real-time PCR-HRM assay was developed with a pair of primers targeting the NCCR, and mutational patterns of NCCRs were compared using sequence-confirmed JCV DNA clones and CSF DNAs from PML patients. The NCCR patterns of DNA clones of the archetype JCV and PML-type variants could be differentiated by PCR-HRM. The mutational patterns of the rearranged NCCR clones were similar to those of JCV variants in the original CSF specimens as judged by nested PCR-HRM using pre-amplified targets. In addition, nested PCR-HRM could distinguish NCCR mutations in the JCV DNAs from each specimen at the patient level. These results indicate that the HRM-based assay affords a valuable technique for PML diagnosis and a versatile tool for the rapid scanning of NCCR mutations.

## Introduction

Progressive multifocal leukoencephalopathy (PML) is a rare but fatal demyelinating disease of the central nervous system (CNS) caused by JC virus (JCV), a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* [1–3]. Humans are infected with JCV asymptomatically during childhood, resulting in persistent infection throughout their life. From 50 to 90 % of adults have been reported to be serologically positive for JCV [1–4]. However, in some severely immunocompromised patients, JCV reactivates and causes a lytic infection in the oligodendrocytes, leading to PML [1–4]. PML develops in HIV-positive patients as well as in those that are immunodeficient due to hematological malignancies, chemotherapy, transplantation, lymphocyte depletion or the treatment of autoimmune disorders with immunosuppressive agents, including monoclonal antibodies, such as natalizumab, rituximab, and efalizumab [1–3, 5, 6].

The detection of JCV DNA in the cerebrospinal fluid (CSF) by PCR is a reliable and less-invasive diagnostic marker of PML [7, 8]. The rapid and specific quantification of JCV DNA using a real-time PCR technique has become the current diagnostic standard [5]. However, because of its sensitivity, real-time PCR has a risk of false-negative results due to DNA contamination of samples [9]. Clinical isolates of JCV can be classified into two groups 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 [10–12]. Nonpathogenic JCV strains isolated from the urine of healthy individuals contain a consistent NCCR sequence known as the archetype [13]. In contrast, JCV isolates from PML patients are characterized by hypervariable mutations within the NCCR [12, 14]. The changes in the NCCR sequences are thought to be related to the activation of virus replication during disease progression [12, 14, 15]. These mutated sequences are thought to be derived from the archetype NCCR via deletions and/or duplications [16, 17], leading to the alteration of promoter activity [18, 19].

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There have been a number of reports on the nucleotide sequence analysis of the rearranged NCCRs to differentiate patient-dependent JCV variants for PML diagnosis and/or to examine the molecular pathogenesis of JCV [14, 19–29]. However, since multiple variants are frequently released into the CSF of many PML patients, NCCR typing requires molecular cloning of viral DNA, nucleotide sequencing, and *in silico* analysis of mutations [14, 19–24]. Thus, the analysis of NCCR patterns is labor-intensive, time-consuming, and highly expensive, making it difficult to be undertaken routinely.

High-resolution melting (HRM) analysis is a rapid, sensitive, closed-tube technique for the detection of DNA sequence variations. It relies on fluorescence melting curves obtained from the transition of double-stranded DNA to single-stranded DNA as a result of temperature increases [30]. HRM is used as a high-throughput and low-cost method for mutation scanning and genotyping in clinical practice and risk assessment, including the characterization of viral genomes [31]. This study was undertaken to develop and evaluate an HRM-based analysis technique to distinguish the NCCR patterns of JCV variants.

## Materials and methods

### CSF specimens

The study protocol was approved by the Ethical Committee for Biomedical Science in the National Institute of Infectious Diseases (approval number 388 and 389). CSF specimens were collected by lumbar puncture from 163 patients after informed consent from the patients or their family members was obtained, and then transferred from the respective hospitals to the Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, for the clinical testing of JCV DNA as a part of routine medical practice. Twelve of the subjects were diagnosed with PML on the basis of neurological symptoms, magnetic resonance imaging patterns, and the detection of JCV DNA in the initial CSF testing. One hundred fifty-one patients were negative for CSF JCV as judged by real-time PCR testing.

### DNA extraction and real-time PCR

Total DNA was 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 [24, 32].

### Cloning and sequencing of NCCRs

The NCCRs were amplified by nested PCR using CSF DNAs from 12 PML patients and were cloned into a plasmid vector as reported earlier [24]. Sequencing of 96 NCCR clones (8 clones/patient) from both sides of the insert was performed using universal primers, and the nucleotide sequences were compared to that of the archetype CY strain recovered from a healthy Japanese individual (GenBank: AB038249.1) [33] using the CLC Genomics Workbench 6.0.1 software program (CLC bio, Aarhus, Denmark) as described previously [24]. In each specimen, at least 5 of the 8 NCCR clones had identical sequences and were used as representative clones for HRM typing.

### HRM analysis of cloned NCCRs

The viral genome of archetype JCV (CY strain) was provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. A pair of primers, JCHRM-F (5′-CCT CCT AAA AAG CCT CCA CG-3′) and JCHRM-R (5′-AGA AGC CTT ACG TGA CAG C-3′), which correspond to nucleotides 5064-5083 and 282-300 within the circular genome of JCV CY strain, respectively, was designed to anneal to highly conserved sequences adjacent to the NCCR. Custom-synthesized and cartridge-purified primers were obtained from Life Technologies (Gaithersburg, MD). Real-time PCR amplification and HRM analysis were carried out in a single tube and a single run using a LightCycler Nano System and ResoLight, a DNA-intercalating fluorescent dye (Roche, Penzberg, Germany) according to the protocols supplied by the manufacturers. For HRM analysis of the plasmid-cloned NCCRs, the reaction was performed in a total volume of 20 μL containing 10 μL of the LightCycler 480 High Resolution Melting Master (Roche, Penzberg, Germany), 0.8 μL each of the 10 μM forward and reverse primers, 2 μL of 25 mM magnesium chloride (Roche), and 2 μL of the plasmid DNA (10 ng/reaction). Amplification and real-time fluorescence detection were performed using a LightCycler Nano Instrument (Roche), and the cycling conditions were 95 °C for 10 min, followed by 30 cycles of 95 °C for 10 s, 60 °C for 25 s, and 72 °C for 15 s. The optimal cycle conditions and magnesium chloride concentration were determined by pilot experiments to prevent background noise due to nonspecific amplification. HRM analysis was performed immediately after cycling by first heating to 95 °C, cooling to 40 °C, heating again to 65 °C (all at 4 °C/s), and then melting at 0.05 °C/s with continuous acquisition of fluorescence until 95 °C. The HRM data were analyzed using the LightCycler Nano Software (Roche) following the manufacturer's instructions. The melting curves were normalized and temperature shifted to allow samples to be directly compared. Difference plots were generated by selecting the reference DNA, converting the melting profile to a baseline, and normalizing the melting profiles of the other samples against this sample.

The specificities of the amplified products were confirmed by the conventional direct sequencing method.

HRM analysis of JCV DNA in CSF samples

For mutation scanning of small amounts of viral DNA in clinical specimens, the NCCRs were pre-amplified by conventional PCR using CSF DNAs from 12 PML patients and 151 CSF JCV-negative patients. To prevent over-amplification, the JCV-positive CSF DNAs were diluted to less than $10^3$ copies per reaction and were used as PCR templates. The JCV-negative CSF DNAs were directly subjected to PCR without dilution. The outer primers, A1 (5′-TCC ATG GAT TCC TCC CTA TTC AGC ACT TTG T-3′) and A2 (TTA CTT ACC TAT GTA GCT TT-3′), have been described previously [28]. PCR was performed using a high-fidelity DNA polymerase with proofreading activity in a 50-μL reaction mixture as reported previously [24, 34]. Amplification was performed in a thermal cycler (PCR Thermal Cycler Dice; Takara, Shiga, Japan) with initial denaturation at 98 °C for 1 min, followed by 40 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product was diluted tenfold with PCR-grade water and subjected to the real-time PCR-HRM assay as described above.

Nucleotide sequence accession numbers

NCCR sequences were submitted to the DNA Data Bank of Japan (DDBJ) and were assigned the accession numbers GenBank/EMBL/DDBJ: AB841277–AB841306.

## Results

Nucleotide sequence analysis of the NCCRs of JCV variants in CSF specimens from PML patients

The first series of analyses was conducted to examine the NCCR patterns of JCV DNA detected in CSF specimens from PML patients, using a conventional cloning and sequencing approach (Table 1). The viral loads in CSF specimens of 12 PML patients ranged between $2.06 \times 10^3$

**Table 1** PML patients and representative NCCR patterns of JCV variants detected in CSF specimens

| Patient no. | Underlying disease | CSF viral load (copies/mL) | NCCR clone | Nucleotide sequence composition[a] | | |
|---|---|---|---|---|---|---|
| | | | | Deletion[b] | Insertion | Single-base difference[c] |
| 1 | HIV infection | $2.06 \times 10^3$ | Os-1-1 | 60-78 | 81-108, 49-57 | NA |
| 2 | HIV infection | $2.26 \times 10^3$ | Tk-5-2 | 108-123, 128-175 | NA | NA |
| 3 | HCV-related liver disease | $3.06 \times 10^3$ | Tk-16-4 | 91-159 | 158-194, 59-66, 60-92, 252-258 | T91C, C159A, G226A |
| 4 | HIV infection | $2.58 \times 10^4$ | Ng-21-2 | 203-252 | NA | NA |
| 5 | HIV infection | $4.27 \times 10^4$ | Tk-24-1 | 116-178 | 181-205, 46-75, 98-113, 179-206, 47-113 | NA |
| 6 | HIV infection | $2.69 \times 10^5$ | Ir-30-1 | 54-76 | NA | C85G |
| 7 | Primary immunodeficiency syndrome | $5.21 \times 10^5$ | St-29-4 | 37-38, 41-60, 141-169, 220-257 | 77-142 | NA |
| 8 | HIV infection | $5.36 \times 10^5$ | Hs-32-2 | 124-130 | 133-141, 40-107, 108-121, 30-34 | T107A, G112T, G141A, C159A, G217A |
| 9 | NA | $9.93 \times 10^5$ | Fo-39-1 | 112-167, 200-201 | 103-114, 168-199, 202-219, 30-111, 103-114 | NA |
| 10 | HIV infection | $1.49 \times 10^8$ | Ac-46-1 | 76-168, 189-224 | 37-86, 169-185, 163-166, 68-86 | NA |
| 11 | HIV infection | $3.15 \times 10^8$ | St-58-2 | 94-182 | 183-209, 49-91, 181-191, 49-91, 181-207 | A208T, G217A |
| 12 | Acute lymphoblastic leukemia | $4.85 \times 10^8$ | Os-47-1 | 88-178, 213-220 | 179-198, 108-150, 71-76 | NA |

PML, progressive multifocal leukoencephalopathy; NCCR, non-coding control region; JCV, JC virus; CSF, cerebrospinal fluid; HCV, hepatitis C virus; NA, not applicable

[a] The nucleotide numbers corresponding to the nucleotide positions within the JCV genome of the archetype CY strain are shown

[b] "60-78" indicates that 5′and 3′ nucleotide positions 60-78 are deleted

[c] "T91C" indicates that T at the nucleotide position 91 in the archetype NCCR is C

**Table 2** Frequency and sequence differences in the rearranged NCCRs cloned from CSF specimens of PML patients

| Patient no. | NCCR clone | Accession no. | Frequency | Size (bp) | Nucleotide sequence composition[a] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Deletion | Insertion[b] | Single-base difference[c] |
| 1 | Os-1-1 | AB841277 | 6/8 | 285 | - | - | - |
| | Os-1-7 | AB841278 | 1/8 | 285 | NA | NA | T222C |
| | Os-1-3 | AB841279 | 1/8 | 285 | NA | NA | A151G, G163A |
| 2 | Tk-5-2 | AB841280 | 7/8 | 203 | - | - | - |
| | Tk-5-1 | AB841281 | 1/8 | 203 | NA | NA | C56A |
| 3 | Tk-16-4 | AB841282 | 6/8 | 283 | - | - | - |
| | Tk-16-2 | AB841283 | 1/8 | 283 | NA | NA | A211G |
| | Tk-16-10 | AB841284 | 1/8 | 283 | NA | NA | A92G |
| 4 | Ng-21-2 | AB841285 | 7/8 | 217 | - | - | - |
| | Ng-21-5 | AB841286 | 1/8 | 217 | NA | NA | A56G |
| 5 | Tk-24-1 | AB841287 | 7/8 | 370 | - | - | - |
| | Tk-24-8 | AB841288 | 1/8 | 370 | NA | NA | A293G |
| 6 | Ir-30-1 | AB841289 | 8/8 | 244 | - | - | - |
| 7 | St-29-4 | AB841290 | 5/8 | 244 | - | - | - |
| | St-29-7 | AB841291 | 2/8 | 264 | NA | 43-60 | NA |
| | St-29-2 | AB841292 | 1/8 | 264 | NA | 43-60 | T91C |
| 8 | Hs-32-2 | AB841293 | 6/8 | 356 | - | - | - |
| | Hs-32-8 | AB841294 | 1/8 | 356 | NA | NA | C90G, T205G |
| | Hs-32-10 | AB841295 | 1/8 | 355 | NA | NA | del29, T205G |
| 9 | Fo-39-1 | AB841296 | 6/8 | 365 | - | - | - |
| | Fo-39-5 | AB841297 | 1/8 | 365 | NA | NA | A152G |
| | Fo-39-7 | AB841298 | 1/8 | 365 | NA | NA | T353C |
| 10 | Ac-46-1 | AB841299 | 5/8 | 228 | - | - | - |
| | Ac-46-8 | AB841300 | 1/8 | 228 | NA | NA | C116T |
| | Ac-46-2 | AB841301 | 1/8 | 230 | NA | 232-233 | NA |
| | Ac-46-5 | AB841302 | 1/8 | 189 | 62-100 | NA | NA |
| 11 | St-58-2 | AB841303 | 6/8 | 329 | - | - | - |
| | St-58-3 | AB841304 | 2/8 | 242 | 122-140, 151-167, 172-177, 240-284 | NA | C149G, A150G, A179G |
| 12 | Os-47-1 | AB841305 | 7/8 | 237 | - | - | - |
| | Os-47-9 | AB841306 | 1/8 | 248 | NA | 88-90, 159-163, 156-158 | NA |

NCCR, non-coding control region; CSF, cerebrospinal fluid; PML, progressive multifocal leukoencephalopathy; NA, not applicable

[a] The NCCR sequences were compared to those of the frequent patterns in each CSF specimen (minus symbols)

[b] The nucleotide numbers corresponding to the nucleotide positions within the JCV genome of the archetype CY strain are shown as the sequences were partially deleted in some frequent patterns

[c] "T222C" indicates that T at the nucleotide position 222 in the frequent NCCR pattern is C

and $4.85 \times 10^8$ copies/mL. The DNA fragments containing the NCCR were amplified by nested PCR, and their nucleotide sequences were analyzed after plasmid cloning. The NCCR patterns of the representative clones in each specimen are shown. When compared to the archetype sequence, all patterns had deletions, mainly in the C (nucleotides 60-114) and/or D (nucleotides 115-180) regions within the NCCR. Partial deletions were also observed in other regions within the NCCRs of 7 of the 12 clones. In 9 of the 12 NCCRs, short fragments were inserted into the deleted regions, resulting in duplications. Single-nucleotide differences within the NCCR were also identified in 4 of the 12 NCCRs. Table 2 shows a comparison of the nucleotide sequences between the representative and low-frequency NCCR patterns. In all PML patients, except patient 6, multiple NCCR patterns were identified in the same CSF specimen. The sequence compositions of these NCCRs differed among patients. These

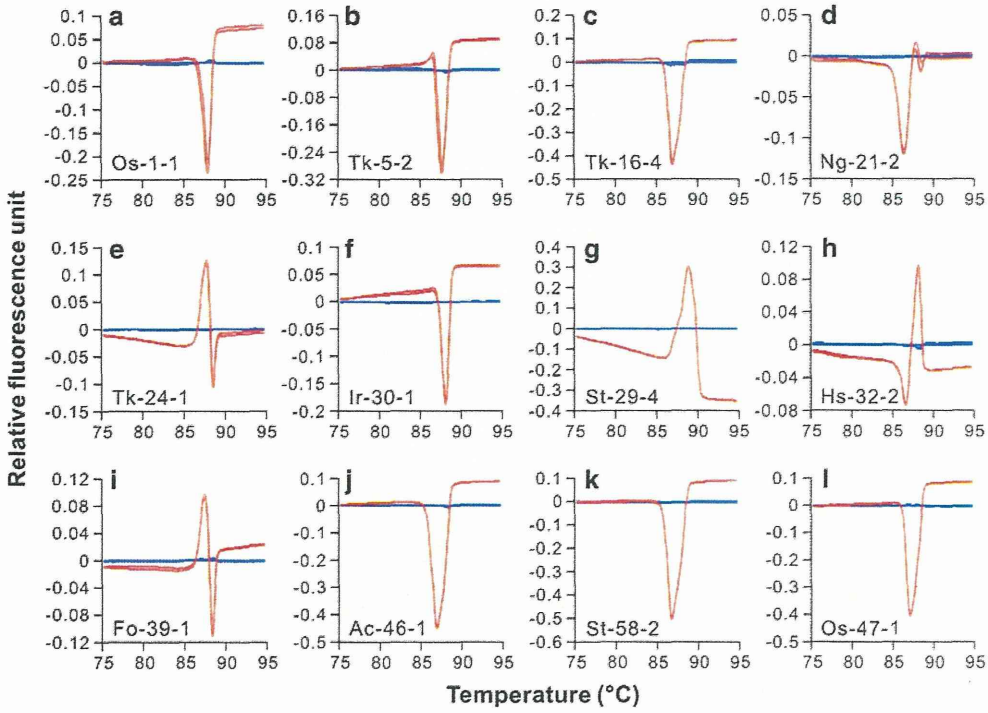


**Fig. 1** Comparisons of HRM profiles between the archetype and rearranged NCCRs. The real-time PCR-HRM assay was performed on the archetype NCCR (blue lines) and the rearranged NCCRs listed in Table 1 (red lines) in duplicate. Differences in fluorescence were determined by comparison with the data of the archetype NCCR. Similar results were obtained from two other experiments (color figure online)

data suggest that the NCCRs of JCV from each individual exhibited patient-dependent mutational patterns.

## Detection of NCCR mutations by real-time PCR-HRM analysis

The next series of analyses was carried out to examine whether the real-time PCR-HRM assay could detect the mutations in the sequence-determined NCCRs. The archetype and representative NCCRs in Table 1 were amplified by real-time PCR, and the temperature-shifted difference plots of each product were determined by HRM analysis. Baseline data were obtained from the archetype NCCR (Fig. 1, blue lines). When the rearranged NCCRs were used as PCR templates, HRM profiles clearly deviated from the baseline in the positive and/or negative direction on the y-axis during the temperature shifts (Fig. 1, red lines). Figure 2a shows the results of the HRM profiles for each NCCR with reference to the rearranged NCCR sequence. When the data from the NCCR clone Os-1-1 were set as a baseline, the HRM profiles of these sequences appeared to differ from those of the other clones (Fig. 2a). In addition, the HRM profiles of the NCCRs differed from each other when any NCCR clones were set as the baseline (data not shown). The experiments shown in Fig. 2b were performed to determine whether the real-time PCR-HRM assay could discriminate between the minor sequence differences in NCCR clones derived from the same CSF specimen. The real-time PCR-HRM analyses were performed using the representative and other DNA clones as PCR templates (Table 2). The HRM profiles of the minor NCCRs were compared with reference to those of the representative clones as a baseline. Although the HRM profiles seemed to be similar, the NCCR patterns could be discriminated in all template combinations except NCCR clones Tk-16-10 and Tk16-4 (94.4 %). These data demonstrate that the real-time PCR-HRM can be used for the differentiation of DNA sequences of the archetype and rearranged NCCRs.

## Scanning of NCCR mutations within the JCV genome detected in CSF specimens from PML patients by real-time PCR-HRM analysis

To analyze low-copy JCV DNA in clinical specimens, the real-time PCR-HRM assay was improved by the use of a nested PCR technique (hereafter "nested PCR-HRM"). When the pre-amplified products of conventional PCR using outer primers were used as templates, nested PCR-HRM was capable of detecting and scanning at least 20 copies of NCCR fragment per reaction. The nested PCR-HRM analysis was performed on JCV-positive and -negative CSF DNAs (12 and 151 specimens, respectively). Baseline data were obtained from the archetype NCCR (Fig. 3, blue lines). The assay detected JCV NCCR in all JCV-positive specimens, and HRM patterns of JCV NCCRs from these samples (Fig. 3, red lines) appeared to differ from those of the archetype JCV (Fig. 3, blue lines).

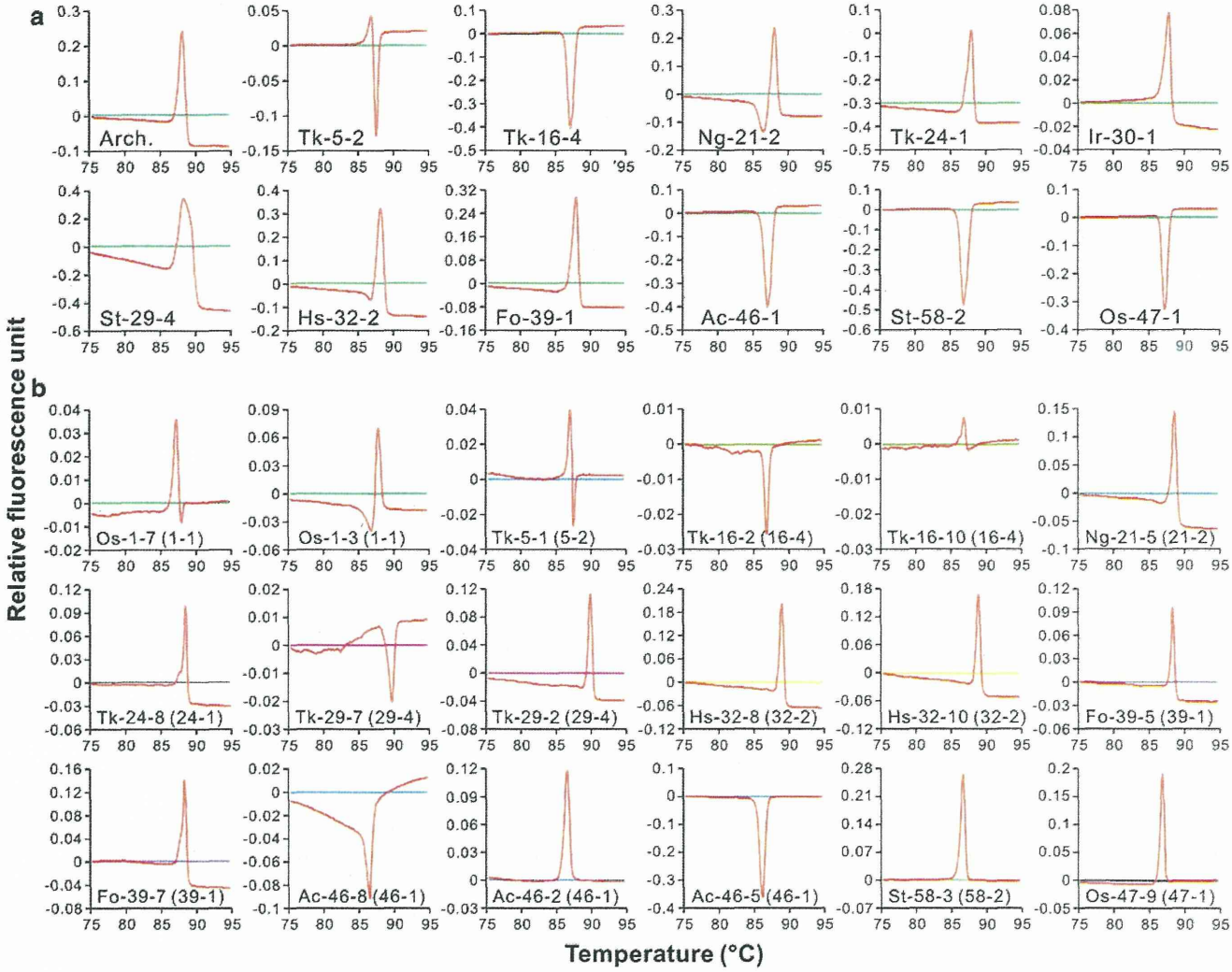


**Fig. 2** Comparisons of HRM profiles among the rearranged NCCRs cloned from different specimens or the same specimen from PML patients. The single-step PCR-HRM analyses were conducted using the archetype NCCR (Arch.) and the rearranged NCCR clones listed in Table 2 as described for Fig. 1. In panel a, the baseline data were obtained from NCCR clone Os-1-1 (green lines), and the HRM profiles of the representative NCCRs from different patients are compared (red lines). In panel b, the HRM profiles of the rearranged NCCRs (red lines) were calculated with reference to those of the representative NCCRs cloned from the same patient (other colors). The scale on the y-axis differs from that in panel a. The baseline data are shown in parentheses (e.g., "1-1" indicates that the baseline data are obtained from clone Os-1-1 (color figure online)

In contrast, no amplification signals were obtained from any JCV-negative CSF specimens. It was also found that the HRM profiles of JCV variants in each specimen were similar to those of the cloned NCCRs (Fig. 1, red lines). The experiments shown in Fig. 4 were conducted to assess whether the nested PCR-HRM is able to distinguish the NCCR patterns of JCV DNA variants in clinical specimens. The nested-PCR HRM analyses were performed on CSF DNAs from PML patients after obtaining baseline data from each clinical sample as well as from the archetype JCV. The HRM profiles of the JCV variants from patients 2 to 4 (Fig. 4a, red lines) were different from those from patient 1 (Fig. 4a, green line). The analyses shown in Fig. 4b were performed to determine whether the NCCR patterns could be numerically distinguished by the nested PCR-HRM assay in all combinations of CSF DNAs. The maximum values of the fluorescence differences from the baselines were determined as indicated in Fig. 4a. When the HRM data of CSF DNAs from each PML patient were used for the baseline, the HRM patterns of the NCCRs were different from each other. These data demonstrate that the nested PCR-HRM assay can distinguish the NCCR patterns of JCV variants in clinical specimens without the cloning and sequencing of viral DNA.

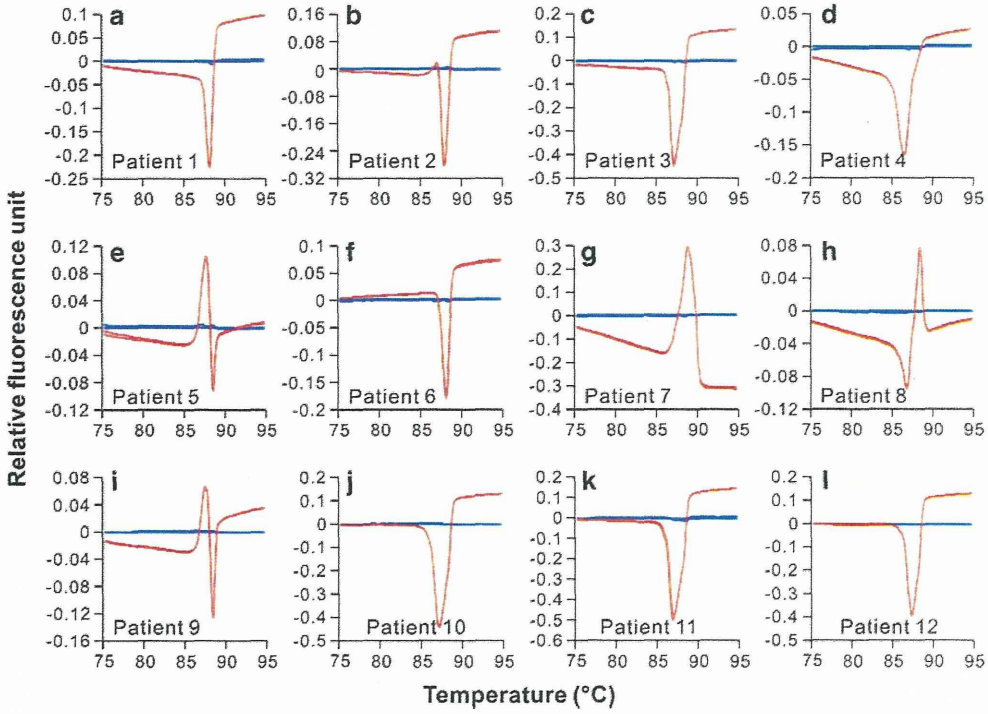


**Fig. 3** Comparisons of HRM patterns of NCCRs between the archetype JCV and JCV variants in CSF specimens from PML patients. The nested PCR-HRM assay targeting the NCCR was performed using the archetype JCV genome (blue lines) and JCV DNAs in CSFs from the patients listed in Table 1 (red lines) in duplicate. The differences in fluorescence were determined by comparison with the data of the archetype JCV. Similar results were obtained from two other experiments (color figure online)

## Discussion

In this study, two types of HRM assays were established in combination with single-step and nested PCRs for the NCCR scanning of cloned JCV DNA and JCV variants, respectively, in CSF specimens. The HRM analyses developed in this study are thought to be unique in that the virus variants can be discriminated by the patient-specific and hypervariable mutations in the NCCRs without nucleotide sequencing. Because of highly variable mutations, it is difficult to distinguish rearranged NCCRs using real-time PCR with a specific oligonucleotide probe. Recently, Ryschkewitsch *et al.* have reported a probe-based real-time PCR assay targeting a partial sequence within the archetype NCCR, which is frequently deleted in the rearranged NCCRs of PML-type JCV variants [35]. This probe-based PCR is highly sensitive and quantitative, and is similar to the PCR-HRM developed in this study in that both of these aim to distinguish the archetype JCV from the JCV variants with rearranged NCCRs. However, when we analyzed the primer and probe sequences used for the probe-based PCR *in silico*, it was noticed that several rearranged NCCRs of PML-type JCV contain target regions as shown in this study (clones Os-1-1, Os-1-7, Ng-21-2, Ng-21-5, and Ir-30-1) and in a previous report by Gosert *et al.* [19]. Thus, the possibility cannot be excluded that the probe-based PCR detects the rearranged NCCR as the archetype. If these NCCR patterns are dominant in the clinical specimens, the detected virus may be judged to be either the archetype or a mixture of the archetype and PML-type viruses. In addition, the probe-based PCR assay cannot detect rearranged NCCRs, while the current PCR-HRM assays can differentiate the mutational patterns of the rearranged NCCRs.

An important aspect of the single-step PCR-HRM assay is that it is able to compare the NCCR patterns of cloned JCV DNA in about 1 hour. The differences in NCCR patterns can be measured rapidly and semi-automatically without sequencing and *in silico* analyses. The nucleotide sequencing analyses of NCCRs are generally performed using multiple clones from a single specimen, and the highly variable mutations have to be examined *in silico* by repeatedly aligning partial sequences to the archetype NCCR. Thus, the number of target clones can be reduced by using HRM scanning prior to sequence analysis. In addition, the present assay was able to distinguish the mutational patterns of multiple NCCRs cloned from CSF specimens from the same patient. The single-step PCR-HRM assay developed in the present study may serve as a quick and convenient tool for comparing the mutational patterns of JCV NCCRs, not only as part of PML diagnosis but also in various studies examining JCV variant populations. Although the amplification efficiency of the single-step PCR-HRM is low, the assay possesses one advantage in that the results are not influenced by contamination with small amounts of DNA when using high-copy plasmids as PCR templates.

Since the real-time PCR assay for JCV testing is generally designed to detect highly conserved regions within

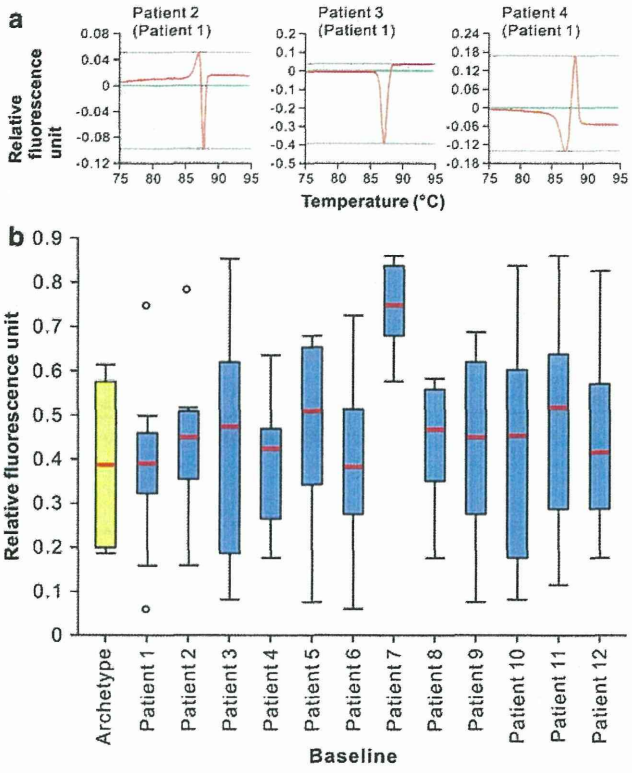


**Fig. 4** Differentiation of HRM profiles of JCV NCCRs among PML patients. The nested PCR-HRM analyses targeting NCCRs were performed as described in Fig. 3. In panel a, the HRM profiles of NCCR patterns in the CSF specimens from patients 2 to 4 (red lines) with reference to those from patient 1 (green lines) are shown to represent the maximum values of fluorescence differences (dotted lines). In panel b, the baseline data were collected from CSF DNA of one PML patient, as indicated below the x-axis (blue), and the fluorescence differences of other CSF samples are shown in box-and-whisker plots ($n = 11$). The HRM profiles of all CSF samples with reference to the archetype JCV DNA are also shown (yellow, $n = 12$). The red horizontal line within each box is the median; the lower and upper boundaries are the 25th and 75th percentiles, respectively; vertical whiskers extend over the range, and open circles show outliers (color figure online)

the viral genome [34, 36–38], sporadic contamination due to sample-to-sample carryover of positive control or JCV-positive specimens during sample processing, DNA extraction, and PCR template preparation is difficult to control by sequencing of the PCR product. As shown in Fig. 4, NCCR typing is thought to be useful to exclude the possibility of DNA contamination from JCV-positive specimens. For PCR amplification of the full-length NCCR, nested PCR was generally conducted due to the low amplification efficiency [14, 19, 28]. In agreement with previous reports, the authors could not find appropriate primers or cycle conditions for highly sensitive single-step PCR. Thus, the nested PCR-HRM analyses were performed to detect small amounts of PCR templates in clinical samples. Although this nested PCR-HRM assay requires the pre-amplification of target sequences by conventional PCR, the NCCR patterns of CSF JCVs can be discriminated in a total of about 2 hours without cloning or sequencing. When NCCR typing is conducted routinely for diagnostic purposes, it is ideal that the procedures involving electrophoresis and plasmid cloning of the PCR products is minimized to eliminate the risk of DNA contamination. Since the HRM profiles of the CSF DNAs from PML patients were similar to those of the cloned NCCRs, the cloning and/or sequencing of target sequences can be omitted for NCCR typing by the use of the nested PCR-HRM. In addition, the absence of nonspecific amplification was confirmed in the current assay using more than 150 JCV-negative CSF samples. Since no specific treatment has yet been established for PML, restoration of the immune system is the only treatment option for the management of PML [1]. False-positive results in the routine PCR testing for JCV DNA may influence therapeutic interventions, especially in moderating immunosuppressive therapies in HIV-negative PML patients. This possibility can be reduced by the confirmation of PML using HRM analysis. Thus, HRM-based scanning serves as a powerful methodology, not only for basic research but also for PML diagnosis, when combined with routine real-time PCR testing.

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

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Short communication

# Favorable outcome after withdrawal of immunosuppressant therapy in progressive multifocal leukoencephalopathy after renal transplantation: Case report and literature review

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ABSTRACT

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease caused by the JC polyomavirus (JCV). Most patients with PML after renal transplantation have had poor outcomes. We describe a patient with PML after renal transplantation who had a good response to the withdrawal of immunosuppressant therapy. We performed quantitative real-time PCR testing for JCV DNA in cerebrospinal fluid (CSF), and assessed mutation of the JC virus genome detected in the CSF. At the same time, we checked cranial magnetic resonance imaging (MRI). Immunosuppressant therapy was discontinued immediately. The MRI scan that followed showed markedly decreased numbers of high intensity signals, and the results of real-time PCR for JCV DNA in CSF became negative. The patient had no other neurological deficits. Withdrawal of immunosuppressant treatment has a beneficial effect on the course of PML after renal transplantation, and quantitative PCR may facilitate the immediate withdrawal of immunosuppressant agents.



## 1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease caused by the JC polyomavirus (JCV) [13], and survivors often have severe neurological sequelae. PML has been closely associated with profound immunodeficiency in the setting of human immunodeficiency virus (HIV) infection, hematologic malignancies, or autoimmune disorders. Rarely, PML has been reported in solid-organ transplant recipients. The estimated incidence in recipients of renal transplants is 0.027% [10]. Most patients with PML after renal transplantation have had poor outcomes [7]. We describe a patient with PML after renal transplantation who had a good response to the withdrawal of immunosuppressant therapy.

## 2. Case report

A 51-year-old woman had undergone living-donor renal transplantation at the age of 22 years because of chronic renal failure with glomerular nephritis. At 27 years of age, hemodialysis was resumed because of chronic allograft nephropathy. At 45 years of age, renal transplantation was performed again. The titer of panel reactive antibody (PRA) class I against human leukocyte antigen of the donor was as high as a rate of 60.1%. The patient received intravenous immunoglobulin (500 mg/kg/day, 5 days) and plasmapheresis (5 days) with oral basiliximab (40 mg twice) to prevent antibody-mediated rejection. Neutrophilic infiltration of glomeruli and peritubular capillaries was confirmed histologically. Subsequently, cyclosporine (100 mg/day), mycophenolate mofetil (MMF, 1000 mg/day), and prednisone (10 mg/day) were given for 5 years.

In February 2012, she noticed numbness on the left side of the face and the left limbs and was admitted to our hospital. Neck stiffness was absent, and she was fully oriented. Except for mild weakness and involuntary movements in the left forearm, other neurological findings were normal. The results of routine serum laboratory tests were normal, including renal function, and HIV was negative. Initial cranial magnetic resonance imaging (MRI) on day 2 after admission showed abnormally high signal intensity in the right frontal and temporal lobes (Fig. 1a and b). The white cell count and the protein concentration in cerebrospinal fluid (CSF) were 5 cells/mm$^3$ and 44 mg/dl, respectively. Quantitative real-time PCR testing for JCV DNA in CSF was positive, and the JCV DNA load was 12,623 copies/ml. On nucleotide sequence analysis, apparent mutations (deletions, insertions, or both) characteristic of PML-type virus [9] were found in the non-coding control region of the JCV genome in CSF (Fig. 2). PML was therefore diagnosed. The weakness

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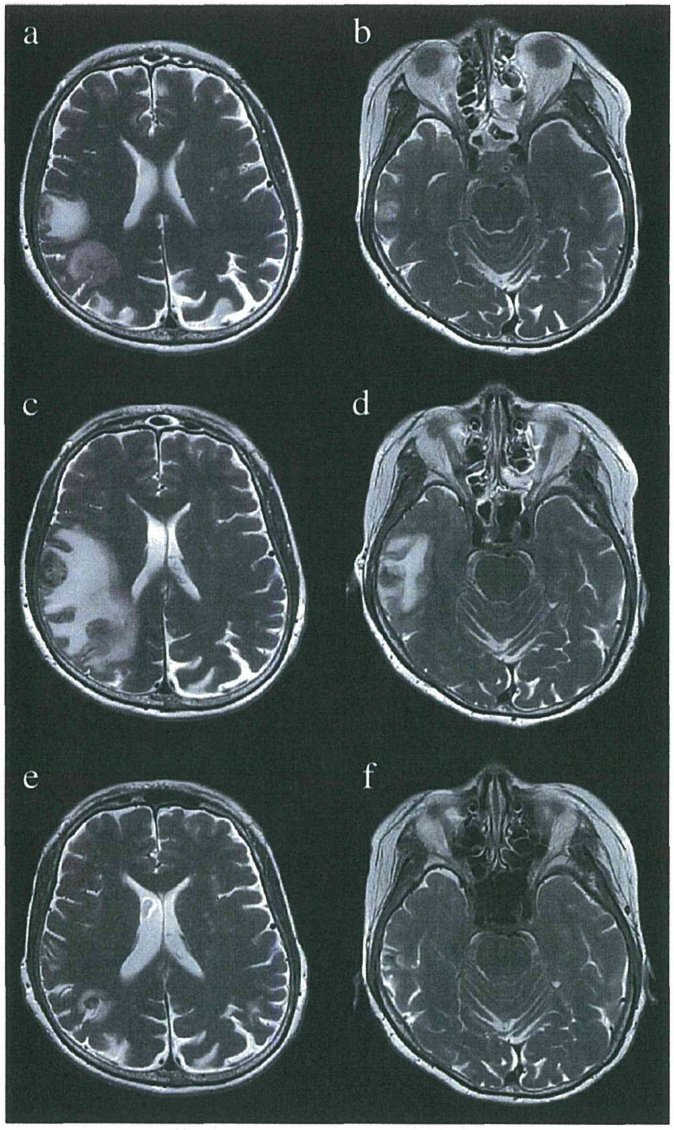


**Fig. 1.** Serial magnetic resonance (MR) findings. Initial MR T2-weighted images obtained on day 2, showing abnormally high signal intensity in the right lateral and parietal lobes (panels a and b). Lesions with abnormally high signal intensity on images obtained at the second MR examination, performed on day 23 (panels c and d). Third MR T2-weighted images obtained on day 200, showing that the abnormally high signal intensity had resolved (panels e and f).

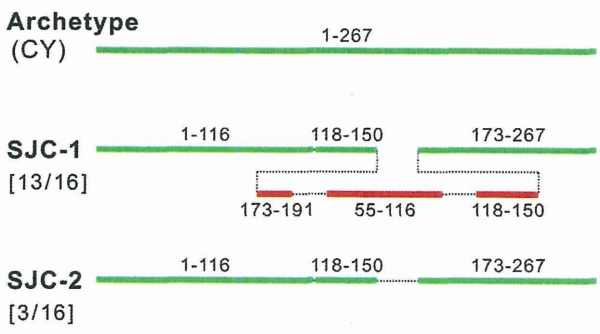


**Fig. 2.** Mutation of the JC virus genome detected in the CSF. The non-coding control region was cloned and sequenced, and the rearrangement patterns of each clone (designated as SJC-1 and SJC-2) were compared with the sequence of the archetype (nonpathogenic) CY strain. The green and red lines indicate the fragments identical to the archetype virus and the duplicated sequences, respectively. The nucleotide numbers corresponding to the archetype NCCR are shown above or below the solid lines. Frequencies of NCCR patterns are shown in square brackets.

and involuntary movements of the forearm progressed, and a second MRI scan on day 9 showed increased numbers of abnormal high-intensity signals (Fig. 1c and d). On day 10 after admission, MMF was discontinued. On day 25, cyclosporine was withdrawn, and the dose of prednisone was decreased to 5 mg/day. One month after the withdrawal of these drugs, the involuntary movements decreased, and the weakness had resolved. On day 200 after admission, the third MRI scan showed markedly decreased numbers of high-intensity signals (Fig. 1e and f), and the results of real-time PCR for JCV DNA in CSF became negative. In April 2013, the patient had no other neurological deficits. After that prednisone (5 mg/day) was continued. In November 2012, renal function deteriorated (serum creatinine: 3.6 mg/dl), and she received cyclosporine (10 mg/day), prednisone (60 mg/dl), and mizoribin (100 mg/day). In July 2013, neurological deterioration was not evident, but the CSF titer of JCV DNA on quantitative real-time PCR had increased to 1068 copies/ml. Both cyclosporine and mizoribin were withdrawn, and prednisone (5 mg/day) was continued. In August 2013, JCV DNA became negative. In November, she was reintroduced to hemodialysis, and no neurological deterioration occurred.

## 3. Discussion

To date, PML has been managed by mefloquine or highly active antiretroviral treatment (HAART) for HIV-positive cases or by the withdrawal of immunosuppressants, but the benefits of these measures have been limited. In our patient, the withdrawal of both cyclosporine and MMF and a decrease in the dose of prednisone reduced the severity of PML as well as cranial lesions on MRI, and the results of tests for JCV genome in CSF became negative.

Previously, six patients with PML after renal transplantation who had good outcomes have been reported (Table 1) [4,15,8,2,5,12]. The interval from renal transplantation to the onset of PML in our patient was 5 years, similar to previous patients (median, 4.25 years; range, 1 month to 10 years). This interval is longer than that of general cases of PML after renal transplantation (median, 30 months) [11]. The interval from the withdrawal of immunosuppressant therapy to the onset of clinical improvement was shorter in our patient (1 month) than in the other patients (median, 3 months; range, 1 to 11 months). The relatively prompt improvement in our patient might be ascribed to the fact that the immunosuppressant therapy was discontinued immediately after the early diagnosis of PML, performed using a highly sensitive quantitative PCR approach. MMF (n = 5), tacrolimus (2), cyclophosphamide (2), or cyclosporine (1) was withdrawn in the 7 reported patients including our patient [4,15,8,2,5,12]. The early withdrawal of MMF in most patients was probably related to the fact that current guidelines issued by the United States Food and Drug Administration recommend that MMF is initially withdrawn [14].

In one patient with a favorable course, the JCV DNA load in serum was evaluated, and the viral genome was not detected in serum 6 weeks after stopping immunosuppressant therapy (Table 1); however, the viral load in CSF was not assessed [15]. We analyzed the JCV DNA load in CSF by quantitative PCR and diagnosed PML without the need for an invasive brain biopsy, and the JCV genome in CSF disappeared with a reduction in the severity of PML. A study of real-time PCR in patients with HIV-positive PML showed that JCV DNA levels in CSF turned out to be negative in 4 patients with benign or asymptomatic PML [3]. The JCV DNA level in our patient was 4.10 log copies/ml. A JCV DNA level exceeding 3.64 log copies/ml in CSF on real-time PCR has been associated with a shorter survival time in HIV-positive PML [1]. However, another study showed that viral load values of >4.68 log in CSF were associated with shorter survival in HIV-positive PML [6]. This difference might account for different proportions of subjects who received highly active antiretroviral therapy (HAART) in these studies (former vs. later study; 38/61, 62% vs. 5/12, 42%). HIV-negative PML is characterized by a very high rate of JCV replication in the CNS [3], and mortality is as high as 84% [7]. The good outcome in our patient may be attributed to

**Table 1**
Clinical features of previously reported cases of PML developing after renal transplantation in patients who had good outcomes.

| | Patient 1 [15] | Patient 2 [2] | Patient 3 [8] | Patient 4 [12] | Patient 5 [5] | Patient 6 [4] |
|---|---|---|---|---|---|---|
| Age/sex | 13–14/M | 47/M | 52/F | 28/M | 28/M | 42/M |
| Immunosuppressant | MMF, PSL, rapamycin | MMF, PSL, FK-506 | MMF, PSL | CPA, PSL | CPA, PSL | MMF, PSL, FK-506 |
| Duration from renal transplantation to the onset of PML | 3.5 yr | 5 mo | 20 yr | 10 yr | 10 yr | 1 mo |
| Clinical features | Somnolence | Focal seizure, cognitive decline | Aphagia, hemiparesis, apraxia, cognitive decline | Hemiparesis, vague sensation, dysarthria | Disorientation, hemiparesis | Coma, seizure, cognitive decline |
| Diagnostic method for PML | PCR (serum) | Brain pathology | Brain pathology | Brain pathology | Brain pathology | Neuroimages |
| JCV DNA load (copies/ml) | 4012 (serum) | ND | Not detected (CSF) | ND | ND | ND |
| Cranial MRI lesions | Thalamus, brainstem, parietal WM | Parietal WM, basal ganglia | Temporal, parietal, and frontal WM | Frontal, parietal, and occipital WM* | ND | Occipital WM |
| Therapy for PML | Withdrawal of MMF, cidofovir | Withdrawal of MMF, FK-506 | Withdrawal of MMF, decreased dosage of PSL | Withdrawal of CPA and PSL, Ara-C | Withdrawal of CPA, Ara-C | Withdrawal of MMF, PSL and FK-506 |
| Outcome | | | | | | |
| Clinical features | Disappeared | Disappeared | Decreased | Decreased | Decreased | Disappeared |
| Cranial MRI lesions | Disappeared | Decreased | NA | ND | ND | Decreased |
| JCV DNA load (copies/ml) | Not detected | ND | ND | ND | ND | ND |
| Duration from PML therapy to clinical improvement | 6 weeks | 4 weeks | 3 mo | 11 mo | NA | 4 mo |

PML: progressive multifocal leukoencephalopathy, JCV: JC virus, PCR: polymerase chain reaction, MRI: magnetic resonance imaging, CSF: cerebrospinal fluids, WM: subcortical white matter, MMF: mycophenolate mofetil, PSL: prednisolone, CPA: cyclophosphamide, FK-506: tacrolimus, M: male, F: female, NA: not available, ND: not done, mo: months, yr: years and *: computed tomography.

the early withdrawal of immunosuppressant therapy before further JCV replication in the CNS. Withdrawal of immunosuppressant treatment has a beneficial effect on the course of PML after renal transplantation, and quantitative PCR may facilitate the immediate withdrawal of immunosuppressant agents.

## Disclosure

The authors report no conflicts of interest related with our paper.

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**Short Communication**

# Stability of JC Virus DNA in Cerebrospinal Fluid Specimens Preserved with Guanidine Lysis Buffer for Quantitative PCR Testing

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**SUMMARY**: Quantitative PCR testing for JC virus (JCV) DNA in the cerebrospinal fluid (CSF) is one of the diagnostic standards for progressive multifocal leukoencephalopathy (PML). The present study was conducted to examine its reliability using CSF specimens that had been preserved with guanidine lysis buffers in commercial nucleic acid extraction kits under different conditions. When CSFs were mixed with guanidine buffers, JCV DNA levels were not statistically reduced even after storage for 1 month at room temperature or for 3 months at −80°C, compared with the control samples. In addition, the JCV DNA level was not decreased in a mixture of CSF and guanidine thiocyanate buffer incubated for 3 days at 56°C. These data suggest that CSF specimens mixed with commercial guanidine buffers can be stored without refrigeration, more safely handled, and directly subjected to JCV DNA testing for PML.

The JC virus (JCV) is a small DNA virus belonging to the family *Polyomaviridae* and genus *Polyomavirus* (1-3). It is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (1-3). PML is mainly diagnosed in patients with immunodeficiency, including those with HIV infection (1-3). The detection of JCV DNA in the cerebrospinal fluid (CSF) by PCR is a reliable and less-invasive diagnostic marker of PML, particularly when combined with typical magnetic resonance imaging patterns (1-3). The amount of JCV DNA in CSF has been reported to be associated with the severity of PML (4,5). Thus, JCV DNA testing in CSF using a sensitive and quantitative PCR technique has become one of the current diagnostic standards (6).

Real-time PCR testing for viral DNA in CSF is generally performed on DNA extracts prepared by either manual or automated methods using commercially available extraction kits, most of which contain guanidinium salts as one of their components (7). Guanidinium salts such as guanidine hydrochloride (Gdn-HCl) and guanidine thiocyanate (Gdn-SCN) are widely used for cell lysis and protein solubilization (7). These compounds inhibit nuclease activities (8,9) or inactivate infectious pathogens (10-14). If viral DNA in CSF is stable when mixed with a solution containing guanidine such as Gdn-HCl and Gdn-SCN, the specimen could be stored and transported without cooling and directly used for DNA extraction. Furthermore, CSF mixed with a guanidine solution can be considered to be a noninfectious material. The present study was undertaken to examine the stability of JCV DNA in CSF specimens preserved with guanidine lysis buffer.

The study was approved by the Ethical Committee for Biomedical Science in the National Institute of Infectious Diseases, Tokyo, Japan (application no. 340). All the experiments were conducted in accordance with the ethical standards of the Declaration of Helsinki. All the CSF specimens were collected by lumbar puncture from patients suspected of having PML and were transferred from the respective hospitals to the Department of Virology 1, National Institute of Infectious Diseases, for real-time PCR testing for JCV DNA (15). Because all the specimens had been frozen without centrifugation at the hospitals, they were considered to contain leukocytes. The specimens were stored at −80°C until further analyses. Total DNAs were extracted from aliquots of the CSF specimens using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) and were subjected to real-time PCR testing for JCV DNA, as described previously (15-17).

To prepare a CSF pool for the experiments, JCV-negative CSF specimens from 71 patients were combined in a single tube (approximately 0.5 mL/sample). The median values of CSF cell counts and protein levels in the samples were 3.0 cells/μL (range, 0-95.0 cells/μL) and 48.0 mg/dL (range, 23.0-226.0 mg/dL), respectively. A JCV-positive CSF sample from an AIDS-related PML patient was diluted by more than 100-fold with JCV-negative CSF. In the JCV-positive patient, although the CSF cell count was normal (1 leukocyte/μL), a relatively high level of the JCV genome ($2.7 \times 10^6$ copies/mL CSF) was detected (15). Thus, it was thought that cell-free virions were dominant in this CSF pool. The copy number of JCV DNA in this CSF pool was adjusted to $2.0 \times 10^4$ copies/mL, which is similar to the median JCV load in CSFs of 69 PML patients in Japan (15). The CSF pools were aliquoted (200 or 140 μL) into 1.5-mL screw-cap plastic microcentrifuge tubes (Ina-Optica Co. Ltd., Osaka, Japan) and frozen at −80°C until use.

The column-based nucleic acid extraction kits and guanidine lysis buffers were all purchased from Qiagen. A 200-μL aliquot of the frozen CSF pool was thawed at 37°C in a block incubator (CTU-Mini; Taitec, Saitama, Japan), left untreated or mixed with an equal volume of

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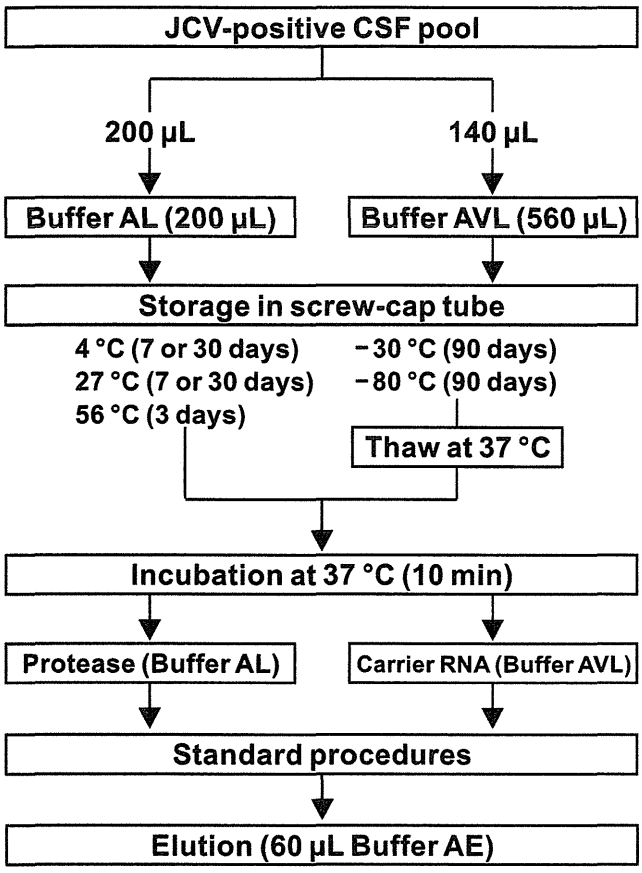


Fig. 1. Schematic representation of the handling procedures for DNA extraction and the storage conditions of the mixtures of CSF and guanidine. Aliquots of the JCV-positive CSF pool were incubated with either Buffer AL or Buffer AVL. After incubation at different temperatures and for different durations, total DNA was extracted using a QIAamp DNA Blood Mini Kit or QIAamp Viral RNA Mini Kit as described in the text.

lysis buffer containing Gdn-HCl (Buffer AL), and stored with protection from exposure to light. Whether DNA recovery is decreased by visible light during storage in Buffer AL or Buffer AVL remains unclear. However, because the laboratory equipment was routinely irradiated with ultraviolet light to prevent DNA contamination before and after examination, we placed samples in lightproof boxes for long storage periods.

The handling procedures of the CSF guanidine solutions and storage conditions are summarized in Fig. 1. The frozen samples were thawed at 37°C and mixed thoroughly after storage. All the samples were incubated at 37°C for 10 min and then vortexed for 1 min. Subsequently, protease solution was added to the mixture, and total DNA was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's protocol. Alternatively, 140-µL aliquots of the frozen CSF pool were thawed at 37°C and were left untreated or mixed with 560 µL of lysis buffer containing Gdn-SCN (Buffer AVL) and subsequently incubated under similar conditions. Total DNA was then extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's protocol, with the exception that carrier RNA was added to the lysate after incubation at 37°C for 10 min and that DNA was eluted in 60 µL of Buffer AE. Although Buffer AL and Buffer AVL were supplied in the QIAamp DNA Blood Mini Kit and QIAamp Viral RNA Mini Kit, respectively, both the kits used the same extraction columns and wash buffers. Thus, after the lysates were passed through the columns, DNAs could be extracted using a common procedure. In the control experiments without additional incubation, the frozen aliquots of the CSF pool were thawed at 37°C just before use, and total DNAs were immediately extracted by standard procedures using the QIAamp DNA Blood Mini Kit and QIAamp Viral RNA Kit. The extraction kits were routinely checked for their quality by adding plasmid DNA solution to the lysis buffer (control experiments). The contamination of control plasmid DNA into test samples can be detected using specific primers and probe (15).

The JCV DNA copy number in each sample was determined in duplicate using real-time PCR targeting the JCV large T gene, as described previously (15–17). The absence of DNA contamination was confirmed by means of negative controls. The absolute copy numbers of JCV DNA in each sample were determined. The experiments were repeated six times, and the amounts of JCV DNA in the extracts under the respective conditions were statistically compared using the nonparametric Steel test for multiple comparisons. All $P$-values less than 0.05 were considered to be statistically significant.

The absolute copy numbers of JCV DNA in the CSF pool, as determined by the standard procedures for the QIAamp DNA Blood Mini Kit and QIAamp Viral RNA Kit, were $1.92 \times 10^4$ and $3.6 \times 10^4$ copies/mL, respectively. When CSFs were mixed with Buffer AL containing Gdn-HCl, JCV DNA levels were statistically reduced after incubation at 56°C for 3 days (Fig. 2A). However, there were no statistically significant differences between the control and Buffer AL-incubated samples stored under other conditions. In CSFs mixed with Buffer AVL containing Gdn-SCN, the amounts of JCV DNA were not statistically reduced after incubation under all conditions tested, although the viral DNA level was statistically lower when the specimens were incubated without lysis buffer at 27°C for 1 week and DNA was extracted by the standard procedure (Fig. 2B). No thermal deposition of guanidinium salt was observed in any lysate during incubation at 4°C for 1 month.

The results of the present study demonstrated that if CSF specimens are mixed with commercially available guanidine lysis buffers, they can be used for quantitative PCR testing for JCV DNA even after storage for 1 month at room temperature. In addition, it was also shown that lysates could be frozen to meet longer storage requirements. The target fragments of JCV DNA were demonstrated to be stable in the presence of Gdn-HCl or Gdn-SCN and other buffer components. However, JCV DNA levels were impaired in CSFs incubated with Buffer AL at 56°C for 3 days. This temperature is recommended for the proteinase K digestion step in the QIAamp DNA Blood Mini Kit protocol (56°C for 10 min) and is used to examine the stability of JCV DNA at a higher temperature. The present data suggest that JCV DNA in Buffer AL tends to be degraded at relatively high temperatures. It is important to examine the stability of purified viral DNA under more precise conditions in future studies. It was also found that JCV

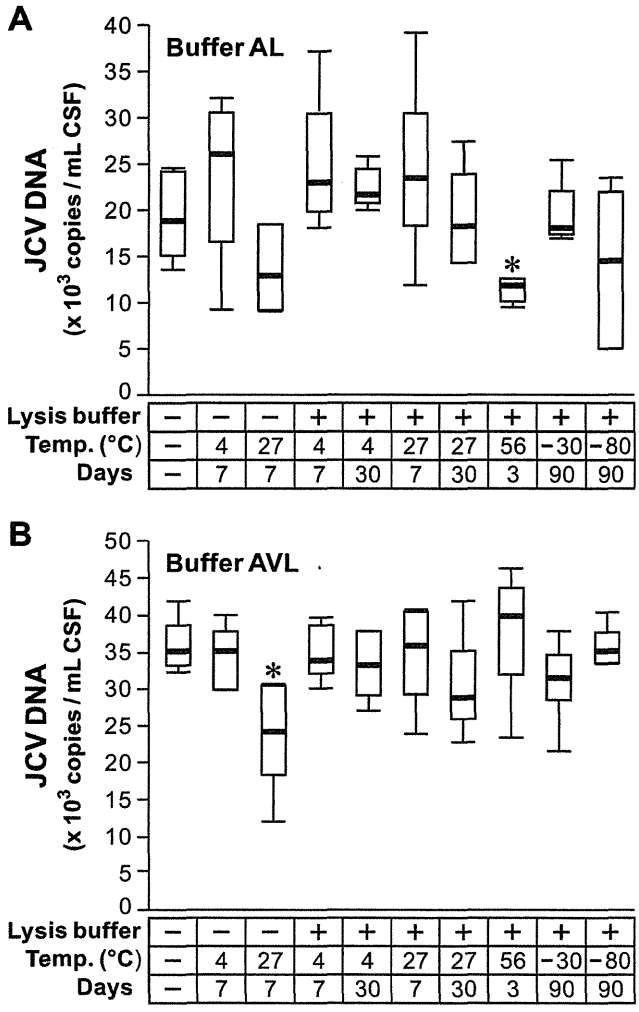


Fig. 2. Availability of CSF specimens preserved with guanidine-containing lysis buffers for the quantitative PCR testing for JCV DNA detection. Aliquots of the JCV-positive CSF pool were incubated with (+) or without (−) either Buffer AL (A) or Buffer AVL (B) under the different conditions, and total DNA was extracted as described in the legend to Fig. 1. When CSFs were incubated without lysis buffer, the samples were mixed with lysis buffer after time periods and were subjected to DNA extraction. The absolute copy numbers of the JCV genome in each DNA extract were determined using a real-time PCR method. The experiments were repeated independently 6 times, and the results are shown in box-and-whisker plots. The thick horizontal line within each box is the median; the lower and upper boundaries are the 25th and 75th percentiles, respectively; vertical whiskers extend over the range; and no outliers were detected. Statistically significant differences ($P < 0.05$) are indicated by asterisks (comparison to the first box from the left).

DNA in CSF can be extracted using the QIAamp Viral RNA Kit designed for RNA extraction from liquid samples. The data obtained in the present study suggest that the QIAamp Viral RNA Kit containing Buffer AVL can be used to extract JCV DNA from liquid materials according to the manufacturer's instructions.

Compared with the results presented in Fig. 2A and 2B, it seems likely that the median copy numbers of JCV DNA in Buffer AVL have less variation under different conditions than those in Buffer AL. These results may be related to the higher initial amount of the obtained JCV DNA in Buffer AVL ($3.6 \times 10^4$ copies/mL) than that in Buffer AL ($1.92 \times 10^4$ copies/mL), probably because of the addition of carrier RNA in the procedure. To examine the copy number of JCV DNA for PML diagnosis, it is important to obtain the amount of JCV DNA with less variation from samples containing the same amounts of JCV DNA under various conditions. Based on the above-mentioned findings, it seems likely that Buffer AVL is more applicable to the JCV DNA sample in CSF.

In addition, the current protocol using Buffer AVL is advantageous not only for sample storage as a part of routine JCV testing but also for the transportation of CSF specimens from outlying clinics to laboratories, without the need for a refrigerant. Considering the ability of Gdn-SCN to inactivate infectious agents, it is thought that the use of guanidine lysis buffer can be advantageous in decreasing the infectivity of pathogens. The results of the present study are thought to demonstrate that the use of a guanidine lysis buffer is a safe and convenient method for storing and handling CSF specimens. Furthermore, the preservation and storage of cell-free JCV suspension with guanidine solution may be useful for the in vitro high-throughput quantification of JCV DNA in a large number of samples.

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**Conflict of interest** None to declare.

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