A granule cell neuron-associated JC virus variant has a unique deletion in the VP1 gene

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The human polyomavirus JC (JCV) typically infects glial cells and is the aetiological agent of progressive multifocal leukoencephalopathy (PML), which occurs in immunosuppressed individuals. The full-length sequence of a granule cell neuron-tropic JCV variant, JCVGCN1, associated with lytic infection of granule cell neurons and cerebellar atrophy in a human immunodeficiency virus-infected patient with PML was determined and compared with the sequence of the JCV isolate from the classic PML lesions present in the hemispheric white matter of the same individual (JCVHWM). A unique deletion was found in the C terminus of the VP1 gene of JCVGCN1, which encodes the major capsid protein, resulting in a frame shift and a total change of the C-terminal amino acid sequence of this protein. This deletion was not present in JCVHWM, suggesting that this mutation may be instrumental in facilitating entry or replication of JCV into granule cell neurons.

The human polyomavirus JC (JCV) is the aetiological agent of progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971). This disease occurs in immunosuppressed individuals, for example in people with AIDS or leukaemia, or in organ-transplant recipients. JCV causes a lytic infection of oligodendrocytes and a restrictive infection of astrocytes. Until recently, this virus was thought to have a host-cell range limited to human glial cells, as a productive infection of neurons had never been observed. However, we have described a lytic infection of cerebellar granule cell neurons by JCV in a human immunodeficiency virus (HIV)-infected patient with PML (Du Pasquier et al., 2003; Tyler, 2003). This neuronal infection resulted in focal loss of granule cell neurons leading to cerebellar atrophy. This was associated with a clinically apparent cerebellar syndrome, which was distinct from PML, as classic demyelinating lesions were found only in the hemispheric white matter of the cerebrum, and not of the cerebellum, of this patient.

To estimate the JCV load in cerebellum and hemispheric white matter, a quantitative PCR was performed on DNA extracted from fresh-frozen samples as described previously (Du Pasquier et al., 2006); this indicated that the JCV load in the cerebellum and hemispheric white matter was 3·45 × 10² and 1·88 × 10² copies (µg DNA)⁻¹, respectively.

We then attempted unsuccessfully to rescue JCV by cocultivation of autopsy brain and cerebellum samples with human SVG astroglial cell and human astrocyte progenitors as described previously (Major et al., 1985). Therefore, we sought instead to clone full-length JCV sequences from cerebellum and hemispheric white matter. To achieve this goal, long PCR was performed on cellular DNA extracted from these two locations [as described by Koralnik et al. (1999)] using primer pair Eco7 (5’-GAATTCCACTACC-CAATCTAAATGAGGAT-3’, nt 1721–1750) and Eco12 [5’-TGGAATTCTGGCCACACTGTAACAAG-3’, nt 1729–1704, reverse complement (RC); Agostini & Stoner, 1995]. A full-length JCV clone was obtained from the hemispheric white matter (JCVHWM). Due to the low copy number of JCV DNA in the cerebellum, long PCR amplification failed to amplify a full-length product from this location. We therefore designed primers according to the sequence of JCVHWM to sequence the JCV strain present in granule cell neurons (JCVGCN1) in small overlapping fragments.

We analysed the regulatory region (RR) sequences (from nt 5118 to 276; see Fig. 1a) of JCVHWM and JCVGCN1. PCR amplification of the JCV RR was performed using primer pair JCS4913 (5’-TGGTCCCCATGCAGATCTAC-3’, nt 4913–4936) and JCR755 (5’-GCCCGGGAGCTCCATG-3’, nt 755–738 RC). As shown in Fig. 1(a), the predominant JCV RRs found in hemispheric white matter and granule cell neurons comprised a tandem-repeat pattern with two incomplete 98 bp elements, the first of which had an intact 23 bp insert and the second a truncated 23 bp and an intact 66 bp insert. In addition, JCVHWM had a deletion in the late promoter. These data confirmed and expanded our previous analyses performed with PCR primers encompassing a smaller area of the RR (Pfister et al., 2001).

The coding region of JCVHWM and JCVGCN1 contained 105 identical point mutations, including 22 resulting in amino acid changes compared with the JCV Mad 1 reference strain. Of these, 21 have been reported separately as JCV subtype 3 (Agostini et al., 1997; Loeb & Dorries, 1988). In addition,
there was a new point mutation in both JCV\textsubscript{HWM} and JCV\textsubscript{GCN1}, resulting in an amino acid change from Gly (Mad 1) to Ala (JCV\textsubscript{HWM} and JCV\textsubscript{GCN1}) at position 62 of the VP2 protein (see Table 1 for details).

JCV\textsubscript{HWM} had a unique deletion of 18 bp starting at nt 2711 (compared with the JCV Mad 1 sequence) leading to the putative deletion of 6 aa (SQSQCF), encompassing aa 648–653 in the C terminus of the T protein, which was not present in JCV\textsubscript{GCN1} (Fig. 1b). Such a deletion in the T gene has not been reported previously. To rule out the possibility that this deletion was caused by an artefact of the long PCR, amplification of the DNA extracted from the brain hemispheric white matter was performed using PCR primers that amplified a 106 bp fragment encompassing this area of the T gene. Amplified products were distinctly smaller than the Mad 1 control DNA and sequencing of four clones confirmed that they all had this deletion. However, this in-frame deletion in the variable domain of the T protein did not seem to affect the phenotype of the JCV\textsubscript{HWM} isolate, as the clinical, radiological and histological presentation of lesions in the hemispheric white matter was classic for PML.

Interestingly, JCV\textsubscript{GCN1} had a unique deletion of 10 bp starting at nt 2503 (compared with the JCV Mad 1 sequence), resulting in an open reading frame shift and a complete change of the deduced last 10 aa of the C terminus of the VP1 protein, as well as the addition of 3 aa, from RYGQLQTKML to SCRQKCCNQP (Fig. 1c). To rule out the possibility that this deletion was caused by a PCR or sequencing artefact, amplification of the DNA extracted from the cerebellum was performed using the PCR primer pair CJ52465 (5'-CAGGAGACCCAGATATGATGAGAT-3', nt 2465–2489) and CJ5278 (5'-TGGTTATACTTTATTTCAATGATATATT-3', nt 2578–2547 RC); nucleotide numbering based on the sequence of JCV\textsubscript{HWM}), which amplify a 104 bp fragment encompassing the deleted area of the VP1 gene, compared with a 114 bp fragment for the non-deleted VP1 gene. Sequencing of eight clones confirmed that they all contained this 10 bp deletion. Using

Fig. 1. Sequence comparison of JCV\textsubscript{HWM} and JCV\textsubscript{GCN1}. (a) The JCV\textsubscript{HWM} and JCV\textsubscript{GCN1} RR\textsubscript{s} had a tandem-repeat pattern, including two incomplete 98 bp elements. The first element had a 23 bp insert and the second had a 66 bp insert and a truncated 23 bp insert. In addition, JCV\textsubscript{HWM} had a 53 bp deletion from nt 207 to 259 in the late promoter region. (b) Alignment of the C-terminal portion of the T gene of JCV prototype Mad 1, JCV\textsubscript{HWM} and JCV\textsubscript{GCN1}. An in-frame 18 bp deletion was present in the JCV\textsubscript{HWM} C terminus and the corresponding missing 6 aa are highlighted in bold. Numbers correspond to the Mad 1 sequence. (c) A novel open reading frame results from a 10 bp deletion in the C-terminal portion of the JCV\textsubscript{GCN1} VP1 gene. Alignment of the C terminus of the VP1 gene of JCV prototype Mad 1, JCV\textsubscript{HWM} and JCV\textsubscript{GCN1} indicated a 10 bp deletion in JCV\textsubscript{GCN1}. The novel amino acid sequence resulting from this frame shift is highlighted in bold italics. The numbers correspond to the Mad 1 sequence. The TAA termination codons used for the different isolates are highlighted in bold.
these PCR primers, JCVGCN1 was the only strain found in this patient’s cerebrospinal fluid (CSF) sample (Fig. 2a). This was verified by sequence analysis of 3/24 cloned fragments of identical size. In contrast, this strain was only detectable 107 days later as a minor species in his peripheral blood mononuclear cells (PBMCs) (Fig. 2a, PBMC #2, lower band, indicated by an arrow). In this sample, only 1/52 clones had the VP1 deletion, verified by sequencing.

Extending our analyses to other patients, we also found JCVGCN1-type VP1 in 3/12 (25 %) CSF samples from PML patients. Two patients had both the intact and the deleted VP1 gene, whilst one patient had the deleted JCVGCN1 VP1 only. This patient presented with prominent PML lesions restricted to the subcortical hemispheric white matter on magnetic resonance imaging associated with a bilateral pyramidal syndrome. The cerebellar white matter had no PML lesions on magnetic resonance imaging. However, this patient also had an unexplained severe static and kinetic cerebellar syndrome with appendicular and gait ataxia. She died of progressive neurological disease and an autopsy was not performed. Therefore, we can only speculate that the VP1 deletion mutant strain detected in her CSF had infected her granule cell neurons and caused her cerebellar dysfunction, similar to our index case. A representative display of amplified products from PML CSF samples is shown in Fig. 2(b). CSF samples from seven PML patients showed only the intact VP1 gene. However, in one patient (Fig. 2b, lane 8, faint lower band, indicated by an arrow), the VP1 deletion coexisted with an intact VP1 gene (Fig. 2b, lane 8, upper band) in the same sample. DNA sequencing determined that the lower band was identical to the JCVGCN1 VP1. This patient had classic PML lesions in the white matter of the cerebellum and a cerebellar syndrome. Therefore, it was not possible to determine whether the presence of a VP1 deletion mutant in his CSF was clinically relevant in this case.

To determine whether VP1 deletion mutants could also be found in the blood of other individuals, we screened stored PBMCs or plasma DNA of PML patients available in our laboratory. Of 25 JCV PCR-positive samples, eight (32 %) showed an intact VP1 gene only, whereas 17 (68 %) had two bands on the gel, a major upper band for the intact VP1 and a fainter lower band corresponding to the deleted form of VP1. Sequence analysis was performed on amplified PCR products of one of these patients and showed that 1/8 clones had a GCN1 deletion identical to that described in our index case. The deleted VP1 form was not found in isolation in the PBMCs of any PML patient. There was no association between the presence of the GCN1 mutation in blood and a cerebellar syndrome.

While JCV can enter a variety of cell types, the restricted host-cell range of this virus is attributable mostly to the

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<th>Protein</th>
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*Arrows indicate the codon change from Mad 1 to JCVHWM and JCVGCN1.
†As the 10 bp deletion starts from the last A of this codon in JCVGCN1, the open reading frame of the C-terminal portion of VP1 is shifted ahead by 1 bp and this codon is changed from AGA in JCVHWM to AGT in JCVGCN1. For details, see Fig. 1.
cellular requirements for transcription of the viral genome (Raj & Khalili, 1995). The JCV RR, which is required for viral expression and DNA replication, contains numerous binding sites for cellular proteins. Some of these proteins, such as NF1 class D, are predominantly expressed in glial cells (Sumner et al., 1996). If the RR of JCVGCN1 is responsible for its ability to grow in granule cell neurons, one would have expected to find a uniquely different RR sequence in these cells. However, in the present case, the RR of JCVGCN1 had a classic tandem-repeat pattern, as seen in PML isolates such as JCVHWM isolated in the classic PML brain tumours in children. Therefore, researchers have tried to determine whether polyomaviruses could have an aetiological role in medulloblastomas. Indeed, JCV induces an abortive infection of granule cell neurons in neonatal hamsters, causing medulloblastomas (Kim et al., 2003; Ressetar et al., 1990). These tumours arise precisely from the granule cell layer of the cerebellum (Kim et al., 2003) and account for 20% of brain tumours in children. Therefore, researchers have tried to determine whether polyomaviruses could have an aetiological role in medulloblastomas. In one study, investigators reported that 22/23 medulloblastomas (96%) contained JCV DNA sequences, and JCV T antigen was detected by immunohistochemistry in 4/16 (25%) samples tested (Krynska et al., 1999). However, these results were not confirmed by others (Kim et al., 2002; Weggen et al., 2000). Therefore, the availability of a granule cell neuron-tropic virus 40 and on studies of mouse polyomavirus (Stehle et al., 1994, 1996), the capsid of JCV is formed from 72 pentamers of VP1 protein, which are associated on their inner surface with either the VP2 or the VP3 protein. Although the exact site of interaction of JCV VP1 protein with its receptors remains unknown, it is unlikely that the GCN1 mutation, located in the C terminus, leads to a direct alteration of this site, as this portion of the VP1 protein is not exposed on the outer surface of the capsid.

In contrast, the C-terminal arms of the VP1 protein form the principal interpentamer contacts by extending away from the subunit of origin and reaching out to a subunit of an adjacent VP1 pentamer. Therefore, each pentamer extends five C-terminal arms to surrounding pentamers and receives five invading arms in return (Stehle et al., 1996). This lattice effectively acts as the ‘grout’ that keeps the pentamers together. The deletion of the VP1 gene appears to change the deduced tertiary structure of the VP1 C terminus dramatically, as resolved by EXPASY (Expert Protein Analysis System), resulting in the loss of a β-pleated sheet in this area (data not shown). Therefore, we hypothesize that this mutation results in an alteration of the properties of the viral capsid. However, classic JC virions assembled in crystal structure were seen by electron microscopy in the granule cell neurons of this patient (Du Pasquier et al., 2003). This finding suggests that the VP1 deletion may not prevent formation of the capsid, but rather may alter post-viral entry events such as uncoating, transport and replication of JCV DNA, which in turn could enable a productive infection in granule cell neurons. Similarly, a change in phenotype and host-cell range was demonstrated in a mouse polyomavirus isolate that had a single amino acid change in the VP1 protein (Bauer et al., 1995; Freund et al., 1991).

JCVGCN1 was found in 25% of CSF samples and 68% of PBMC samples from our PML patients. These results suggest that this deletion may originate outside of the central nervous system, perhaps at the level of the bone marrow. Nevertheless, these data indicate that VP1 deletion mutants frequently co-exist as a minor species with intact JCV strains in the blood of patients with PML.

Finally, our findings open up a new avenue of investigation, which may shed some light on the pathogenesis of medulloblastomas. Indeed, JCV induces an abortive infection of granule cell neurons in neonatal hamsters, causing medulloblastomas (Kim et al., 2003; Ressetar et al., 1990). These tumours arise precisely from the granule cell layer of the cerebellum (Kim et al., 2003) and account for 20% of brain tumours in children. Therefore, researchers have tried to determine whether polyomaviruses could have an aetiological role in medulloblastomas. In one study, investigators reported that 22/23 medulloblastomas (96%) contained JCV DNA sequences, and JCV T antigen was detected by immunohistochemistry in 4/16 (25%) samples tested (Krynska et al., 1999). However, these results were not confirmed by others (Kim et al., 2002; Weggen et al., 2000). Therefore, the availability of a granule cell neuron-tropic
strain of JCV would be extremely valuable to clarify the potential role of this virus in human cerebellar tumours.

Since the publication of our index case, we and others have reported a productive infection of granule cell neurons by JCV in other patients (Gahaffar et al., 2003; Koralnik et al., 2005). We are now investigating whether this mutation confers a different phenotype on JCV in vitro.

Acknowledgements

This study was supported in part by NIH grant R21 NS 051124 to I. J. K. We are grateful to Eugene Major, PhD, and the staff of his laboratory for his help in attempting to culture the JCV isolates in vitro.

References


