Brain Pathology 9: 33-44(1999)

RESEARCH ARTICLE

Identification in Human Brain Tumors of DNA Sequences Specific for SV40 Large T Antigen

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Simian virus 40 (SV40) sequences have recently been identified in a variety of human neoplasms, including mesothelioma, osteosarcoma, and brain tumors, but significant discrepancies exist regarding the frequency at which this occurs. The SV40 genome is 70% homologous to JC and BK, two related polyomaviruses that are highly prevalent in humans and which may cause in immune-compromised patients progressive multifocal leukoencephalopathy (PML) and cystitis, respectively. We have established a specific and sensitive method to identify SV40 sequence in DNA extracted from histological sections, using PCR followed by Southern hybridization to probes specific to the large T region. We found SV40 large T antigen sequences in all brain tumor types investigated. High frequencies were found in low-grade astrocytomas, anaplastic astrocytomas and secondary glioblastomas derived thereof (13/22, 59%) while somewhat lower frequencies were found in gemistocytic astrocytomas (9/28, 32%) and oligodendrogliomas (3/12, 25%). Primary glioblastomas, giant cell glioblastomas, and gliosarcomas, which clinically develop de novo, contained SV40 sequences in 11-25% of cases. Presence of viral DNA was also observed in pediatric brain tumors, including ependymomas (9/16, 56%), choroid plexus papillomas (6/16, 38%), and medulloblastomas (5/17, 29%). In 8 tumor biopsies with SV40 sequences, the adjacent normal brain tissue was also analyzed but was devoid of viral DNA in all but one case. BK and JC virus sequences were rarely detected, the overall frequencies being 3% and 2%, respectively. It remains to be shown whether the presence of SV40 contributes significantly to malignant transformation or whether certain human neoplasms provide a microenvironment that favors viral replication in humans with latent SV40 infection.

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Introduction

Simian virus 40 (SV40) is a small double stranded DNA virus, and the SV40 large T antigen (Tag) is the primary viral gene product responsible for SV40 replication and SV40 mediated cell transformation (43). It binds to and inactivates the products of several tumor suppressor genes including p53 and pRB (16). The transforming activity of SV40 large T is also linked to its ability to induce the expression of the insulin-like growth factor 1 (42, 46). It further alters the integrity and karyotype stability of the host cell genome, inducing numerical and structural chromosomal aberrations (43, 51, 56). SV40 is highly oncogenic in hamsters and induces a variety of tumors, including ependymomas, choroid plexus papillomas, osteosarcomas, soft tissue sarcomas, mesotheliomas, and lymphomas, depending on the route of inoculation (35). Transgenic mice carrying SV40 large T antigen under transcriptional control of the viral enhancer develop choroid plexus papillomas (39), and retroviral mediated transfer of SV40 large T antigen into neural transplants in rats induced primitive neuroectodermal tumors histologically indistinguishable from human cerebellar medulloblastomas (15).

The natural host of SV40 is the Macaque monkey and infection of SV40 to humans does not usually occur unless there is close contact with infected monkeys or their tissues (47, 48, 53). SV40 was accidentally intro-

Oligonucleotide sequences	Positions	Tm annealing	Tm hybridization	PCR product
SV40				
SVTAGP1: 5'-TTAGCAATTCTGAAGGAAAGTCCTTG-3'	nt 4388-4413	53°C		156 bp
SVTAGP2: 5'-AGCAGTGGTGGAATGCCTTTCATGAGG-3'	nt 4517-4543			
or				
SVTAGP1: 5'-TTAGCAATTCTGAAGGAAAGTCCTTG-3'	nt 4388-4413	49°C		126 bp
SVTAGP3: 5'- ACCTGTTTTGCTCAGAAG-3'	nt 4496-4513			
SVPROBE: 5'- ATGTTGAGAGTCAGCAGTAGCC-3'	nt 4452-4473		52°C	
BK				
BKTAGP1: 5'-TTGACTAAGAAACTGGTGTAGATC	nt 2519-2542	50°C		187 bp
BKTAGP2: 5'-AGAGTGGGAGTCCTGGTGGAGTTCC-3'	nt 2681-2705			
BKPROBE: 5-'AATCTTCATCCCATTTTTCA-3'	nt 2656-2675		49°C	
JC				
JCTAGP1: 5'-TGAGGAATGCATGCAGATCTAC-3'	nt 4228-4249	51°C		248 bp
JCTAGP2: 5'-TTTGCAGGGCATTTTGTTTTTTAC-3'	nt 4452-4475			
JCPROBE: 5-'GTTGGGATCCTGTGTTTTCAT-3'	nt 4303-4323		52°C	

Table 1. Primers and probes used for detection of SV40, BK and JC large T antigen sequences.

duced into human populations between 1955 and 1963 through SV40-contaminated poliovaccine and adenovirus 3 and 7 vaccines which were prepared using monkey kidneys (8, 19, 40, 47). Millions of children and adults in the United States, Canada, and Europe were inoculated. In the USA alone, over 98 million people, or 62% of the population, had received one or more doses of the vaccine during the period when a proportion of the vaccine was contaminated with SV40 (47). Some children treated with SV40-contaminated oral poliovaccine excreted SV40 for several weeks (34), indicating that SV40 has the capacity to replicate in humans. SV40 can transform human cells (43) and cause chromosomal aberrations, aneuploidy, and point mutations in human cells *in vitro* (51).

Between 1970 and 1990, several laboratories reported the presence of sequences identical to SV40 (19, 24, 25, 33, 45, 60) and expression of SV40 T antigen (49, 52, 55, 63, 66) in human tumors, including brain tumors, but other studies showed negative results (10, 13, 20, 22). This discrepancy could be due to inadequate methods, *i.e.*, restriction enzyme analyses, DNA-DNA hybridization, Southern blotting, and immunohistochemistry, which are not sensitive enough to detect low levels of SV40 sequences and which are not specific enough to distinguish SV40 from other polyomaviruses (8). Using PCR followed by Southern hybridization with SV40 specific probe, Bergsagel *et al*. (4) in 1992 provided convincing evidence of the presence of SV40-like sequences in human brain tumors: 10 of 11 (91%) ependymomas and 10 of 20 (50%) choroid plexus papillomas contained SV40 T antigen sequences. Martini *et al.* (32), Lednicky *et al.* (29), and Suzuki *et al.* (54) further confirmed the presence of SV40 sequences in a variety of human brain tumors. However, other groups using similarly sensitive methods failed to detect SV40 sequences in human brain tumors (12, 23, 58). Conflicting results were also obtained regarding the presence of SV40 sequences in other human tumors, especially in mesotheliomas (6, 7, 40, 53). Some studies are difficult to interpret since the primers used to amplify SV40 sequences also amplify other polyomavirus sequences, *i.e.*, BK and JC viruses, which are highly homologous to each other and to SV40. Since JC and BK viruses are ubiquitously distributed throughout the human population with about 80% of adults having antibodies against these viruses (1, 14, 18), it is important to establish a method that unequivocally distinguishes SV40 DNA from that of JC and BK viruses.

To improve the specificity and sensitivity of the method, we used specific primers to amplify SV40, BK, and JC large T antigen sequences separately, and PCR products were subsequently hybridized with virus-specific probes. Using this method, we screened a total of 199 human brain tumors with different histogenesis and clinical behavior. The results clearly show that sequences identical to SV40 large T are frequently present, while BK and JC DNA is rarely found in human brain tumors.

Materials and Methods

Tumor samples. DNA was extracted from formalin fixed, paraffin embedded brain tumor specimens as previously described (62). Biopsies included 28 primary (*de novo*) glioblastomas (WHO Grade IV, 16 males and

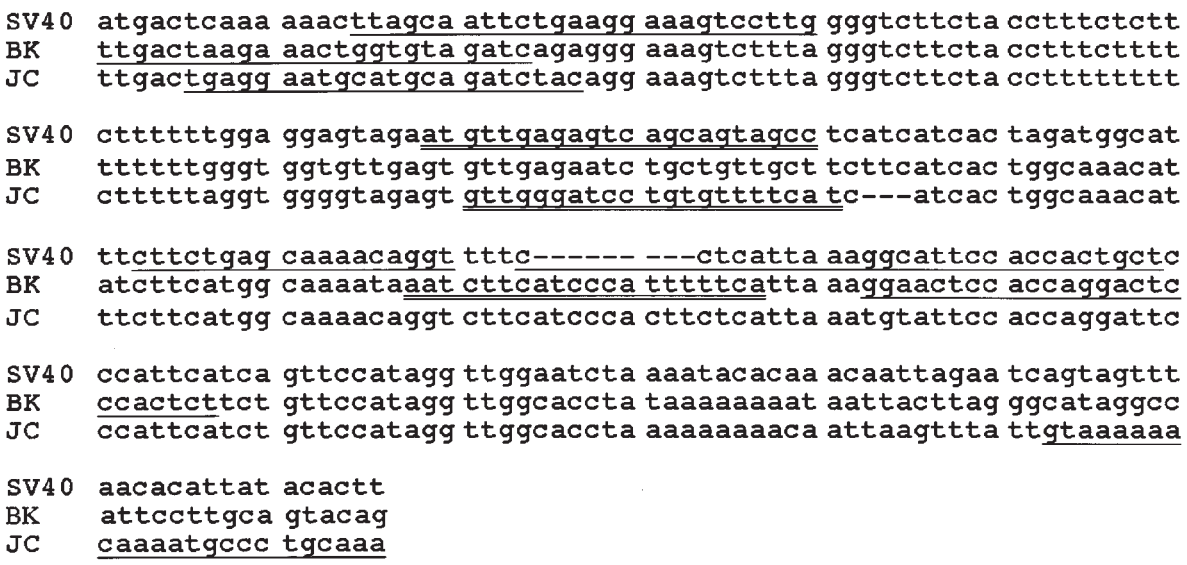

Figure 1. Nucleotide sequences of primers (underlined) and probes (double underlined) used for the detection of SV40, BK and JC virus T antigen. The regions shown are from nucleotide 4374-4620 for SV40, from 2519-2774 for BK (MM strain) and from 4223-4475 for JC viruses.

12 females; mean age 58.1 years, range 34-73 years), 22 secondary glioblastomas (WHO Grade IV) which developed through progression from low-grade (16 cases, 8 males and 8 females; mean age 35.1 years, range 23-52 years) or anaplastic astrocytomas (6 cases, 2 males and 4 females; mean age 33.5 years, range 23-45 years), 18 giant cell glioblastomas (WHO Grade IV, 11 males and 7 females; mean age 40.2 years, range 9-58 years), 20 gliosarcomas (WHO Grade IV, 12 males and 8 females; mean age 55.9 years, range 32-76 years), 17 gemistocytic astrocytomas (WHO Grade II, 10 males and 7 females; mean age 38.8 years, range 8-62 years), 11 anaplastic gemistocytic astrocytomas (WHO Grade III, 9 males and 2 females; mean age 38.7 years, range 23-67 years), 16 choroid plexus papillomas (10 males and 6 females, 14 choroid plexus papillomas, WHO Grade I, and 2 anaplastic choroid plexus papillomas, WHO Grade II; mean age 14.4 years, range 1-65 years), 16 ependymal tumors (7 males and 9 females; 8 anaplastic ependymomas, WHO Grade III, 7 ependymomas, WHO Grade II, and 1 myxopapillary ependymoma, WHO Grade I; mean age 24.8 years, range 1-62 years), 17 medulloblastomas (WHO Grade IV, 13 males and 4 females; mean age 20.4 years, range 1-60 years), and 12 oligodendrogliomas (WHO Grade II, 8 males and 4 females; mean age 43.0 years, range 2-70 years). For secondary glioblastomas, their less malignant precursor lesions, i.e., 16 low-grade astrocytomas (WHO Grade II) and 6 anaplastic astrocytomas (WHO Grade III) were also analyzed. Choroid plexus papillomas, ependymomas, and primary and secondary glioblastomas were obtained from patients treated in the Department of Neurosurgery, University Hospital Zürich, Switzerland. Clinical data and source of histologic sections for gemistocytic astrocytomas and giant cell glioblastomas were reported previously (41, 61). Gliosarcomas were obtained from the Department of Neurosurgery, University Hospital Zürich, Switzerland (11 patients), the Institute of Pathology and Molecular Immunology, University of Porto, Portugal (7 patients), the Neuropathology Department, The Radcliffe Infirmary, Oxford, UK (one patient), and the Pathology Department, University of Ribeirao Preto, Sao Paulo, Brazil (one patient).

PCR Primers for SV40, BK, and JC large T antigen sequence amplification. SV40, BK and JC viruses show extensive nucleotide sequence homology. The nucleotide sequences of the large T antigen region of the three viruses were examined and primer pairs specific for each virus were designed using the computer program Oligo-4.0 (National Bioscience Inc, Plymouth, MN, USA), to give as many mismatches as possible among the three viruses. The sequence comparison of the targeted regions of the three viruses and the location of primers and probes are shown in Fig. 1. PCR primer and probe sequences, PCR annealing temperatures, size

	No. of tumors analyzed	No. of positive cases SV40	BK	JC
Astrocytic brain tumors				
Secondary glioblastoma	22	13 (59%)	0	0
Low-grade or anaplastic astrocytoma	22	13 (59%)	1 (5%)	0
Gemistocytic astrocytoma	28	9 (32%)	0	0
Primary (de novo) glioblastoma	28	7 (25%)	1 (4%)	0
Gliosarcoma	20	4 (20%)	1 (5%)	1 (5%)
Giant cell glioblastoma	18	2 (11%)	0	0
Non-astrocytic brain tumors				
Ependymoma	16	9 (56%)	0	0
Choroid plexus papilloma	16	6 (38%)	0	1 (6%)
Medulloblastoma	17	5 (29%)	1 (6%)	0
Oligodendroglioma	12	3 (25%)	2 (17%)	2 (17%)

Table 2. SV40, BK and JC large T antigen sequences in human brain tumors.

of PCR products, and hybridization temperatures are summarized in Table 1.

PCR-Southern hybridization. PCR was performed in a total volume of 10 μl, consisting of 1.5 μl of DNA template, 0.5 U of *Taq* DNA polymerase (Sigma, St Louis, MO, USA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of both sense and antisense primers, 10 mM Tris-HCl, pH 8.3, and 50 mM KCl in the RoboCycler Gradient 96 (Stratagene, La Jolla, CA, USA). PCR was carried out at 95°C for 5 min, then 45 cycles of denaturation at 95°C for 1 min, annealing at 53°C (for SV40, primer pair SVTAGP1-SVTAGP2), 49°C (for SV40, primer pair SVTAGP1-SVTAGP3), 50°C (for BK) or 51°C (for JC) for 1 min, and polymerization at 72°C for 1 min, followed by a final extension of 5 min at 72°C. PCR was performed 2-5 times for all the samples. Only those samples giving a clear hybridization signal in at least two independent PCR-Southern hybridizations were considered positive. The following precautions were taken to prevent a possible contamination: (i) tissue was scrapted from paraffin sections using a new blade for each sample; (ii) all plastic and glass ware was autoclaved; (iii) plastic tips containing cotton wool in the upper part were used for pipetting; (iv) PCR setup and post-PCR work were carried out in separated rooms or different work areas in the laboratory; (v) a negative (no template) and a positive (plasmid) control were included in every PCR amplification.

Five μl of the PCR product were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. After transfer to a nylon membrane (GeneScreen Plus, Dupont NEN, Boston, MA, USA), the PCR product was hybridized overnight at 52°C (for SV40 and JC) or 49°C (for BK) with an oligonucleotide probe (Figure 1, Table 1) labeled with a DIG Oligonucleotide 3'-End Labeling Kit, according to the manufacturer's instructions (Boehringer Mannheim, Germany). The membrane was subsequently washed in 2xSSC, 0.1% SDS and 0.1xSSC, 0.1% SDS, twice each for 5 min at the hybridization temperatures. The DIG-labeled DNA was then detected using a DIG Luminescence Detection Kit with CSPD as a substrate (Boehringer Mannheim, Germany).

Restriction enzyme digestion and sequencing of the PCR products. To confirm the specificity of PCR products amplified by SV40 primers, PCR products were purified with a PCR purification kit or gel extraction kit (QIAGEN Inc, Hilden, Germany). The purified DNA was digested with the restriction enzyme *Sty* I at 37°C for 2 h and separated on a 2% agarose gel to check the fragments. DNA sequencing was also carried out using a Sequenase PCR Product Sequencing Kit (USB, Cleveland, OH, USA) on PCR products amplified by primers for SV40, BK and JC.

Results

Specificity of PCR primers and oligonucleotide probes. To confirm the specificity of this method, plasmids containing SV40 sequence (P11-4cG) (31), BK sequence (pBK-Dik) (28) and JC sequence (pJC-MAD-1) (28) were subjected to PCR amplification using various pairs of primers. Only the corresponding plasmid-primer pair produced PCR products with the expected size (Fig. 2). After transfer, PCR products were further hybridized and rehybridized to different probes. The SV40 and JC probes recognized only PCR products derived from SV40 and JC plasmids, respectively, while the BK probe bound to PCR products from BK plasmid and, to a very small extent, to those from the JC plasmid (Fig. 2).

Sensitivity of the PCR-Southern hybridization system. To assess the sensitivity of this method, a series of

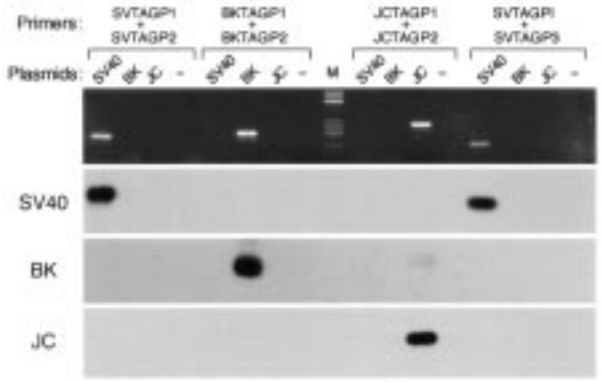

Figure 2. Specificity of PCR primers and oligonucleotide probes. SV40, BK and JC plasmids were subjected to PCR amplification. Only the corresponding plasmid-primer pair produced PCR products with the expected size (dark panel), demonstrating a high primer specificity. PCR products were hybridized and rehybridized to different probes (light panels). The SV40 and JC probes recognized only PCR products derived from SV40 and JC plasmids, respectively. The BK probe bound to PCR products from BK plasmid and, to a much lesser extent, to JC plasmid. M, DNA marker; -, negative control (no template DNA).

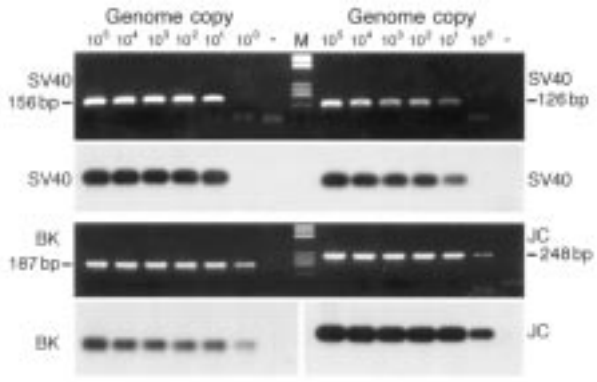

Figure 3. Sensitivity of the PCR-Southern hybridization system. Various amounts of plasmids containing 10^5 to one genome copies of the viral DNA were amplified and separated on a 2% agarose gel and stained with ethidium bromide (dark panel). PCR products were subsequently transferred to a nylon membrane and hybridized to different virus probes (light panel). The detection limit of this system after 45 cycles of PCR amplification is 1-10 genome copies for SV40 and one genome copy for BK and JC. M, DNA marker; -, negative control (no template DNA).

dilutions of the control plasmids containing 10^5 to one genome copies of the virus DNAs were used in a 10 μl PCR reaction. Of the PCR product, 5 μl were separated on a 2% agarose gel and stained with ethidium bromide. PCR products were subsequently transferred to a nylon membrane and hybridized to different viral probes. After 45 cycles of PCR, the detection limit of this system was 1-10 genome copies for SV40 and one genome copy for BK and JC (Fig. 3).

Detection of SV40, BK, and JC T antigen sequences in human brain tumors. Single or multiple bands have been observed on the agarose gels after PCR amplification of SV40. After Southern hybridization, only one band with the predicted size was obtained (Fig. 4A). SV40 large T antigen sequences were detected in all types of brain tumors analyzed, although at markedly different prevalence (Table 2). For secondary glioblastomas, the SV40 status was identical to that in the corresponding less malignant lesions (low grade or anaplastic astrocytoma). In 8 tumor biopsies containing SV40 sequences (1 primary glioblastoma, 3 low-grade astrocytomas, 1 secondary glioblastoma, 1 gemistocytic astrocytoma, 2 gliosarcomas), we analyzed adjacent normal brain tissues on the same histologic sections. Except for one case (a gliosarcoma), the normal brain tissue did not contain SV40 (Fig. 4B).

BK and JC sequences were only occasionally identified, the overall frequency being 3% and 2%, respectively (Table 2).

Restriction enzyme digestion and DNA sequencing to confirm PCR product specificity. To confirm the specificity of PCR products, PCR products from two samples (one secondary glioblastoma and one low-grade astrocytoma) amplified with SV40 primers (SVTAGP1-SVTAGP2), were purified and subjected to digestion by *Sty* I (restriction site only present in SV40). Two fragments with the predicted sizes (135 bp and 21 bp) were produced in both cases (data not shown).

Furthermore, direct DNA sequencing was performed on PCR products from some tumor samples, including one primary glioblastoma, 4 secondary glioblastomas, 2 low-grade astrocytomas, one gemistocytic astrocytoma, one giant cell glioblastoma, 3 choroid plexus papillomas and one ependymoma positive for SV40 sequence. All sequences obtained were identical to the SV40 wild-type Tag sequence (Fig. 5). One medulloblastoma and one oligodendroglioma positive for BK and one choroid plexus papilloma positive for JC were also sequenced. The sequences obtained were identical to the published wild-type BK and JC sequences respectively.

Relationship between the presence of SV40 Tag and other genetic alterations. We correlated the presence of

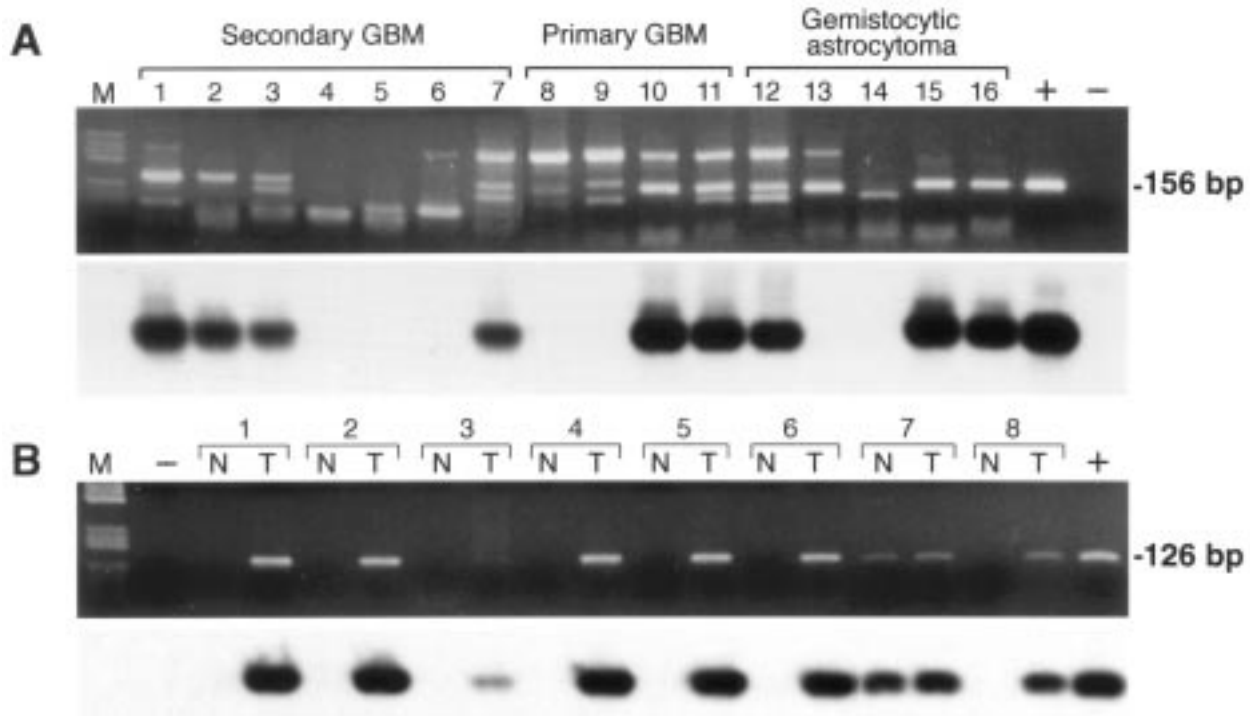

Figure 4. PCR-Southern hybridization screening of human brain tumors for the presence of SV40 T antigen sequence. Single or multiple PCR bands were observed on the agarose gels (dark panels). After Southern hybridization, only one band with the predicted size appears on the blot (light panels). (A) Detection of SV40 T antigen sequence from various tumors. Lanes 1-7 represent DNAs from secondary glioblastomas, lanes 8-11 from primary glioblastomas, lanes 12-14 from gemistocytic astrocytomas and lanes 15-16 from anaplastic gemistocytic astrocytomas. Lanes 1, 2, 3, 7, 10, 11, 12, 15 and 16 are positive for SV40. (B) Detection of SV40 T antigen sequence in normal brain (N) and brain tumor (T) tissue from the same histological section. DNA was isolated from a primary glioblastoma (lane 1), low grade astrocytomas (lanes 2-4), secondary glioblastoma (lane 5), gemistocytic astrocytoma (lane 6) and gliosarcomas (lanes 7, 8). Only tumor tissue, and not normal brain tissue contains SV40 sequences, except for one gliosarcoma (lane 7). M, DNA marker; +, SV40 plasmid; -, negative control (no template DNA).

SV40 Tag sequences with genetic alterations previously reported in this series of brain tumors, *i.e.*, primary and secondary glioblastomas (57, 62), giant cell glioblastomas (41), and gemistocytic astrocytomas (61). SV40-positive and SV40-negative primary glioblastomas, giant cell glioblastomas, and gemistocytic astrocytomas showed a similar frequency of *p53* mutations, *PTEN* mutations and *EGFR* amplification. In secondary glioblastomas, there was a tendency toward higher frequency of *p53* mutation and p53 protein accumulation in SV40-positive cases (85%) than in SV40-negative cases (44%), but the difference was not statistically significant (P=0.074).

Relationship between the presence of SV40 Tag sequences and the age of patients. To investigate whether patients who may have received the SV40-contaminated vaccines between 1955-1963 scored positive for SV40 more frequently, we divided the samples into two groups: one from patients born before 1923 (15 patients) or after 1965 (21 patients) and the other from 92 patients born between 1923 and 1965 (32). The frequency of SV40 sequences was similar in both groups (12/36, 33% *vs.* 34/ 92, 37%; P=0.8381).

Discussion

The etiology of sporadic brain tumors is still largely unknown. Epidemiological studies failed to detect an unequivocal causative link with environmental and lifestyle factors, with the exception of therapeutic irradiation (27). Several viral oncogenes have the capacity to induce tumors of the central nervous system in experimental animals, including v-*src* large T, polyoma middle T, and SV40 large T antigen (15, 21, 39). Among these, SV40 is the only virus that has been repeatedly implicated in the etiology of human brain tumors (3, 5, 8, 60), but an unequivocal pathogenic role has not yet been established. Previous studies vary considerably in the reported frequency of SV40 DNA sequences in CNS

neoplasms and this may be at least in part due to the use of methods that cannot clearly distinguish SV40 from related DNA viruses.

Using a highly sensitive and specific PCR-Southern hybridization method to identify SV40, BK, and JC viruses (Figs. 2 and 3), this study provides evidence that sequences identical to SV40 large T antigen are present in a variety of human brain tumors. Consistent with studies by Bergsagel *et al.* (4) and Lednicky *et al.* (29), SV40 sequences were detected in embryonal (medulloblastoma), pediatric (choroid plexus papillomas, ependymomas) and diffuse astrocytomas of adults, including glioblastomas and oligodendrogliomas (Table 2). Among astrocytic brain tumors, secondary glioblastomas which progressed from low-grade or anaplastic astrocytomas were most frequently infected and their SV40 status was similar to that of the corresponding less malignant precursor lesion. This suggests that SV40 DNA infection occurred during early stages of tumor development rather than being associated with glioma progression.

In this study, primary glioblastomas, gliosarcomas, and giant cell glioblastomas, which all develop *de novo* with a short clinical history, showed a lower frequency of SV40 sequences than gliomas with a longer clinical history. This could suggest that tumor tissues simply attract SV40 already present in the body and that tumors that develop slowly have a greater chance of being infected. This view is supported by an experimental study which shows that 7 out of 7 brain tumors induced transplacentally in rats by *N*-nitrosoethylurea, and infected by SV40 after birth, contained SV40 sequences, while only 3 of these contained SV40 DNA in the normal brain tissue. This suggests that brain tumors provide a microenvironment favoring infection with and replication of SV40 (19). This hypothesis is also supported by the present and earlier studies (32, 54), which show that SV40 sequences are rarely found in the adjacent normal brain structures (Fig. 4B).

We screened only a small region of the large T antigen and therefore the results do not support the conclusion that copies of the entire SV40 genome are present in the positive cases, though some reports suggest that this is indeed the case (29, 45, 50). Lednicky *et al.* (29) screened choroid plexus and ependymal tumors for three different regions of SV40 and found that they were present in each case. Furthermore, an infectious SV40 wild-type strain was rescued by transfection of DNA from a human choroid plexus carcinoma into permissive monkey kidney cells (29). Other studies with osteosarcomas (30) and papillary thyroid carcinomas (38) also

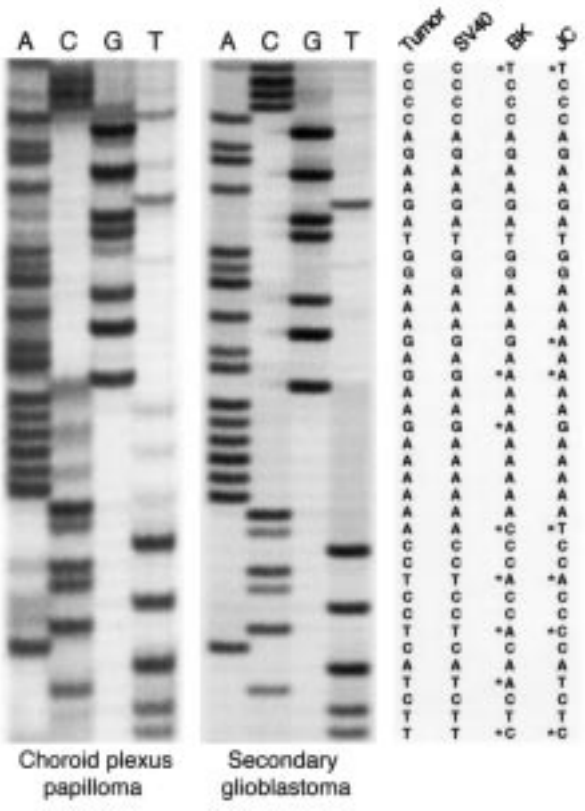

Figure 5. DNA sequencing of PCR products from a choroid plexus papilloma (left) and a secondary glioblastoma (right) positive for SV40 sequence. The nucleotide sequences from both samples are identical to SV40 wild type sequence in this region. For comparison, sequences of the same region for BK and JC are shown on the right. Asterisks indicate bases different from those of SV40.

suggest the presence of the whole SV40 genome in these tumors.

This study revealed a tendency for SV40-positive glioblastomas to contain a *p53* mutation and p53 protein accumulation at higher frequencies than SV40-negative glioblastomas, but the difference was not statistically significant (P=0.074). Carbone *et al.* (9) showed a significant association between Li-Fraumeni patients with osteosarcomas and the presence of SV40 sequence (31%), compared to the presence of SV40 sequence in Li-Fraumeni patients who did not develop osteosarcomas (6%). Family members with this syndrome contain one functional *p53* allele and are prone to develop a variety of tumors if the remaining *p53* allele becomes inactivated. This raises the possibility that the presence of SV40 large T antigen, which binds and inactivates p53 protein, is particularly harmful in family members carrying a *p53* germline mutation or in tumors carrying

a *p53* mutation and one wild-type allele.

There is evidence that SV40 is capable of replicating in humans. Adult volunteers who were intranasally infected with SV40-contaminated respiratory syncytial virus stocks developed a subclinical SV40 infection (36). Children who received an oral dose of contaminated poliovaccine excreted SV40 in their stools for at least 5 weeks following vaccination (34). Furthermore, Martini *et al.* (32) reported the presence of SV40 sequences in peripheral blood cells and sperm fluid. These findings suggest that SV40 may spread in the human population through different routes. In line with other studies (17, 32), we found that the frequency of SV40 sequences was similar in tumors from patients with and without possible exposure to SV40-contaminated poliovaccines. Similarly, SV40-positive osteosarcomas (9), choroid plexus papillomas and ependymomas (4) were identified in children who definitively did not receive SV40-contaminated poliovaccines. These results suggest that SV40, iatrogenically introduced on a large scale four decades ago, is now commonly present in human populations.

BK and JC, human polyomaviruses related to SV40, also are known to induce brain tumors in experimental animals. Following intracerebral injection in newborn hamsters (59), JC viruses cause brain tumors, mostly cerebellar medulloblastomas. After intracranial inoculation of newborn rats with JC virus Tokyo-1, a strain isolated from the brain of a patient with progressive multifocal leukoencephalopathy (PML), more than 70% of animals developed forebrain tumors (37). Transgenic mice expressing JC virus T antigen under the control of an SV40 regulatory region exhibited extensive pathology, including B-cell lymphoma, osteosarcoma, and choroid plexus papilloma (44). Transgenic mice carrying the early region of JCV.CY, which has been repeatedly isolated from the urine of PML and non-PML individuals, developed medulloblastomas at 9-13 months of age (26). Depending on the route of administration, BK virus is capable of inducing ependymomas, pancreatic islet tumors, and osteosarcomas in hamsters (3).

Similar to the identification of SV40 sequences in human tumors, there have also been conflicting results regarding the presence of BK sequences. Recently, a BK T antigen sequence was reported to be present in glioblastomas (93% of cases), astrocytomas (94%) and all choroid plexus papillomas investigated (11, 32), but other studies showed completely negative results (2, 65). Our data (Table 2) clearly indicate that BK and JC viral sequences are rarely present in human brain tumors. The detection limit of our system is one genome copy for BK and JC plasmids, which is more sensitive than those previously used for the identification of BK and JC viral DNAs (100-1000 copies) (2, 64). Although the sensitivity may be lower when using DNAs extracted from paraffin sections, we should have been able to detect BK and JC in the vast majority of cases if the genome copy numbers of SV40, BK or JC were similar in our biopsies.

In summary, this study shows that sequences identical to SV40 large T antigen are commonly present in human brain tumors and that BK and JC viruses are rarely present. It remains to be shown whether the presence of SV40 contributes significantly to the process of malignant transformation or whether this constitutes a bystander infection due to an intra-tumoral microenvironment that favors viral replication in humans with latent SV40 infection.

Acknowledgments

This work was supported by a grant from the Foundation for Promotion of Cancer Research, Japan. We thank Dr. J.S. Butel, Baylor College of Medicine, Division of Virology, Texas Medical Centre, Houston, Texas, U.S.A., for providing the plasmids pBK-Dik and pJC-MAD-1. Plasmid P11-4cG was obtained from the laboratory of Dr. A. Levine, Department of Molecular Biology, Princeton University, Princeton, New Jersey, U.S.A. We are grateful to Dr. Leila Chimelli, Pathology Department, University of Ribeirao Preto, Sao Paulo, Brazil and Dr. M. V. Squier, Neuropathology Department, The Radcliffe Infirmary, Oxford, UK, for gliosarcoma samples.

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EDITORIAL

Is SV40 a tumorigenic human pathogen?

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Has SV40 crossed the species barrier from the macaque to become a tumorigenic human pathogen? SV40, a close simian relative of the human polyomaviruses JC and BK, was introduced into a very large segment of the human population in the USA and Europe through inadvertent contamination of polio vaccines grown in monkey kidney cells in the late 1950s (16). However, direct evidence that this infection is being perpetuated in the human population has not been forthcoming. While excretion of JCV and BKV is common (1, 13, 21), studies of urine for the presence SV40 have been unrewarding (17), and circulation of SV40 in the human population, if it occurs, has not yet been convincingly demonstrated. Evidence for seropositivity to SV40 obtained in some early studies may have been due to unrecognized crossreactions between antibodies to BKV or JCV capsid antigens and the homologous epitopes in SV40.

Nevertheless, the work of Huang et al. (5) suggests that SV40 may now be endemic in the human population, and that the viral T antigen may be at least a co-factor in the induction of a wide variety of astrocytic and non-astrocytic brain tumors. The SV40 DNA early region encoding the viral oncoprotein, large T antigen, was found in 11-59% of tumors (36% overall). DNA was extracted from 199 formalin-fixed, paraffin-embedded brain tumor specimens. This work supports and extends PCR findings from other research groups (2, 4, 9). While the authors are properly cautious in not claiming an etiologic role for SV40 at this time, the results clearly imply that this widely studied virus of high oncogenic potential in animal models is more than a mere brain tumor passenger. If the polyomaviruses are involved in the etiology of some human brain tumors, experience from animal models suggests that it may more likely be a heterologous virus such as SV40, rather than one of the viral species for which humans are the natural hosts. By a similar logic, it is reasonable that the human pathogen JCV causes experimental brain tumors in owl and squirrel monkeys (11, 12). Significantly, JCV and BKV DNAs were only rarely detected in human brain tumors (2% and 3%, respectively) by Huang et al. (5).

In 1971 JC virus was proposed as the etiologic agent of the fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML) (15), A report the following year that SV40 occurred in two PML brains in the USA (23) has never been confirmed by other isolations in this country, and a case reported in Germany of PML due to SV40 was recently demonstrated by in situ and PCR methods to be associated with JCV infection (20). The methods available 30 years ago consisted primarily of viral culture. The pitfalls posed by endogenous viruses such as SV40 harbored in primary cultures of monkey kidney cells were known at that time (3), and must still be emphasized. In our own experience during recent studies of BKV infection in the kidney of an AIDS patient with endstage renal disease (18), SV40 was first obtained upon culture of the kidney tissue extract in primary rhesus monkey kidney (RMK) cells (unpublished data). However, in the inoculum prepared directly from the renal biopsy, BKV, rather than SV40, was detected by PCR.

The PCR methods available today, capable of amplifying an agent directly from tissue without culture or cloning, are extremely powerful. For this reason they present real dangers, again especially for detection of SV40. The SV40 promoter and associated DNA sequences are widely employed in the plasmid vectors used as molecular biology reagents (22). Other potential sources of contamination in the laboratory may be less obvious. Several years ago, false positives for SV40 large T antigen were turning up in our PCR controls which were eventually traced to contamination in the dNTPs provided by a prominent molecular biology supplier. When this contamination was pointed out to the company, the problem was promptly acknowledged and corrected. Clearly, rigorously controlled conditions are crucial, as appears to be the case in these studies.

How can results like the present ones be verified? The state and extent of the SV40 genomes present in brain tumors should be clarified. There is already evidence to suggest that SV40 strains obtained from choroid plexus papillomas and ependymomas have a wild-type regulatory region configuration (“archetype”) rather than the 72-bp enhancer repeats found in the widely used laboratory strain 776 (9). Coding region variation should be defined also. In the case of BKV several genotypes have been identified which provide the basis for serological distinctions (6). For JCV only a single serotype has been identified, but genotypes assignable to geographic regions (Europe, Asia, Africa) have been recognized (1). As many as seven JCV genotypes and additional subtypes exist (7). It is possible that

natural variation in the SV40 genome (8, 14, 19) could be used to validate findings such as those reported here by Huang et al. (5), as has been done for osteosarcomas (10). If the ultimate source of some of these SV40-related DNA sequences was indeed batches of contaminated polio vaccine administered in the late 1950s, then it may be possible to use distinctive single nucleotide polymorphisms to tie the observed SV40 sequences in individual brain tumors to their respective antecedents in particular vaccine lots (if still available for testing).

Has an agent linked to more than 1/3 of all human brain tumors been found? SV40 infection and its consequences in humans remain enigmatic, and continued rigorous examination of this possibility is needed.

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