Advances in Brief

Detection of JC Polyomavirus DNA Sequences and Cellular Localization of T-Antigen and Agnoprotein in Oligodendrogliomas

Luis Del Valle, Sahnila Enam, César Lara, Carlos Ortiz-Hidalgo, Christos D. Katsetos, and Kamel Khalili


Abstract

Purpose: Productive infection of the human neurotropic polyomavirus JCPyV in oligodendrocytes leads to the development of progressive multifocal leukoencephalopathy, a fatal demyelinating disorder of the central nervous system. In addition to its role in viral infection, JCPyV T-antigen can transform cells in vitro and induce tumors in experimental animals in the absence of viral DNA replication and late gene expression. The goal of this study is to examine the presence of JCPyV DNA sequences and viral antigens in a series of human oligodendrogliomas.

Experimental Design: A total of 20 well-characterized oligodendrogliomas were examined for detection of the JCPyV genome by PCR and immunohistochemistry for expression of viral proteins.

Results: Gene amplification has revealed the presence of JCPyV DNA sequences corresponding to the NH2-terminal of T-antigen in 15 of 20 samples. DNA sequences corresponding to late regions, which are responsible for production of the capsid protein, VP1, were detected in 14 of 20 samples. Sequencing of the viral control region determined the presence of JCPyV Mad-4 or JCPyVC in these tumors. By immunohistochemistry, T-antigen expression was detected in the nuclei of tumor cells from 10 samples that also contained corresponding DNA sequences by PCR. Eleven of 20 tumors exhibited immunoreactivity for the late auxiliary gene product, agnoprotein. None of the samples showed immunoreactivity for the capsid proteins, ruling out productive infection of neoplastic cells by JCPyV.

Conclusions: Collectively, these observations provide new evidence in support of the association of the oncogenic human neurotropic JCPyV and oligodendrogliomas.

Introduction

Oligodendrogliomas are common glial tumors of the CNS3 with an unpredictable clinical behavior. These slow-growing neoplasms arise from the cortex and the white matter of the cerebral hemispheres (1–3). According to the latest WHO classification of tumors of the nervous system, oligodendrogliomas are divided into two main groups, the low-grade and classical (WHO grade II) and the high-grade or anaplastic (WHO grade II). The pathological diagnosis of these tumors is based on the recognition of salient histological features (4–6). Despite a phenotypical resemblance to oligodendrocytes, neoplastic cells in oligodendrogliomas do not generally express markers of differentiated oligodendrocytes (7, 8) but may express similar to other glial tumors or oligodendrocyte progenitor cell antigens (9–11). Frequently, subpopulations of tumor cells express GFAP (for review see Ref. 3).

A significant percentage of oligodendrogliomas exhibit combined loss of chromosomes 1p and 19q (12–16). Clinically, 1p/19q loss is considered to be a strong predictor of a favorable response to chemotherapy and a better prognosis, particularly in anaplastic tumors (17–22). However, the etiology and pathogenesis of oligodendrogliomas remains presently unknown.

Recent studies from several laboratories point to the association of polyomaviruses, including JC polyomavirus JCPyV, with a variety of human brain tumors (for review see Ref. 23). JCPyV is the established etiological infectious agent of PML, a subacute and fatal demyelinating disease of the CNS (24, 25). Since the first description of PML by Åström and Richardson in 1958 (26), there have been several reports on the association of the demyelinating disease with concomitant brain tumors. The first of these cases was a 58-year-old male suffering from chronic lymphocytic leukemia and PML; the postmortem examination incidentally revealed a concurrent oligodendroglioma (27). In 1996, Rencic et al. (28) described the detection of JCPyV genome and the expression of viral early protein, T-antigen in the brain of an immunocompetent patient with an oligoastrocytoma.

In more recent studies, JCPyV DNA sequences have been found in several types of brain tumors, including medulloblastomas (29, 30), ependymomas, astrocytomas, anaplastic astrocytomas (31), and glioblastoma multiforme (32). Although the

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1 This work was made possible by grants awarded by NIH (to K. K.).
2 To whom requests for reprints should be addressed, at Phone: (215) 204-0678; Fax: (215) 204-0679; E-mail: kamel.khalili@temple.edu.
3 The abbreviations used are: CNS, central nervous system; GFAP, glial fibrillary acidic protein; PML, progressive multifocal leukoencephalopathy.
etiological role of JCPyV in these tumors at present remains unknown, several laboratory studies provide compelling evidence for the tumorigenicity of JCPyV in experimental animals (23). For example, JCPyV can induce brain tumors of neuroectodermal origin when intracerebrally inoculated in rodents (33, 34) and monkeys (35, 36).

The JCPyV genome is composed of a circular, closed, double-stranded DNA of 5130 nucleotides in size (37). The viral genome can be divided in three regions, a control region, which contains two 98-bp repeats that are the initiation sites of viral replication, an early coding region, and a late coding region. The viral genome can be divided into three regions, a control region, which contains two 98-bp repeats that are the initiation sites of viral replication, an early coding region, and a late coding region. The early region encodes for the transactivator proteins large and small T-antigen, and the late region encodes for the capsid proteins VP-1, VP-2, and VP-3 as well as the auxiliary agnoprotein (38). Primary infection occurs in early childhood and is subclinical. Furthermore, ~80% of the adult population of the world has been exposed to the virus and exhibit specific antibodies against JCPyV (39, 40).

It is well established that the demyelinating lesions of PML are the result of cytolytic destruction of oligodendrocytes upon productive infection with JCPyV. Infection of oligodendrocytes begins with association of JCPyV with the cell membrane, which leads to internalization of viral particles and release of the viral genome in the nuclei (41). The cell type-specific expression of the viral genome at the early stage through the interaction of various host proteins leads to production of the viral early protein, T-antigen (42). Because of the transforming abilities of T-antigen, which is mediated by its interaction with various tumor suppressors, such as p53 and Rb, it is believed that nonproductive infection of the cells by JCPyV may result in the transformation of the cells and the development of neoplasia. In this study, we have examined a series of morphologically defined tumor samples to determine the association of JCPyV genome and the expression of early and late viral proteins in human oligodendrogliomas.

Materials and Methods

Clinical Samples. A total of 20 formalin-fixed, paraffin-embedded surgical and postmortem samples comprising 18 classical oligodendrogliomas (WHO grade II) and 2 cases of anaplastic oligodendrogliomas (WHO grade III) were collected from the pathology archives of the American British Cowdray Hospital in Mexico City, Mexico and the Royal London Hospital, Whitechapel (London, United Kingdom). Table 1 describes the clinical data from these patients, including their origin, gender, and age at the time of surgical resection.

DNA Extraction and PCR Amplification. DNA was extracted from several sections of 10-μm thickness from each of the tissue samples by using the QIAamp Tissue Kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA). To prevent any contamination, we used a dedicated microtome for this study. Furthermore, the block and blade-holder were periodically autoclaved, and a fresh, disposable blade was used for each specimen. The sections were handled with disposable, one-time use applicators.

PCR amplification was performed on DNA extracted from the tumors by using four individual sets of primers numerically designated as described by Frisque et al. (Ref. 38; GenBank Accession No. J02226): PEP1 and PEP2 (nucleotides 4255–4274 and 4408–4427, respectively), which amplify sequences in the NH2-terminal region of JCPyV T-antigen VP2 and VP3 (nucleotides 1828–1848 and 2019–2039, respectively), which amplify a portion of the VP1 capsid gene sequence, AGNO1 and AGNO2 (nucleotides 280–298 and 438–458, respectively), which amplify a region within coding region of JCPyV agnoprotein and CR1, CR2, and CR3 (nucleotides 4987–5006, 238–257, and 5101–5121, respectively), which amplifies the control region of JCPyV. Amplification was carried out on 500 ng of template DNA with AmpliTaq DNA Polymerase (Perkin-Elmer) in a total volume of 50 μl. Hot-start PCR using AmpliWax PCR Gem 100 (Perkin-Elmer) according to the manufacturer’s instructions was performed in the presence of 2.5 mM MgCl2 and 0.5 mM of each primer (Oligos, Etc., Guilford, CT). A Perkin-Elmer Gene Amp 9600 PCR System was used exclusively for this study with denaturation at 95°C for 90 s, followed by 45 cycles of denaturation at 95°C for 15 s, annealing for 30 s, and extension at 72°C for 30 s. The annealing steps were performed at temperatures of 55°C for the phosphoenolpyruvate primers, 52°C for the TC primers, and 54°C for the VP primers. As a termination step, the extension time of the last cycle was increased to 7 min. Samples amplified in the absence of template DNA served as a negative control, whereas inclusion of serial dilutions of the plasmid pBJC containing the JCPyV (Mad-4 regulatory region) genome as template served as a positive control for each procedure.

Southern Blot Hybridization. Southern blot analysis was performed by using 15 μl of each PCR reaction separated by 2% agarose gel electrophoresis, after which DNAs were transferred from the gel to a nylon membrane (Hybond-N; Amersham) in 20× SSC by using a semidry transfer apparatus (Turboblot; Schleicher & Schuell). The blots were prehybridized in 0.5% SDS/10 mM EDTA/5× SSC/5× Denhardt’s solution/0.1 μg/μl salmon sperm DNA for 4 h followed by hybridization in the same solution containing 5×105 cpm/ml [γ-32P]ATP end-labeled oligonucleotide probe overnight at 65°C. To remove nonspecific binding, blots were washed twice in 2× SSC/0.1% SDS at 55°C for 5 min each and then three to five times in 0.1× SSC/0.1% SDS at 55°C for 3 min/wash before autoradiography. Probes used for Southern blotting included JCPyV probe (nucleotides 4303–4327) to detect fragments amplified with phosphoenolpyruvate primers, VP probe (nucleotides 1872–1891) for those amplified with VP primers, agno probe (nucleotides 425–445) for sequences amplified with the agno 1 and 2 primers, and CR probe (nucleotides 62–81) for sequences of the control region of JC polyomavirus.

Sequencing of PCR-amplified Fragments. Amplified DNA fragments initially identified by Southern blot hybridization wereexcised from preparatory agarose gels stained with ethidium bromide, and DNA was purified by using the QIAquick PCR Purification Kit according to the manufacturer’s instructions (Qiagen). After extraction, the DNA was sequenced by automated fluorescent DNA cycle sequencing using the Applied Biosystems Prism 377 DNA Sequencer-XL.

Histological and Immunohistochemical Analysis. The tissue that had been previously formalin-fixed and paraffin-embedded was sectioned in a microtome at 4-μm thickness and stained with H&E for histological diagnosis. Immunohisto-
Table 1  Diagnosis of the tumor is based on WHO criteria for classification of brain tumors, as specified in “Materials and Methods.”

Gender and age at the time of surgical resection are shown. Country of origin of the samples is indicated. TuJ1 (BIII), class III β-tubulin; SY-38, synaptophysin; NF, neurofilaments; T-Ag, T-antigen; −, negative immunoreactivity; +, 1–30% cell positivity; ++, 31–60% cell positivity; ++++, >61% cell positivity; f, indicates focal positivity; n/d, samples were not in appropriate condition for PCR amplification analysis.

<table>
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<th>SY-38</th>
<th>NF</th>
<th>p53</th>
<th>T-Ag</th>
<th>Agno</th>
<th>Peps</th>
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<th>Control Region</th>
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chemistry was performed using an avidin-biotin-peroxidase complex system according to the manufacturer’s instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories, Inc., Burlingame, CA). The protocol includes deparaffinization in xylene and rehydration of the tissue through descending grades of alcohols up to water. Nonenzymatic antigen retrieval was performed by heating the sections to 95°C in 0.01 M sodium citrate buffer (pH 6.0) for 40 min in a vacuum oven. After a 30-min cooling period, the slides were rinsed in PBS and incubated in methanol/3% H2O2 for 20 min to quench endogenous peroxidase. Sections were then rinsed with PBS and blocked with 5% normal horse or goat serum in 0.1% PBS/BSA for 2 h at room temperature. Primary antibodies, including viral proteins and cellular markers, were incubated overnight at room temperature in a humidifier chamber. The primary antibodies used in this study included: a rabbit polyclonal antibody against agnoprotein (kindly provided by Dr. M. Safak, Center for Neuровirology and Cancer Biology, Temple University, Philadelphia, PA; 1:500 dilution); a rabbit polyclonal antibody against JCPyV capsid protein VP-1 (kindly provided by Dr. Walter Atwood, Brown University, Providence, RI; 1:1000 dilution); a mouse monoclonal antibody for the detection of SV40 T-antigen, which cross-reacts with JCPyV T-antigen (Clone pAb416, 1:100 dilution; Oncogene Science; Ref. 43); a mouse monoclonal antibody against p53 (Clone DO-7, 1:100 dilution; Dako); a mouse monoclonal antibody specific for GFAP (Clone 62F, 1:100 dilution; Dako); and a mouse monoclonal antibody against Synaptophysin (Clone MAB332, 1:500 dilution; Chemicon Int.). Positive controls included a normal brain for GFAP, and Synaptophysin, demyelinating brain lesions from a patient with PML for the viral proteins T-antigen, VP-1 and agno, and a colon adenocarcinoma specimen for p53. After rinsing the sections in PBS, biotinilated antimouse or antirabbit secondary antibodies were incubated for 1 h at room temperature and rinsed in PBS. The tissue was subsequently incubated with avidin-biotin-peroxidase complexes for 1 h at room temperature, and finally, the sections were developed with a 3,3’-diaminobenzidine substrate (Sigma, St. Louis, MO), counterstained with hematoxilin, and coverslipped with Permount (Fisher, Pittsburgh, PA). To assess the fraction of immunolabeled cells in specimens from each patient case, the labeling index (LI) defined as the percentage of positive cells out of the total number of tumor cells counted was determined.

Results

Tumor Morphology. A collection of oligodendrogial tumors, including 18 oligodendrogliomas and 2 anaplastic oligodendrogliomas, was obtained from the Pathology Archives of the American British Cowdray Hospital in Mexico City, Mexico, and the Royal London Hospital in London, United Kingdom. The tumors were histopathologically characterized according to the latest World Health Organization Classification of Brain Tumors (3, 44). In general, oligodendrogliomas (WHO grade II) were characterized by homogeneous groups of cells surrounded by a clear halo, a distinct capillary network, and a variable number of calcifications (Fig. 1A). Certain tumors contained microgemistocytes. Subpopulations of GFAP-positive tumor cells were present in 14 of 20 specimens. Most tumors samples were synaptophysin negative, however, synaptophysin-like expression was observed in rare neoplastic cells in 2 of 20 cases. The diagnosis of anaplastic oligodendroglioma (WHO grade III) was based on the presence of nuclear atypia, mitotic cells (Fig. 1B), hypertrophy of tumor blood vessels, and foci of tumor necrosis (criteria reviewed in Ref. 5). None of the tumors showed evidence for the presence of myelin, as examined by luxol fast blue staining (Fig. 1C); however, the neoplastic cells retain the cytoplasmic expression of early neuroecto-
dermal markers such as class III β-tubulin (Fig. 1D). Table 1 summarizes results of immunohistochemistry.

**Detection of JCPyV DNA Sequences in Tumor Samples.** Gene amplification of JCPyV DNA sequences in the tumor samples was performed by PCR using specific pairs of primers that recognize four regions of the viral genome. These include the control region, the NH2-terminal of large T-antigen, the gene encoding agnoprotein, and the region corresponding to the VP-1 capsid encoding sequence (Fig. 2). PCR amplification followed by Southern blot using JCPyV-specific DNA probe revealed the presence of viral DNA sequences corresponding to the T-antigen coding region in 13 of 18 (72.2%) oligodendrogliomas that were analyzed. The 2 anaplastic oligodendrogliomas were both positive for the presence of JCPyV early gene sequences. The viral DNA sequence corresponding to the capsid coding region was found in 12 of 18 (66.6%) oligodendrogliomas and in both of the anaplastic oligodendrogliomas. Evaluation of the tumor samples for the presence of DNA from the viral auxiliary gene, which is located in the late region of the JCPyV genome, revealed its detection in 10 of the oligodendrogliomas (55.5%) and in the 2 available anaplastic oligodendrogliomas. To determine the presence of the JCPyV control region, we used a pair of primers that are derived from the region flanking the 98-bp enhancer promoter of the viral genome. Results from the PCR amplification showed detection of the viral control sequence in 7 of 18 oligodendrogliomas that were examined. Surprisingly, none of the anaplastic oligodendrogliomas were positive for the presence of the JCPyV control region. Fig. 2 illustrates the representative Southern blot for the detection of the various regions of the JCPyV genome.

**Immunohistochemical Localization of T-Antigen and Late Viral Protein Agno in Neoplastic Cells.** The presence of viral protein T-antigen was observed in the nuclei of 8 of 18 oligodendrogliomas (44.4%) and in both cases of anaplastic oligodendroglioma (Fig. 4A, see also Table 1). Interestingly, not
all of the samples that possessed the sequence corresponding to JCPyV early genome showed expression of T-antigen. As indicated in Table 1, only 6 from the 13 cases that were positive for the NH2-terminal of T-antigen were immunoreactive to anti-T-antigen antibody, suggesting that either expression of T-antigen is extinguished in the neoplastic cells, or mutations in the other regions of the viral early genome affected the production of this protein in the tumor cells. Alternatively, this may be attributable to the potentially weak cross-reactivity of the SV40 antibody for JCPyV T-antigen. Because T-antigen by association with p53 increases the stability of p53 and causes the inactivation of this tumor suppressor, in parallel, we examined the level of p53 gene expression by immunohistochemistry. As shown in Fig. 4B and summarized in Table 1, 9 of 18 oligodendrogliomas and both anaplastic tumors showed nuclear immunopositivity for p53. Only 6 of 9 p53-positive samples showed expression of T-antigen, suggesting that an alternative pathway such as mutations in the p53 gene may contribute to the stability of p53 in the absence of T-antigen. Also, we noted that only 4 samples that contained JCPyV early DNA sequences and exhibited T-antigen expression contained intact viral control sequences of either Mad-4 (3 samples) or JCPyV CY (1 sample; see Table 1). Thus, it is possible that expression of the early genome in those samples that were negative for the control region sequence is mediated by the adjacent cellular promoter, where the viral genome is positioned. We found no evidence for the presence of other polyomaviruses control sequences, including SV40 and BK in the tested samples (data not shown).

Examination for the expression of viral capsid proteins by immunohistochemistry indicated no evidence of the expression of the capsid proteins, including VP-1 (data not shown). This observation verifies that JCPyV is not at the lytic infection cycle, and there is no productive viral infection in the tumor cells. Examination of the viral late auxiliary protein agnoprotein showed the presence of this protein with a granular pattern in the cytoplasm of the neoplastic cells (Fig. 4, C and D). Agnoprotein was found in 10 of 18 oligodendrogliomas (55.5%) and in 1 of 2 anaplastic oligodendrogliomas (see Table 1). We also noted that 3 samples that contained sequences corresponding to the agno gene were negative for the expression of the agnoprotein by immunohistochemistry.

Discussion
Because JCPyV is widely spread among the human population worldwide (23, 46) and can enter oligodendrocytes and
efficiently express its genome, our observations, along with several other reports linking JCPyV with various human brain tumors, are of particular interest, pointing to a potential role for JCPyV as a primary inducer or cofactor in the development of CNS neoplasia. More direct evidence of the etiologic role of JCPyV in the genesis of brain tumors stems from several experimental animals where either a virus particle or a segment of the viral genome was introduced into the animal by infection or transgenic approaches (for review see Ref. 23). The transgenic animals verify to the ability of the viral early protein, T-antigen, to induce a cascade of events that causes uncontrolled proliferation of various cells of neural origin. For example, the use of the JCPyV CY early genome spanning the viral control sequence and T-antigen as a transgene led to the development of an animal model for primitive neuroectodermal tumors (47). This observation led us to hypothesize that activation of the viral gene expression under conditions that are not appropriate for the replication of the virus can lead to deregulated proliferation of the host and the development of tumors. Several mechanisms can participate in the tumorigenicity of T-antigen. Earlier studies indicated the ability of JCPyV to associate with p53 and the Rb family of tumor suppressor proteins (47). Results from a series of in vivo studies indicated the ability of pRB2/p130 to interact with JCPyV T-antigen and hamper the ability of T-antigen to induce tumor formation in nude mice (48). Examination of other pathways such as the Wnt signaling pathway revealed the potential participation of β-catenin and LEF-1 in the development of JCPyV-induced murine medulloblastoma (49). Although the mechanism for T-antigen tumorigenicity in oligodendrocytic cell lineage remains to be investigated, detection of p53 in tumor samples with a high level of T-antigen may point to the interaction of T-antigen with p53 in these tumor cells.

Fig. 4 Immunohistochemical detection and localization of viral proteins. The JCPyV early protein T-antigen is detected by immunohistochemistry in the nuclei of some neoplastic oligodendrocytes (A). The tumor suppressor protein p53 is localized in the nuclei of some but not all neoplastic oligodendrocytes (B). Immunohistochemistry against the accessory agnoprotein demonstrates intense reactivity in the cytoplasm of neoplastic cells (C). At a higher magnification, the cytoplasmic presence of agnoprotein is evident, but a small nuclear component is also observed (D). A–C, original magnification: ×40. D, original magnification: ×100.
Results from sequencing of the control region of two samples revealed the presence of JCPyV CY in the tumor cells. This is a novel finding that demonstrates, for the first time, the ability of JCPyVC Y in human brain tumors. JCPyVC Y has been repeatedly detected in the kidneys and urine of people with and without the PML (50, 51) leading us to believe that this type of JCPyV circulates in the population. It has been suggested that during viral replication, rearrangement within the archetype transcriptional control region yields the Mad configuration, which is a potentially more active form of the virus in lytic infection. Isolation of JCPyVC Y in the oligodendrocytes is of particular interest in light of the earlier results demonstrating the ability of JCPyVC Y to cause tumors in animal models. These results will encourage additional investigation of oligodendrogliomas and their association with JCPyV.

Acknowledgments

We thank the present and past members of the Center for Neurovirology and Cancer Biology for their support and sharing of ideas and reagents. We also thank Dr. Mahmut Safak for his technical support in designing the sequences used for agno peptide synthesis and antibody production, Dr. Jennifer Gordon for her technical support and insightful comments on PCR analysis, and Dr. Jennfian F. Geddes (Department of Histopathology and Morbid Anatomy, Queen Mary, University of London, United Kingdom) for her generous supply of oligodendrogliomas. We also thank Cynthia Schriver for editorial assistance and preparation of this manuscript.

References


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