Detection of reactivation and size variation in the regulatory region of JC virus in brain tissue

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Abstract

Aims—To develop a sensitive and specific polymerase chain reaction (PCR) based system for detecting genomic variation in JC virus. To apply this system to formalin fixed, paraffin wax embedded brain tissue from patients with and without progressive multifocal leucoencephalopathy (PML).

Methods—A pair of primers (JC1 and JC2) were designed to be complementary to the early and late regions of JC and BK polyomaviruses, respectively. A third primer (JC3), internal to JC1 and JC2, was designed to be specific for JC virus. The specificity of JC3 was investigated by amplifying plasmids with BK or JC virus genomes. Sensitivity was estimated by titration of a plasmid containing JC virus genome. Seven brains from patients with PML (PMLB) and 30 from patients without PML (non-PMLB) were amplified using JC1 and JC2, followed by JC1 and JC3. Amplification of the β globin gene was used as an amplification control.

Results—Amplification with JC1 and JC2 was common for JC and BK viruses, but with JC1 and JC3 it was specific for JC virus. The sensitivity of the system was 25 copies of JC plasmid per 10 μ l of digested tissue. Five out of seven PMLB and 28 of the 30 non-PMLB amplified for β globin, but only the PMLB gave a signal with polyoma primers. Hyper-variation of the length of the regulatory region of the JC isolates in the PML tissues was consistent with the presence of multiple strains of JC.

Conclusions—Variation in the regulatory region of JC virus can be specifically and sensitively detected from routinely processed, paraffin wax embedded brain tissue. Variation in the regulatory region is common in PML derived JC strains, but JC virus was not detectable in non-PMLB tissue.

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Primary infection with JC virus is common, with most individuals seroconverting by adulthood.¹ Most infections are apparently clinically asymptomatic, but in a few people, usually when immunocompromised, sufficient replication of JC occurs in the central nervous system to cause progressive multifocal leucoencephalopathy (PML).²

Recently, Mori *et al* used immunohisto-

chemistry, in situ hybridisation, and the polymerase chain reaction (PCR) to show that JC virus can be detected in 30-40% of elderly Japanese patients without PML.^{3,4} The presence of JC virus in such a large proportion of brains from elderly patients has important implications for understanding JC replication, and may also be associated with the deterioration of cognitive functions with increasing age. The replication and reactivation of JC in the brains of people without PML is, however, controversial.³⁻⁷

Our aim was to develop a PCR based system for detection of JC virus from routinely processed brain tissue, with a high degree of sensitivity and specificity. In addition, we aimed to use the variation in the size of the regulatory region to provide information on divergence from the archetypal JC virus.

Methods

Necropsy brain tissue was available from seven patients with a diagnosis of PML on clinical and established histological criteria (table 1). Necropsy brain tissue was also available from a further 30 caucasian patients with no histological features of PML.

Group 1 under 50 years old (average age 37 years, seven men, three women), cause of death: trauma n = 3, myocardial infarction n = 2, bronchopneumonia n = 2, renal failure n = 1, subarachnoid haemorrhage n = 1, sudden death n = 1.

Group 2 over 50 years old but not seriously debilitated for more than a few days before death (average age 84 years, two men, eight women); cause of death: bronchopneumonia n = 5, pulmonary embolism n = 2, cerebrovascular accident n = 2, disseminated cancer n = 1.

Group 3 over 50 and debilitated for several months before death (average age 81 years, three men, seven women); cause of death: bronchopneumonia n = 5, chronic pulmonary abscess n = 2, massive pulmonary embolism n = 1, congestive cardiac failure n = 1, cerebrovascular accident n = 1.

Table 1 Clinical details of PML patients

Case No	Sex	Age	Underlying conditions
B	M	34	AIDS
C	M	24	Severe combined immunodeficiency
D	F	66	Chronic lymphocytic leukemia
E	M	67	Hodgkin's disease
F	M	82	Dementia, no other known predisposing cause
G	M	68	Sarcoidosis
H	F	57	No known predisposing cause

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Figure 1 Map of polyomavirus genome showing site and orientation of the primers JC1, JC2, and JC3. Primer sequences are shown; the nucleotides which are not complementary to the genome of BK virus (strain MM) are marked above by an asterisk.

PRIMER DESIGN AND SPECIFICITY
Primers for polyomaviruses were designed using the European Molecular Biology Library (EMBL) database, such that JC1 and JC2 were complementary to the early and late regions of all known JC and BK strains. JC3 was complementary to the late region of JC virus, internal to JC1 and JC2, and had multiple mismatches with BK virus (fig 1).
Two plasmids pGEM JC and pGEM BK, containing genomes of JC and BK virus, respectively, were amplified with JC1 and JC2, using 5 ng of each plasmid and 25 cycles (94°C for 90 seconds, 49°C for 120 seconds, 72°C for 120 seconds of PCR). A second round of amplification was carried out on 2 µl of the amplification product using primers JC1 and JC3 for a further 25 cycles and the products from this were run on a 1% agarose gel.

SAMPLE PREPARATION AND AMPLIFICATION
All tissue samples were fixed in formalin for four weeks (except for PML sample B, which was fixed for 24 hours), and embedded in paraffin wax. Sections (5 µm) were cut from each block to a final tissue dimensions of 2 cm² by 5 µm, and the tissue was dewaxed by two washes of xylene followed by two washes of ethanol in a 1.5 ml Eppendorf tube. Overnight digestion was carried out in a buffer volume of 200 µl (TRIS 50mM, EDTA 1mM, Tween 20 (Sigma) 0.5%, pH 8.5) with proteinase K (final concentration 200 µg/ml) at 37°C. After digestion the proteinase K (Boehringer Mannheim) was denatured by heating to 94°C for 10 minutes, and 10 µl of each sample underwent 45 cycles of amplification (94°C for 90 seconds, 49°C for 120 seconds, 72°C for 120 seconds, total reaction volume of 50 µl in Cetus PCR

Figure 2 Amplification of plasmids containing polyoma sequences with JC1/JC2 and JC1/JC3 primers. Lanes 1 and 2, amplification of pGEM JC and pGEM BK with JC1/JC2. Lane 3, amplification of pGEM JC with JC1/JC3. Lane 4, no product from amplification of pGEM BK with JC1/JC3. Lane 5, pBR322 cut with msp1 as a molecular weight marker.

Figure 3 Sensitivity titration of pGEM JC in proteinase K digested tissue. Lanes 1 to 5, 25 000, 2500, 250, 25, five copies, respectively. Lane S is marker VIII (Boehringer Mannheim) as a molecular weight marker.

buffer), using primers for the β globin gene Bext 1 and Bext 2, and in a parallel reaction tube using JC1 and JC2. The amplified products (15 µl) were electrophoresed on a 2% agarose gel and the β globin and polyoma signals were visualised under ultraviolet light with ethidium bromide staining.
Each sample amplified with JC1 and JC2 was then reamplified for 25 cycles using 2 µl of the first round amplification product, primers JC1 and JC3, and the above cycling steps. This product (20 µl) was run on a 5% polyacrylamide gel and stained with ethidium bromide. A sensitivity titration was carried out by adding dilutions of the JC plasmid (25 000, 2500, 250, 25 and five copies) to 10 µl of a proteinase K digest of non-PMLB specimen and amplified by JC1/JC2 for 45 cycles followed by JC1/JC3 for 25 cycles with the above cycling steps.

Results
Primer pair JC1 and JC2 amplified pGEM BK and pGEM JC equally well giving a product of about 380 base pairs. Primer pair JC1 and JC3 proved specific for the PGEM JC derived product (fig 2).
The sensitivity titration showed that as little as 25 copies of JC virus could be detected in 10 µl of digestion mix (fig 3). Five of the seven PMLB and 28 of the 30 non-PMLB (10 from group 1, nine from each of groups 2 and 3) gave a 335 base pair positive signal with Bext 1 and 2 (data not shown). Of the five PMLB which amplified with the β globin primers, three gave a product with JC1/JC2, and subsequently with JC1/JC3. The remaining two gave a product only on the second round amplification with JC1/JC3 (table 2).

Table 2 Results of β globin and polyoma amplification of PML samples run on a polyacrylamide gel

Case No	β globin	Polyoma first round	Polyoma second round
B	+	+	+
C	+	+	+
D	+	+	+
E	+	-	+
F	+	-	+
G	-	-	-
H	-	-	-

Figure 4 Polyacrylamide gel electrophoresis of amplification products of PML brain tissues using primers JC1/JC2 (lanes A1, B1, C1 and D1), and internal amplification of first round products using primers JC1 and JC3 (lanes A2, B2, C2, D2, E2 and F2). Lane S is pBR322 cut with *msp1* as a molecular weight marker. Sample A was pGEM plasmid with a JC virus insert. Samples B to F were PML brain tissues from five different subjects.

None of the non-PMLB gave an amplification product with the polyomavirus primers. Polyacrylamide gel electrophoresis showed substantial variation in the size of the amplified products from the seven PMLB and showed multiple bands in lanes D2, E2, and F2 (fig 4).

Discussion

JC virus was first isolated from the brain of a patient with PML.⁸ Repeated isolation and identification from PMLB has established JC virus reactivation as the cause of the characteristic cytodical changes seen in oligodendrocytes in this rare demyelinating disorder. PML usually develops in patients with severe primary immune deficiency or a variety of lymphoproliferative disorders and has been increasingly diagnosed in patients with AIDS.²

The presence of JC virus in brain tissues of patients without PML is controversial. Meinke *et al* were unable to demonstrate JC DNA in the brains of four patients with multiple sclerosis using DNA-DNA reassociation kinetics.⁵ A Southern blot analysis of brain and renal necropsy tissues from 30 non-PML subjects detected JC virus DNA in the renal tissue of three, but not from brain tissues.⁶ This apparent absence of polyomavirus in non-PMLB tissues was challenged by the detection of JC viral DNA and the common papovaviral capsid protein in the brains of four out of 10 elderly patients using *in situ* hybridisation and immunohistochemical staining.³ Most of the patients in this recent study were, in contrast to the findings of previous studies, debilitated with pneumonia or neoplastic disease before death. These observations were extended, by the same research group to 33 elderly patients, using PCR followed by Southern blot analysis.⁴

To address this issue, we decided to use the highly sensitive technique of nested PCR for the detection of JC viral DNA.⁹ Sequence analysis of JC virus isolates from PMLB has shown a double stranded circular genome similar to other polyomaviruses, with an early and late region, separated by a regulatory region containing the origin of replication.¹⁰ Comparison between isolates of JC virus from PMLB shows a great degree of similarity for the early and late regions, but extensive variation in the regulatory region.¹¹ This degree of variation was assumed to be common for all

JC virus isolates, but sequencing of JC virus from the urine of eight non-immunocompromised subjects identified an isolate which was present in all, possessed a well conserved regulatory region, and probably represents the wild type or archetypal JC.¹² This has been confirmed,^{13,14} and it therefore seems that primary infection is due to the wild type virus, with subsequent reactivation when the immune environment is suppressed. In favour of this hypothesis is the demonstration by Yogo *et al* that the regulatory region of the PML strains could have evolved from the wild type regulatory region by a series of deletions and amplifications.¹²

For the detection of JC virus, primers can be designed for any part of the genome which is well conserved, to give a product of a defined size. By designing primers which amplify across the regulatory region (fig 1), however, we have a simple method for identifying not only JC virus, but also of rapid comparison of the sizes of the regulatory regions.

Figure 3 shows that JC virus was detected from all five PMLB which were not refractory to amplification, and confirms that hypervariation in the regulatory region is present in all cases. In addition, samples D, E, and F have more than one amplification product. This is compatible with the presence of two strains of JC virus in the same tissue but may also be due to PCR artefact. Two strains of polyomavirus have previously been detected from one person, but from different organs (brain and kidney).¹⁵ During the process of evolving from the archetypal strain to the various PML strains, JC virus presumably passes through a number of mutant forms which were detected by our method. If serial samples were available—for example, biopsy followed by post mortem material—it would be possible to follow the evolution of strains (as in lane F2) to see which one becomes dominant.

Our 30 non-PMLB patients were chosen to include 10 from a group of elderly debilitated subjects. All 30 were negative for JC virus after two rounds of PCR. Amplification efficiency was controlled by amplifying the β globin gene from 28 of these brains. This is important as inhibitors to amplification have been shown in some formalin fixed, paraffin wax embedded material.¹⁶ Our results agree with those of earlier reports, but have not been able to reproduce the results of Mori *et al*^{3,4} which showed JC virus in about 30% of elderly non-PMLB patients. The nine patients in group 3 are comparable with those reported by Mori *et al*, in terms of age and malaise immediately before death, but there are obvious racial and geographic differences in the two studies, which may account for the discrepancy in results. Nested PCR has a very high level of sensitivity, at least comparable with, if not better than, *in situ* hybridisation or PCR followed by Southern hybridisation.⁹ Using nested PCR, our protocol permits detection of 25 copies of JC in 10 μ l of digestion mix, representing between 10 000 and 30 000 cells.^{17,18}

In conclusion, we have developed a highly

sensitive protocol for detecting JC virus, which can also show variation within the regulatory region, and thus may detect the presence of multiple strains in the same sample. Hypervariation of the regulatory region is usual in PML brain tissue. In contrast, JC virus is not common in non-PML brain tissue.

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