

## JC virus granule cell neuronopathy is associated with VP1 C terminus mutants

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The polyomavirus JC (JCV) infects glial cells and causes progressive multifocal leukoencephalopathy (PML). We described a novel JCV-variant with a 10 bp deletion in the C terminus of the VP1 capsid protein, JCV<sub>GCN1</sub>. This mutant was associated with lytic infection of cerebellar granule cell neurons and cerebellar atrophy in an human immunodeficiency virus/PML patient. This condition, also observed independently from PML, was named JCV granule cell neuronopathy (JCV GCN). We characterized JCV mutations in cerebrospinal fluid (CSF) of four other JCV GCN patients, and reviewed the literature on 10 reported cases. The strain from one patient harboured the identical GCN1-deletion, while the other patients had novel mutations in the same area, named JCV<sub>GCN2–4</sub>, causing variable changes in VP1 structure. One patient also had wild-type JCV in the CSF. To study the mechanisms leading to JCV GCN, we compared viral replication kinetics from JCV<sub>GCN1</sub> with the prototype JCV<sub>Mad1</sub>, the PML isolate JCV<sub>HWM</sub> and the prototype JCV<sub>Mad1D</sub> engineered with the GCN1-deletion. While all strains replicated at low levels in the medulloblastoma cell line DAOY from a cerebellar neuronal tumour, JCV<sub>Mad1</sub> replicated better in astroglial SVG cells than JCV<sub>Mad1D</sub> or JCV<sub>GCN1</sub> and all strains replicated at higher levels in COS-7 kidney cells, suggesting that the GCN1-deletion confers a disadvantage for viral growth in central nervous system white matter. The GCN1-deletion remained stable after 100 days in culture and VP1 protein was produced in all cell lines, indicating that JCV<sub>GCN1</sub> is replication-competent *in vitro*. These data highlight an important and previously overlooked aspect of JCV-pathogenesis. Detection of GCN-type JCV strains in CSF may help clinicians diagnose JCV GCN.

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## INTRODUCTION

Infection with the polyomavirus JC (JCV) is widespread and 50–86% of healthy adults are JCV seropositive (Knowles *et al.*, 2003; Weber *et al.*, 1997). The virus remains quiescent in immunocompetent people, but reactivates in the setting of immunosuppression. It is the aetiological agent of progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system (CNS) caused by productive and lytic infection of oligodendrocytes and astrocytes (Tan & Koralnik, 2010). PML usually occurs in

patients with AIDS (Berger *et al.*, 1987; Miller *et al.*, 1982), haematological malignancies (Shimizu *et al.*, 1999) and organ transplant recipients (Yogo *et al.*, 1991), as well as individuals with autoimmune diseases treated by immunomodulatory medications (Carson *et al.*, 2009; Clifford *et al.*, 2010; Korman *et al.*, 2009; Schwab *et al.*, 2009).

In the lesions of PML, productive infection can be readily demonstrated in oligodendrocytes and astrocytes. We and others have also observed that JCV can infect neurons. We reported productive and lytic JCV infection of cerebellar

granule cell neurons in a 39-year-old human immunodeficiency virus (HIV)-seropositive patient with cerebellar atrophy who also had classic lesions of PML in the white matter of both frontal lobes, but no demyelinating lesions in the cerebellum (Du Pasquier *et al.*, 2003; Tyler, 2003). JCV infection of granule cell neurons was confirmed in another HIV-seropositive patient with cerebellar atrophy and no clinical or radiological evidence of PML. This patient had a severe cerebellar syndrome characterized by progressive alteration of coordination, gait instability and ataxia. This novel syndrome, distinct from PML, was named JCV granule cell neuronopathy (JCV GCN) (Koralnik *et al.*, 2005). JCV GCN was subsequently reported as well by others in HIV-positive and -negative patients throughout the world, with or without PML (Table 1) (Du Pasquier *et al.*, 2003; Bustamante *et al.*, 2009; Granot *et al.*, 2009; Hecht *et al.*, 2007; Koralnik *et al.*, 2005; Otis & Moral, 2005; Shin *et al.*, 2008; Tan & Brew, 2009).

Whereas the JCV-coding region is extremely conserved the hypervariable non-coding-regulatory region (RR) has been associated with neurotropism and neurovirulence. Full-length molecular characterization of the strain, named JCV<sub>GCN1</sub>, infecting granule cell neurons in our initial patient, showed a 10 bp out of frame mutation in the C terminus of the VP1 major capsid protein, leading to an alteration of the last 13 aa sequence (Dang & Koralnik, 2006). To investigate further the pathogenic mechanism leading to JCV infection of granule cell neurons, we characterized JCV VP1 sequence variation of four additional JCV GCN patients from the USA and Brazil, and studied the phenotype of JCV<sub>GCN1</sub> *in vitro*. Each patient had either identical or similar mutations in the JCV VP1 C terminus, and the JCV<sub>GCN1</sub> molecular clone was replication-competent in several cell lines *in vitro*, but displayed different characteristics than undeleted JCV strains.

## RESULTS

### JCV strains with mutated VP1 C terminus gene (GCN-type JCV) can be found in every JCV GCN case

The clinical characteristics of the JCV GCN cases we have investigated are listed in Table 1 in chronological order of discovery. The clinical presentation of cases 1–3 has been reported in detail (Dang & Koralnik, 2006; Du Pasquier *et al.*, 2003; Hecht *et al.*, 2007; Koralnik *et al.*, 2005; Tyler, 2003), but the authors of each publication were contacted to provide an update on the clinical outcome of their patients.

#### Case report patient #4 (Table 1)

A 25-year-old HIV-infected Brazilian woman with multi-drug resistance to antiretrovirals presented with 2 months history of right-sided clumsiness and unsteady gait. Neurological examination showed a right cerebellar syndrome. Her

absolute CD4<sup>+</sup> T-cell count was 3 mm<sup>-3</sup> and the plasma HIV viral load was 478 130 copies ml<sup>-1</sup> (5.7 log<sub>10</sub>). A computed tomography (CT) scan of the brain demonstrated right cerebellar atrophy (Fig. 1a). Magnetic resonance imaging (MRI) confirmed that this patient exclusively had cerebellar atrophy without PML lesions in cerebellum (data not shown). Cerebrospinal fluid (CSF) analysis was normal and qualitative PCR performed in a clinical laboratory was negative for JCV DNA. Quantitative PCR (QPCR) performed at the Virology Laboratory at Instituto de Medicina Tropical (Sao Paulo, Brazil) was positive for JCV, establishing the diagnosis of JCV GCN. She was treated with antiretroviral medications and 2 months later, the neurological examination was stable, CD4<sup>+</sup> T-cell count was up to 24 mm<sup>-3</sup> and the HIV plasma viral load was down to 916 copies ml<sup>-1</sup> (2.9 log<sub>10</sub>). However, she died soon thereafter from community-acquired pneumonia 4 months after the onset of neurological symptoms. A post-mortem examination was not performed.

#### Case report patient #5 (Table 1)

A 34-year-old HIV-infected Brazilian woman with a history of poor compliance to antiretrovirals presented with 6 months history of dizziness, unsteady gait and right-sided clumsiness, as well as recent cough and fever. Neurological examination showed a predominantly right cerebellar syndrome. Her absolute CD4<sup>+</sup> T-cell count was 116 cells mm<sup>-3</sup> and HIV plasma viral load was 47 492 copies ml<sup>-1</sup> (4.7 log<sub>10</sub>). A CT scan of the brain demonstrated isolated cerebellar atrophy (Fig. 1b) confirmed by MRI (Fig. 1c). CSF analysis was normal and qualitative PCR was negative for JCV DNA. QPCR performed at the Virology Laboratory at Instituto de Medicina Tropical (Sao Paulo, Brazil) was positive for JCV, however, consistent with the diagnosis of JCV GCN. She was restarted on cART and 9 months after her initial presentation, she had stable CT alterations, but her CD4<sup>+</sup> T-cell count had risen to 221 mm<sup>-3</sup> and her HIV viral load became undetectable (<50 copies ml<sup>-1</sup>). She was lost to follow up 18 months after onset of neurological symptoms.

Among 10 reported JCV GCN cases, we could access clinical samples of five cases (1, 2, 3, 4 and 5; Table 1). The JCV VP1 C terminus of each patient was PCR-amplified, cloned and sequenced as described previously (Dang & Koralnik, 2006). GCN-type JCV could be detected in every JCV GCN case available for testing and were named sequentially JCV<sub>GCN1</sub> to JCV<sub>GCN4</sub>. Among these five cases, three (#2, 4 and 5) had no white matter lesions at presentation and therefore, no evidence of PML in addition to JCV GCN (Table 1). GCN-type JCV was the only JCV strain detected in these JCV GCN cases without PML lesions. Alignment of DNA and primary protein sequences of all GCN types, the reference strain JCV<sub>Mad1</sub> and the JCV<sub>HWM</sub> found in the hemispheric white matter of our initial patient are shown in Fig. 2.

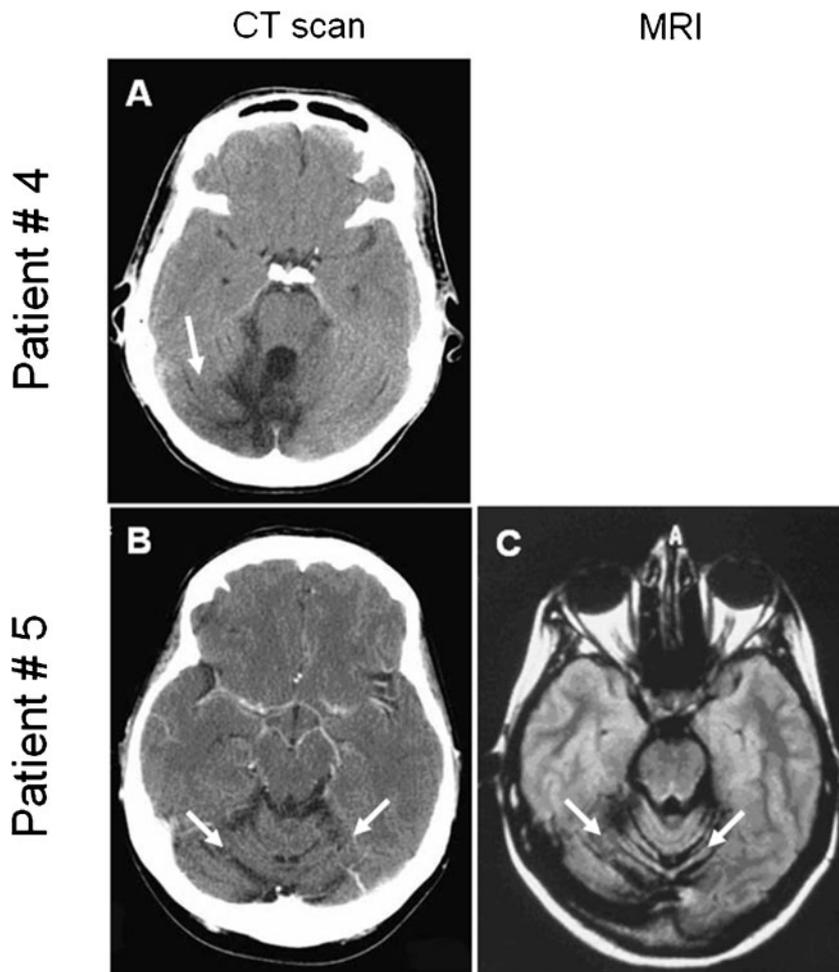
As reported previously, JCV<sub>GCN1</sub> has an out of frame 10 nt mutation, resulting in an alteration of the sequence of the

**Table 1.** Clinical characteristics of all reported JCV GCN cases

MA, Massachusetts; CA, California; NY, New York; cART, Combined Antiretroviral Therapy; NA, not applicable.

Case no.	Geographical location	Age (years)	Gender	Underlying diagnosis	PML	JCV strains	VP1 C terminus gene PCR product size (bp)*	Clinical evolution since onset of neurological symptoms	Reference
1	Boston, MA	39	M	HIV <sup>+</sup>	+	JCV <sub>GCN1</sub> , JCV <sub>HWM</sub>	104, 114	Died 6 months later	Du Pasquier <i>et al.</i> (2003)
2	New York City, NY	43	F	HIV <sup>+</sup>	-	JCV <sub>GCN2</sub>	108	Wheel-chair dependent, on cART, 13 years later	Koralnik <i>et al.</i> (2005)
3	San Francisco, CA	15	M	CD-40 ligand deficiency	+	JCV <sub>GCN3</sub> , JCV <sub>SF</sub>	131, 114	Worsening of cerebellar syndrome. PML lesions in frontal lobes after 21 months with CSF JC viral load $12 \times 10^6$ cps ml <sup>-1</sup> and died after 23 months	Hecht <i>et al.</i> (2007)
4	Sao Paulo, Brazil	25	F	HIV <sup>+</sup>	-	JCV <sub>GCN4</sub>	104	Died 4 months later	
5	Sao Paulo, Brazil	34	F	HIV <sup>+</sup>	-	JCV <sub>GCN1</sub>	104	Lost to follow-up 18 months later	
6	Springfield, MA	52	F	HIV <sup>+</sup>	+	NA	NA	NA	Otis & Moral (2005)
7	Seoul, South Korea	37	F	HIV <sup>+</sup>	-	NA	NA	Bed-ridden 3 years later	Shin <i>et al.</i> (2008)
8	Sydney, Australia	49	F	Sarcoidosis	+	NA	NA	Died	Granot <i>et al.</i> (2009)
9	Sydney, Australia	42	M	HIV <sup>+</sup>	-	NA	NA	Stopped cART and died 2 years later	Tan & Brew (2009)
10	Providencia, Chile	37	M	HIV <sup>+</sup>	+	NA	NA	Alive with severe cerebellar dysfunction 2 years later	Bustamante <i>et al.</i> (2009)

\*Using PCR primers CJS2465 and CJR2578 described in Dang &amp; Koralnik (2006).



**Fig. 1.** Brain imaging studies of two HIV-seropositive patients with JCV GCN. Head CT scan of patient #4 in Table 1 (a) and head CT and MRI of patient #5 in Table 1 (b and c) showing cerebellar atrophy (arrows) and no demyelinating lesions of PML.

last 13 aa of the protein and was found in both our initial case #1 (Table 1) from Boston (Du Pasquier *et al.*, 2003) as well as case #5 from Brazil. JCV<sub>GCN4</sub> from Brazil has the same 10 bp deletion as JCV<sub>GCN1</sub> starting at nt 2503 and

ending at nt 2512 in the VP1 gene. In addition, JCV<sub>GCN4</sub> has five single nucleotide mutations after the 10 bp deletion, leading to 4 aa changes compared with JCV<sub>GCN1</sub>. JCV<sub>GCN2</sub> from New York City has a 6 bp in-frame deletion from nt

**JCV VP1 gene DNA sequence:**

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NT #: 2469                               2503   2512                               2533
JCVMad1: CAGGGGACCCAGACATGATGAGATACGTTGACAAATATGGACAGTTGCAGACAAAAATGCTGTAA
JCVHWM:  ---A-----T-----T-----G-----G-----
JCVGCN1:  ---A-----T-----T-----G-----G-----
JCVGCN4:  ---A-----T-----T-----G-----A-ATG-----G-----
JCVGCN2:  ---A-----T-----T-----G-----G-----A-----G-----
JCVSF:    ---A-----T-----T-----G-----G-----G-----
JCVGCN3:  ---A-----T--GATGAGATATGTTGACAG-----G-----
                                     GATGAGATATGTTGACA
    
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**JCV VP1 protein aa sequence (N-C):**

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aa #: 335       345       354
JCVMad1: GDPDMMRYVDKYGQLQTKML.
JCVHWM:  -----R-----
JCVGCN1: -----S-----CRQKCCNPKPLL.
JCVGCN4: -----R-----YGQRCCNPKPLL.
JCVGCN2: -----R-----
JCVSF:    -----R-----
JCVGCN3: -----R-----
    
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**Fig. 2.** Alignment of DNA and amino acid sequences of VP1 C terminus from JCV strains identified from different JCV GCN cases. The nucleotide and amino acid position numbers correspond to JCV<sub>Mad1</sub> prototype sequence. HWM, Hemispheric white matter; GCN1–4, granule cell neurons strains; SF, San Francisco. Dashed lines denote sequence homology. Line interruption denotes deletion. The sequence underlined and in italics in JCV<sub>GCN3</sub> is the one that is duplicated in the 17 bp insert at nt 2502.

2511 to 2515, resulting in the loss of aa 348 and 349. Finally, JCV<sub>GCN3</sub> from San Francisco has a duplication of a 17 bp sequence inserted at nt 2502, leading to a premature stop codon and a truncated VP1 protein lacking the last 9 aa. This patient also had a wild-type JCV strain in his CSF, named JCV<sub>SF</sub>.

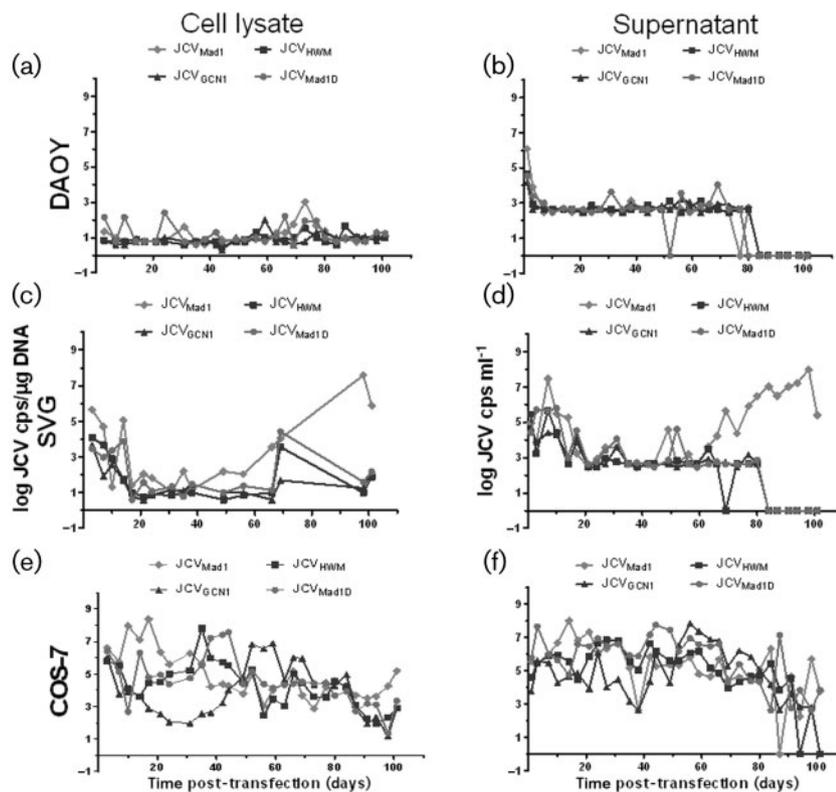
### JCV containing the GCN1 mutation are replication competent *in vitro*

To determine whether mutation of JCV VP1 C terminus altered the kinetics of JCV replication *in vitro* we initially focused on the GCN1 mutation found in our index patient as well as one Brazilian case (Table 1). Prototype JCV<sub>Mad1</sub>, PML isolate JCV<sub>HWM</sub>, JCV<sub>GCN1</sub> and prototype JCV<sub>Mad1D</sub> engineered with the GCN1 deletion were transfected in medulloblastoma cell line DAOY, a tumour arising from the granule cell layer of the cerebellum. In addition, these strains were also transfected in the permissive human astroglial cell line SVG and the monkey kidney cell line COS-7, which have both been transformed with the JCV-related simian virus 40 (SV40) T Ag, and therefore can sustain JCV replication. Cellular and supernatant DNA were harvested over a period of 100 days. Control experiments showed that *DpnI* digestion of input viral DNA resulted in complete digestion of nucleic acids, insuring that QPCR only amplified packaged viral DNA. Viral replication kinetics of the various JCV strains in different cell lines was measured by QPCR and is shown in

Fig. 3. We used a regression model with pairwise contrast and Bonferroni correction for multiple comparisons to analyse differences in viral kinetics over time between the four viral strains in each cell line, and for each strain in the three different cell lines.

The DAOY cells could sustain replication of all four JCV strains at a similar low level, and allowed productive infection that could be detected in the supernatant throughout the experiment. The presence of the GCN1 mutation did not confer any significant advantage or disadvantage compared to the non-deleted strains (Fig. 3a, b). Conversely, in SVG cells however, JCV<sub>Mad1</sub> had the highest peak viral load of all four viral strains reaching 8 logs both in the cells and supernatant. JCV<sub>Mad1</sub> viral load was higher overtime in both cell lysate and supernatant compared with JCV<sub>GCN1</sub> ( $P=0.004$  and  $P<0.0001$ ). Conversely, JCV<sub>Mad1D</sub> engineered with the GCN1 deletion had similar viral load overtime in cell lysate and supernatant as JCV<sub>GCN1</sub> ( $P=1.0$  and  $P=1.0$ ) and lower replication levels than the undeleted JCV<sub>Mad1</sub> ( $P=0.09$  and  $P=0.003$ ) (Fig. 3c, d).

Finally, all four JCV strains could replicate at significantly higher levels overtime in COS-7 cells compared with the other two cell lines. But the JCV<sub>GCN1</sub> initially had the slowest replication kinetic compared with the three other strains and a lower viral load in cell lysate than JCV<sub>Mad1</sub> ( $P=0.03$ ), (Fig. 3e, f).



**Fig. 3.** JCV<sub>GCN1</sub> is replication-competent *in vitro*. Detection of the viral load of undeleted JCV<sub>Mad1</sub>, JCV<sub>HWM</sub>, as well as JCV<sub>GCN1</sub> and JCV<sub>Mad1D</sub> with GCN1 deletion in the cell lysate and supernatant of transfected DAOY (a, b), SVG (c, d) and COS-7 (e, f) cells. DAOY, Human medulloblastoma cell line; SVG, human astroglial cell line; COS-7, monkey kidney cell line.

### GCN1 mutation remains stable overtime *in vitro*

To determine whether the GCN1 mutation persisted over time in various cell lines we amplified the VP1 gene fragment encompassing the mutated area from the cellular DNA after 30 passages *in vitro*. Compared to the undeleted JCV<sub>Mad1</sub> and JCV<sub>HWM</sub>, the JCV<sub>GCN1</sub> and JCV<sub>Mad1D</sub> mutation remained stable after 100 days in culture (Fig. 4a) and the size of the amplified fragment was the same as the one amplified from the input virus (not shown). Furthermore, the JCV VP1 protein could be detected by Western blot from cell lysates of all three cell lines infected with either strain, indicating a complete replication cycle and the presence of mature viral particles in these cells (Fig. 4b).

## DISCUSSION

Although JCV GCN is a recently discovered CNS condition, it has now been recognized in patients from the USA (Du Pasquier *et al.*, 2003; Hecht *et al.*, 2007; Koralnik *et al.*, 2005; Otis & Moral, 2005), Australia (Granot *et al.*, 2009; Tan & Brew, 2009), Chile (Bustamante *et al.*, 2009), South Korea (Shin *et al.*, 2008), and in this report, Brazil. JCV GCN patients were from both genders, their age ranged from 15 to 52 years and all were immunosuppressed, either in the context of HIV infection, congenital or inflammatory conditions. Among all five JCV GCN cases reported in this study and five described by

others (Bustamante *et al.*, 2009; Granot *et al.*, 2009; Otis & Moral, 2005; Shin *et al.*, 2008; Tan & Brew, 2009), half of the cases had co-existent white matter lesions at the time of presentation (case #1, 3, 6, 8 and 10; Table 1). This indicates that JCV GCN can occur separately from, or in association with, PML.

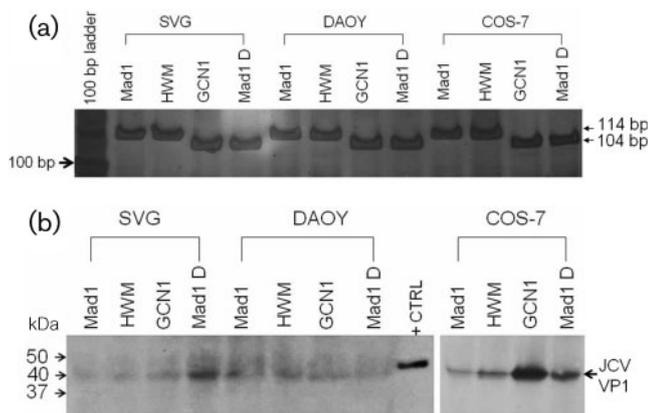
Although clinically recognizable cases of JCV GCN remain rare, the prevalence of JCV infection of granule cell neurons is likely underestimated. A histopathological survey of archival cerebellar samples showed evidence of JCV infection in granule cell neurons of up to 51% of HIV-seropositive PML patients and 3% of HIV-seropositive individuals without PML (Wüthrich *et al.*, 2009). This infection occurred regardless of whether classic PML lesions were also present in the nearby cerebellar white matter. It is therefore possible that many AIDS patients presenting with cerebellar atrophy and a cerebellar syndrome indeed have JCV GCN (Tagliati *et al.*, 1998).

Interestingly, we found three novel GCN-type mutations, all occurring in the same area of the VP1 C terminus as the initial GCN1 deletion (Dang & Koralnik, 2006). JCV-coding region is highly conserved, and these changes had never been described in more than 400 full-length viral sequences deposited in GenBank. It is therefore extremely unlikely that they could have occurred by chance. Furthermore, mutations found in all but the GCN2 variant lead to the loss of a  $\beta$ -pleated sheet in this area as modelled by Expert Protein Analysis System (EXPASY) (data not shown). These changes may probably have important consequences on the structure of the viral capsid, which is formed by 72 pentamers of the VP1 protein, associated on their inner surface with either the VP2 or VP3 protein. Indeed, this region forms the principal interpentamer contact, since each pentamer extends five invading arms to surrounding pentamers and receives five invading arms in return (Stehle *et al.*, 1996). Of note, this area is likely to be exposed on the exterior surface of the capsid, but in a position near the base of the canyons between pentamer knobs, which is remote from the known receptor-binding site on the surface of the pentamers (Neu *et al.*, 2010).

Since mature viral particles were seen by electron microscopy in granule cell neurons of our initial patient (Du Pasquier *et al.*, 2003) these changes may not prevent the formation of the capsid, but rather may influence viral tropism or post-entry events, including uncoating, transport and replication of JCV DNA, which could potentially enable productive infection in granule cell neurons.

Of note, the GCN-type mutations were not located in areas of single nucleotide mutations, leading to amino acid substitutions of the VP1 protein described in a recent study (Gorelik *et al.*, 2011).

Regardless of the exact pathogenetic mechanism, our data suggest that the detection of GCN-type JCV will potentially help clinicians diagnose JCV GCN, using the PCR primers encompassing the area of interest. Interestingly, the patient



**Fig. 4.** The GCN1 deletion is stable and the VP1 protein is expressed *in vitro*. (a) PCR amplification of VP1 C terminus gene using primers CJS2465 and CJR2578. Electrophoresis was performed on 10% TBE polyacrylamide gel followed by silver staining as described previously (Dang & Koralnik, 2006). Result shows that both undeleted JCV strains, JCV<sub>Mad1</sub> and JCV<sub>HWM</sub> (114 bp fragment) as well as JCV<sub>GCN1</sub> and JCV<sub>Mad1D</sub> harbouring the GCN1 deletion (104 bp fragment) remained stable after 30 passages *in vitro* in all cell lines. (b) Western blot detection of a 40 kDa VP1 protein from each JCV strain in all cell lines. DAOY, Human medulloblastoma cell line; SVG, human astroglial cell line; COS-7, monkey kidney cell line; +CTRL, positive control using SV40 virion.

with JCV<sub>GCN3</sub> also had an undeleted wild-type JCV strain in his CSF. The concomitant presence of deleted and undeleted JCV strains was also present in our initial case (Dang & Koralnik, 2006; Du Pasquier *et al.*, 2003), as well as in 25 % of CSF and 68 % of PBMC samples from PML patients, suggesting that the deletion may arise outside of the CNS, perhaps at the level of the bone marrow (Tan *et al.*, 2009).

Studies of JCV pathogenesis in neurons are challenging since JCV only infects humans, and no suitable human neuronal cell line is available. We therefore used for the first time a cell line derived from a human medulloblastoma, a neuronal tumour arising from the cerebellar granule cell layer. The viral load of all JCV strains remained low in this cell line. However, the fact that JCV could replicate at all in cells of neuronal origin which, unlike SVG or COS-7, were not previously transformed and immortalized with the SV40 T-Ag gene, is remarkable. We had initially postulated that the isolates with GCN1 deletion would have a selective advantage in this cell line, which was not verified experimentally. These results highlight the difficulties associated with modelling neuronal infection by JCV *in vitro*. However, our data indicate that the GCN1 mutation does not prevent expression of the VP1 protein, and that the deletion remains stable over a long period of time *in vitro*, without reverting to wild-type. Furthermore, detection of the VP1 protein by Western blot in DAOY cell lysate indicates that despite a low level of viral replication, a full replicative cycle occurred in these cells with production of mature viral particles.

As expected, the prototype JCV<sub>Mad1</sub> outperformed other JCV strains in astroglial cells, whereas the introduction of the GCN1 deletion in this strain conferred the same phenotype as JCV<sub>GCN1</sub>. These results are consistent with the fact that an undeleted VP1 protein provides an advantage for replication in glial cells. It is not clear why JCV<sub>HWM</sub> isolated from a PML lesion, did not replicate significantly better than JCV<sub>GCN1</sub> in this cell line. Both viral isolates have a similar RR with tandem repeat pattern (Dang & Koralnik, 2006), consistent with CNS-adapted strains of JCV. However, JCV<sub>HWM</sub> also has a unique in-frame deletion of 18 bp in the C terminus of the T-Ag gene, with loss of 6 aa (648–653) (Dang & Koralnik, 2006), which may dampen its replication in SVG cells. Finally, all viral strains replicated at higher levels in COS-7 cells, consistent with the fact that the kidney is a major compartment harbouring JCV and excretion in the urine is a common occurrence in healthy and immunosuppressed individuals alike (Arthur *et al.*, 1989; Kitamura *et al.*, 1990; Koralnik *et al.*, 1999; Markowitz *et al.*, 1993).

Altogether, these data underscore the genetic diversity of JCV<sub>GCN</sub> variants and shed light on an important and previously overlooked aspect of JCV pathogenesis.

## METHODS

This study was approved by the BIDMC institutional review board. Patients consent was obtained according to institutional IRB guidelines.

**Construction of JCV<sub>GCN1</sub>.** Due to the low viral copy number, the full-length sequence of JCV<sub>GCN1</sub> in the cerebellum sample of our index patient had been previously characterized after sequencing multiple PCR-amplified fragments covering the entire JCV genome (GenBank accession no. DQ875212) (Dang & Koralnik, 2006; Du Pasquier *et al.*, 2003). Considering that JCV<sub>GCN1</sub> and the JCV strain isolated from the hemispheric white matter of this patient, called JCV<sub>HWM</sub> obtained by long PCR amplification (GenBank accession no. DQ875211), shared the same backbone and differ only in the RR, the C terminus of VP1-coding region, and the C terminus of the T-coding region (Dang & Koralnik, 2006), we used JCV<sub>HWM</sub> genome to reconstruct a full-length clone of JCV<sub>GCN1</sub>. The DNA template corresponding to JCV<sub>GCN1</sub> RR located between two *NcoI* sites (nt 5014–5169–1–334, 490 bp, JCV<sub>NcoI</sub> fragment) was synthesized by BlueHeron. JCV<sub>GCN1</sub> RR target sequence was PCR amplified using primer pair: JGCNco5 (5'-GGAATTCCATGGATTCTC-3', GGAATT-4958–4970 of JCV<sub>HWM</sub>) and JGCNco3 (5'-CTTGTGC-TTTGTTTACTT-3', 257–240 reverse complement sequence of JCV<sub>HWM</sub>, designed by 'Primer Express/version 2.0'; Applied Biosystems). The PCR product was cloned into TA vector (Invitrogen) and sequenced. One positive clone was selected to proceed to the next step. Plasmid containing the JCV<sub>NcoI</sub> fragment was purified from selected positive clone and was digested by *NcoI* (New England Biolabs) to free the JCV<sub>NcoI</sub> fragment. The JCV<sub>NcoI</sub> fragment was recovered from agarose gel (QIAquick Gel Extraction kit; Qiagen) and used to replace the corresponding sequence of JCV<sub>HWM</sub>. The JCV strain obtained after this step was a new JCV strain that had the backbone of JCV<sub>HWM</sub> and RR of JCV<sub>GCN1</sub>, this strain was called JCV<sub>GCN1-RR</sub>.

DNA template of partial JCV<sub>GCN1</sub> genome containing the deleted VP1 gene and intact C terminus of T gene with two single restriction enzyme sites of *PacI* and *Bsu36I* (nt 2369–3056, JCV<sub>HWM</sub> sequence) was also synthesized by BlueHeron (nt 2097–3107, JCV<sub>HWM</sub> sequence, 1099 bp) and named PG<sub>VP1-T</sub>. Primer pair: JV2For (5'-AAGATATTTGGGACACTAACAGGAGGAGA-3', 2097–2126 of JCV<sub>HWM</sub>) and JV2Rev (5'-TTCAGGGCATGGCATAAGCAACC-3', 3187–3165 RC sequence of JCV<sub>HWM</sub>, designed by 'Primer Express/version 2.0') were used to amplify partial JCV<sub>GCN1</sub> sequence containing the C terminus of both VP1 and T gene from PG<sub>VP1-T</sub>. The PCR product was then cloned into TA vector, screened and sequenced. The plasmid containing the PG<sub>VP1-T</sub> fragment was purified and digested by *PacI* (2369 of JCV<sub>HWM</sub>) (Bilcock *et al.*, 1999; Hacıoglu *et al.*, 1997) and *Bsu36I* (3056 of JCV<sub>HWM</sub>) (New England Biolabs). The 696 bp PG<sub>VP1-T</sub> fragment containing 10 bp VP1 deletion and intact C terminus of the T gene was recovered from agarose gel (QIAquick Gel Extraction kit; Qiagen) and then used to replace the counterpart of JCV<sub>GCN1-RR</sub> to obtain the full-length genome of JCV<sub>GCN1</sub>.

**Construction of JCV<sub>Mad1D</sub>.** A similar strategy was used to obtain JCV<sub>Mad1D</sub>, a mutant of the JCV reference strain JCV<sub>Mad1</sub> containing the 10 bp deletion in the VP1 gene found in JCV<sub>GCN1</sub>. DNA template containing the VP1 gene of JCV<sub>Mad1D</sub> with 10 bp deletion in the C terminus was synthesized by BlueHeron (nt 2336–3806 of JCV<sub>Mad1</sub> strain with 10 bp deletion between 2503 and 2512). Primer pair: Mad1D5 (5'-AACCCTACCCAATTTCTTCCTTC-3', 2336–2360 of JCV<sub>Mad1</sub> strain) and Mad1D3 (5'-GCTTATGGGCATGTACTT-AGACTTTCAG-3', 3806–3779 RC sequence of JCV<sub>Mad1</sub>, designed by 'Primer Express/version 2.0') were used to amplify the synthesized template. The PCR product was cloned into TA vector and sequenced. Target JCV<sub>Mad1D</sub> DNA sequence was freed from the vector using *PacI* (position 2373 of JCV<sub>Mad1</sub>) and *PstI* (position 3359 of JCV<sub>Mad1</sub>) (New England Biolabs). The 977 bp target band (with 10 bp VP1 deletion) was recovered from agarose gel (QIAquick Gel Extraction kit; Qiagen) and was then used to replace the counterpart of JCV<sub>Mad1</sub> to obtain full-length genome of JCV<sub>Mad1D</sub>.

Plasmids containing full-length genome of different JCV strains were used to transform *Escherichia coli* INV110 for large-scale preparation, leading to methylation of *DpnI* sites.

**In vitro transfection of four JCV strains into three different cell lines.** In the *in vitro* transfection experiment, a total of four JCV strains; JCV<sub>Mad1</sub>, JCV<sub>Mad1D</sub>, JCV<sub>HWM</sub>, JCV<sub>GCN1</sub> were used to transfect separately three different cell lines: human astroglial SVG (purchased from ATCC), simian kidney cell COS-7 (purchased from ATCC) and human medulloblastoma DAOY (a generous gift from Dr Scott L. Pomeroy, Children's Hospital, Boston, MA, USA). Plasmids containing the full-length JCV genome were digested with *EcoRI* (New England Biolabs), the 5 kb band was recovered from the agarose gel using QIAquick Gel Extraction kit (Qiagen). Transfection was performed with a viral per cell ratio of 10<sup>5</sup> viral genome copies per cell using Effectene transfection reagents from Qiagen in a six-well culture plate. Cells transfected with the JCV genome were then transferred from a six-well plate to P25 flask 3 days post-transfection, and to P175 flasks 6 days post-transfection. JCV-transfected cells were passaged into a new P175 flask at a ratio of 1:5 every 3.5 days, and DNA was extracted from one-fifth of the total cell number at each passage (QIAamp DNA Blood Mini kit; Qiagen). Supernatant was collected at each passage and stored at -20 °C. DNA was extracted from the supernatant using QIAamp DNA Blood Mini kit after cell culture was terminated. The experiment ended after 100 days and 30 passages *in vitro*.

**QPCR detection of JC viral load in the in vitro transfection experiment.** DNA samples extracted from both JCV-transfected cells and the corresponding supernatant were quantified by SmartSpec Plus spectrophotometer (Bio-Rad). To remove any remaining contaminating input JCV DNA, samples were digested with *DpnI* (New England Biolabs) in a volume of 30 µl at 37 °C overnight. Since there are two *DpnI* sites (GA<sup>m</sup>TC, nt 841–844 and 970–973) located within the QPCR target sequence (nt 792–992), any methylated DNA sequence from input viral genome would be digested and could not be used as PCR template. After heat inactivation of *DpnI* at 80 °C for 20 min, 0.5 µg digested DNA was used as template in QPCR analysis. Primer pair: JCD792 (5'-TGGTGGTAGTGCTATTGCTCAGTT-3', position 792–815 of JCV<sub>Mad1</sub> amplifying a conserved sequence in all four JCV strains, and JCD992 (5'-GACGGGCCCAATGCTAG-3', reverse complement sequence of position 974–992 were used for QPCR detection. Probe JCV824 (6FAM-AGATTTTGTGCTGAC-TGGGATCATAAAGTTTCAACA-TAMRA, position 824–858 of JCV<sub>Mad1</sub> strain, all four strains have the same sequence; Applied Biosystems) was used in QPCR. QPCR was performed using standard TaqMan assay condition on ABI 7300 Real-time PCR System. JCV<sub>Mad1</sub> complete genome cloned in pUC18 vector was used as standard. The range of standard curve is from one copy to one million copies.

**Statistical analysis of JC viral load in vitro.** We assessed the distribution of viral load for each of the four virus strains within the three cell lines. We calculated the mean, standard deviation, median, minimum and maximum for each of the viral load distributions. Based on the asymmetry, and non-normality of these distributions, we used the logarithm transformation for the viral load. The log-transformed viral load exhibited symmetry and near normality. Therefore, we used the log-transformed viral load data for multiple regression models. To compare the viral load among the four virus strains in each cell line, we used a regression model where the dependent variable is the log viral load, and the independent variables were the four-level indicator variable for the type of virus, and the time of sample collection. The regression model yielded the adjusted mean level of log viral load for each virus. We then used the model linear contrasts to compute the six pairwise mean differences for the four viruses. We applied Bonferroni adjustment for multiple

comparisons, and reported the adjusted *P*-values for the pairwise comparisons. Similarly, we used the same modelling and testing technique to compare the mean log viral load of each virus strain among the three different cell lines. All of these analyses were conducted using the SAS/STAT version 9 software.

**PCR amplification and detection of mutated C terminus of JCV VP1 gene.** PCR amplification of JCV VP1 C terminus was performed with primers CJS2465 and CJR2578 and amplified sequences were visualized by 10% TBE PAGE and silver staining as described previously (Dang & Koralknik, 2006). The amplified fragments were cloned in TA vector (Invitrogen) and up to eight clones per sample were sequenced.

**Western blot of JCV VP1 protein.** VP1 capsid protein was detected in the cell lysate by Western blot. Based on the viral load data from QPCR, JCV-positive cell samples with a high JCV viral load around mid-point through the experiment were selected for each viral strain for Western blot analysis. A total of three million JCV-transfected cells were resuspended in 100 µl 1 × PBS, then added 50 µl Laemmli Sample Buffer from Bio-Rad, boiled for 5 min. Each boiled sample (15 µl) was loaded onto a 10-well precast SDS-gel from Bio-Rad. Cell lysate (1.5 µl) of SV40-transfected monkey fibroblast diluted to a final volume of 15 µl was used as positive control. JCV VP1 protein was detected by mouse mAb anti-SV40 VP1 protein – PAB597 (generous gift from Dr Walter Atwood), which cross-reacts with JCV VP1 protein. The presence of the protein was revealed with HRP-conjugated goat anti-mouse Ab (GAM-HRP) (Bio-Rad).

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