Medulloblastomas and primitive neuroectodermal tumors rarely contain polyomavirus DNA sequences[1]

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To address the hypothesis that medulloblastoma or supratentorial primitive neuroectodermal tumor (sPNET) can arise through infection by polyomaviruses, we examined genomic DNA isolated from 15 primary medulloblastoma and 5 sPNET biopsy specimens and from 2 medulloblastoma cell lines for the presence of DNA sequences from the polyomaviruses simian virus 40 (SV40), JC virus, and BK virus. These polyomaviruses have oncogenic potential in animals, and their DNA sequences have been detected in other surveys of various solid tumors, including childhood brain tumors. The tumor DNA samples were analyzed by Southern blot hybridization of polymerase chain reaction products that employed probes designed to detect specific polyomavirus sequences. Neither JC virus nor BK virus DNA sequences were detected in any of the specimens. None of the primary medulloblastoma or sPNET specimens contained SV40 sequences. However, SV40 DNA coding and noncoding sequences were detected in the D283-Med (medulloblastoma) cell line. Immunocytochemical studies of D283-Med revealed nuclear expression of SV40 large T antigen. In contrast to childhood ependymomas and choroid plexus tumors, medulloblastomas and sPNETs infrequently express evidence of polyomavirus infection. *Neuro-Oncology 4, 165–170, 2002 (Posted to Neuro-Oncology [serial online], Doc. 01-060, May 1, 2002. URL <neuro-oncology.mc.duke.edu>)*

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[3]Abbreviations used are as follows: BKV, BK virus; JCV, JC virus; PBS, phosphate-buffered saline; PCS, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate; sPNET, supratentorial primitive neuroectodermal tumor; SV40, simian vacuolating virus 40; T-ag, large T antigen.

SV40[3] is a double-stranded DNA virus in the polyomavirus subfamily of *Papovaviridae* that was first discovered as a contaminant of live polio vaccines prepared from rhesus monkey kidney cell cultures (Eckhart, 1991; Sweet and Hilleman, 1960). The tumorigenicity of SV40 has raised public health concerns, especially for recipients of contaminated vaccine (Farwell et al., 1979, 1984; Fraumeni et al., 1963, 1970; Heinonen et al., 1973; Innis, 1968; Mortimer et al., 1981).

Outside its natural simian host range, SV40 can transform a variety of nonhost mammalian cell types that are not permissive for viral replication. Neoplastic transformation is mediated by the SV40 T-ag, a 708–amino-acid protein with several functional domains involved in viral replication.

SV40 T-ag exerts its proliferative effects through its interactions with Rb and P53 and their regulation of cell cycle in a variety of cell lineages (Brinster et al., 1984; DeCaprio et al., 1988; Ludlow et al., 1989, 1990; Saenz Robles et al., 1994; Symonds et al., 1994; Van Dyke

et al., 1987). SV40 is highly oncogenic in hamster cells, inducing ependymomas and choroid plexus tumors as well as osteosarcomas, mesotheliomas, sarcomas, and several types of lymphomas (Cicala et al., 1992; Diamandopoulous, 1972; Dixon et al., 1982; Kirschstein and Gerber, 1962; Lewis and Martin, 1979; London et al., 1978; Nagashima et al., 1984; Padgett et al., 1977; Uchida et al., 1976).

JCV and BKV are polyomaviruses that are closely related to SV40 and can also act as human pathogens, causing progressive multifocal leukoencephalopathy and interstitial nephritis in immunosuppressed hosts, respectively. Like SV40, JCV and BKV express their own large T antigens, but their roles in tumorigenesis are less well established. Although SV40-induced brain tumors in animals are generally of glial or ependymal origin, certain JCV strains induce medulloblastoma-like tumors in greater than 90% of infected Syrian hamsters (Nagashima et al., 1984; Padgett et al., 1977).

For human cancers, SV40 DNA sequences can be detected in most human osteosarcomas and mesotheliomas (Carbone et al., 1994, 1996; Lednicky et al., 1995) and a significant proportion of ependymal and choroid plexus tumors (Bergsagel et al., 1992; Lednicky et al., 1995; Martini et al., 1996). Recently, different groups have reported discordant results regarding the detection of SV40, JCV, and BKV DNA sequences in human medulloblastomas (Hayashi et al., 2001; Huang et al., 1999; Krynska et al., 1999; Weggen et al., 2000).

We examined human medulloblastomas (also known as PNETs of the posterior fossa) and sPNETs for evidence of polyomavirus infection. In contrast to ependymomas and choroid plexus tumors, primary human medulloblastomas and sPNETs do not contain polyomavirus DNA sequences. We detected SV40 DNA sequences and T-ag expression only in the D283-Med medulloblastoma cell line, so we conclude that evidence of polyomavirus infection in human medulloblastomas and sPNETs is rare.

Materials and Methods

Primary Human Medulloblastoma and sPNET Specimens

Primary tumor specimens were obtained at the time of initial surgery from 20 pediatric patients treated at Boston Children's Hospital between August 1984 and January 1998 and were examined by a single neuropathologist. Fifteen tumors were classified as medulloblastomas and 5 as sPNETs, according to World Health Organization criteria.

Genomic DNA was purified from the tumor specimens according to established protocols known to isolate low-molecular-weight DNA species, such as episomal viral DNA (Ausubel et al., 1995; Koralnik et al., 1999). DNA preparations were stored at –20°C until time of analysis. Briefly, each frozen tumor specimen was ground to powder with a prechilled mortar and pestle and resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-Cl [pH 8], 25 mM EDTA [pH 8], 0.5% SDS, 0.1 mg/ml proteinase K [Sigma, St. Louis, Mo.]). Tumor cell lines were harvested by centrifugation after trypsinization, washed with ice-cold PBS, and then resuspended in digestion buffer. Samples were digested at 50°C for 12 to 18 h, then extracted twice with phenol/chloroform/isoamyl alcohol. After centrifugation, DNA-containing aqueous phase material was precipitated with ethanol, washed with 70% ethanol, air dried, and resuspended in Tris/EDTA buffer (pH 8). Residual RNA was removed by digestion with DNase-free RNase (1 mg/ml) and 0.1% SDS at 37°C for 1 h followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation.

PCR

To identify conserved sequences of the polyomavirus T-ag genes of BKV, JCV, and SV40, we used the oligonucleotide primers PYV.for and PYV.rev according to the methods of Bergsagel et al. (1992). These primers amplify conserved sequences from the C terminus of the BKV, JCV, and SV40 T-ag genes. Amplification by PCR was performed with thermocycling parameters (PTC-100, MJ Research, Watertown, Mass.) beginning with initial denaturation at 94°C for 3 min succeeded by 60 cycles of the following: denaturing step at 94°C for 1 min, annealing step at 52°C for 1 min, and extension step at 72°C for 1 min with a final extension step, for 5 min.

The PCR reaction products were then analyzed for polyomavirus-specific sequences by Southern blot hybridization (see below). SV40-specific sequences from the upstream noncoding regulatory region were amplified with nested primer pairs: RA3 and RA4, and RA1 and RA2, using previously described conditions (Lednicky et al., 1995).

For JCV-specific T-ag sequences, the primer pair IKTS and IKTAS amplifies a 235-bp fragment in the middle of JCV T-ag, detectable on Southern blot by hybridization with the JCV-specific T-ag probe IKTP (Table 1). The PCR amplification was performed in a 50-µl reaction consisting of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 units of Amplitaq DNA Gold polymerase (PE Biosystems, Foster City, Calif.) or 5 units of Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.), and 25 pmol of each oligonucleotide primer pair. The amplification was carried out with initial denaturing at 94°C for 4 min succeeded by 40 cycles of the following: denaturing step at 94°C for 30 s, annealing step at 55°C for 1 min, and extension step at 72°C for 1 min with a final extension step for 7 min.

Another JCV-specific primer pair, TC1 and TC2, generates a fragment from the C terminus of the JCV T-ag, detected by hybridization with another JCV-specific T-ag probe (5′-AGTGCAGTTTTCCTGTGTGTCTGCAC-3′) (Table 1). The PCR amplification was performed using 45 cycles of the following: denaturing step at 95°C for 30 s, annealing step at 52°C for 30 s, and extension step at 72°C for 30 s (Krynska et al., 1999).

JCV-specific VP1 gene sequences were amplified with the primer pairs VP11 and VP12 or VP2 and VP3, followed by hybridization with the JCV VP1 probe IKVP1S for VP11/VP12 products (Koralnik et al., 1999) or with another JCV VP1 probe (5′-AGCCAGTGCAGGGCA CCAGC-3′) for VP2/VP3 products (Table 1) (Krynska et

Table 1. PCR primer pairs and Southern blot hybridization probes

β-globin primer pair:	
PCO3	5′-ACACAACTGTGTTCACTAGC-3′
PCO4	5′-CCACTTGCACCTACTTCAAC-3′
Polyomavirus T-ag primer pair:	
PYV.for	5′-TAGGTGCCAACCTATGGACAGA-3′
PYV.rev	5′-GGAAAGTCTTTAGGGTCTTCTACC-3′
Specific T-ag probes:	
BK.probe	GAGAATCTGCTGTTGCTTCTT
JC.probe	GTTGGGATCCTGTGTTTTCAT
SV.probe	ATGTTGAGAGTCAGCAGTAGCC
JCV large T-ag primers:	
IKTS	5′-GCCTTGGAAACCAAATGTGAGG-3′
IKTAS	5′-TGTGGATGCTGTCAACCCTTTG-3′
JCV-specific T-ag probe:	
IKTP	CACATTTTTTGCATTGCTGTGGGTTTTCCTG
JCV large T-ag primers:	
TC1	5′-AACCAGCTTTACTTAACAGTTGC-3′
TC2	5′-CCCATTCTTGACTTTCCTAGAG-3′
JCV-specific T-ag probe:	
	AGTGCAGTTTTCCTGTGTGTCTGCAC
JCV VP1 primers:	
VP11	5′-CAGATACATTTGAAAGTGAC-3′
VP12	5′-CCATTAGAGTGCACATTCATC-3′
JCV VP1 probe:	
IKVP1S	GGACATGCTTCCTTGTTACAGTGTG
JCV VP1 primers:	
VP2	5′-TGTGCACTCTAATGGGCAAGC-3′
VP3	5′-CTAGGTACGCCTTGTGCTCTG-3′
JCV VP1 probe:	
	AGCCAGTGCAGGGCACCAGC
SV40-specific regulatory region nested primers	
Internal:	
RA1	5′-AATGTGTGTCAGTTAGGGTGTG-3′
RA2	5′-TCCAAAAAAGCCTCCTCACTACTT-3′
External:	
RA3	5′-GCGTGACAGCCGGCGCAGCACCA-3′
RA4	5′-GTCCATTAGCTGCAAAGATTCCTC-3′

al., 1999). PCR conditions for primers VP11 and VP12 were identical to those used for primers IKTS and IKTAS. For primers VP2 and VP3, conditions were similar to those for TC1 and TC2, but with an annealing temperature of 52°C. The integrity of all genomic DNA specimens was confirmed by PCR amplification of the β-globin gene using previously described conditions and the primer pair PCO3 and PCO4 (Table 1) (Saiki et al., 1985).

Southern Blot Hybridization Analysis

PCR-amplified DNA products were analyzed by Southern blot hybridization analysis. To identify BKV-specific, JCV-specific, and SV40-specific T-ag sequences, BK.probe, JC.probe, and SV.probe were used, respectively, as previously described (Table 1) (Bergsagel et al., 1992). Detection of JCV-specific sequences with the primer pairs IKTS/AS, TC1/TC2, VP2/VP3, and VP11/VP12 was performed according to the methods of Koralnik et al. (1999). Briefly, probes were prepared by end-labeling specific oligonucleotides with γ-[^{32}P]ATP and T4 polynucleotide kinase using standard methods (Ausubel et al., 1995).

PCR reaction products were fractionated by agarose gel electrophoresis (3% NuSieve: 1% SeaKem agarose gel; FMC Bioproducts, Rockland, Me.), transferred to a nylon membrane (Duralon UV; Stratagene, La Jolla, Calif.; or Turboblot; Schleicher & Schuell, Keene, N.H.), and cross-linked with ultraviolet irradiation (1200 mJ). Blots were prehybridized for 15 min in Quick-Hyb hybridization solution (Stratagene) at 45°C and then hybridized with a minimum of 10^6 cpm/ml of ^{32}P end-labeled oligonucleotide probes in Quick-Hyb for at least 4 h at 42°C. Hybridized filters were washed to a stringency of 0.2× SSC/0.1% SDS at 42°C for BK.probe, JC.probe, and SV.probe. For JCV-specific sequences, blots were washed to a final stringency of 0.5× SSC/0.2% SDS at 45°C for IKTS/AS and VP11/VP12, or in 0.1× SSC/0.1% SDS at 55°C for VP2/VP3 and TC1/TC2 products (Krynska et al., 1999). Washed blots were exposed to a Phosphorimager screen for subsequent image analysis (Molecular Dynamics, Sunnyvale, Calif.).

Cell Culture and Immunocytochemistry

Immunocytochemical detection of T-ag protein was based on previously described methods of DeCaprio et al. (1988). Human medulloblastoma cell lines Daoy and D283-Med and the COS cell line obtained from the American Type Culture Collection (ATCC, Rockville, Md.) were cultivated in Dulbecco's modified Eagle's medium (Sigma, St. Louis, Mo.) with 10% heat-inactivated fetal calf serum (Sigma) in humidified 5% CO_2 at 37°C. The SV40-transformed COS cell line was similarly maintained. Cells were grown on poly-L-lysine–coated coverslips and were fixed in 3.5% paraformaldehyde in PBS for 10 min at 25°C, washed 3 times with PBS, extracted with 1% NP-40 in PBS for 1 min at 25°C, then dehydrated at 4°C in 50% ethanol for 3 min, 100% ethanol for 5 min, and, finally, 50% ethanol for 3 min.

For immunocytochemical staining, the anti–T-ag monoclonal antibody (clone Pab419; Oncogene Research Products, Boston, Mass., and courtesy of J. DeCaprio), which recognizes an amino terminal domain of the SV40 T-ag, also present in the SV40 small T-antigen and also cross-reactive with JCV T-ag, was used. After fixation and permeabilization, coverslips and tissue sections were washed with PBS and blocked with normal goat serum (4% in PBS with 0.1% NP-40; Gibco-BRL, Grand Island, N.Y.). Cells were first incubated with the Pab419 (final concentration 25 μg/ml in blocking buffer) for 1 h at 37°C or overnight at 4°C.

After cells were washed with PBS, they were incubated with either goat antimouse fluorescein isothiocyanate (FITC)- or Cy2-conjugated secondary antibody (diluted 1:200 in blocking buffer; Jackson ImmunoResearch Laboratories, West Grove, Penn.) for 30 to 60 min at room temperature. Cells were counterstained with Hoechst 33342 (10 μM in PBS) for nuclear counterstaining, mounted, and imaged with a NORAN Oz laser-scanning confocal microscope (NORAN Instruments,

Table 2. Detection of polyomavirus DNA sequence

Sample	Tumor type	SV40	JC	BK	β-globin
Daoy	Medulloblastoma cell line	–	–	–	+
D283-Med	Medulloblastoma cell line	+	–	–	+
8400	Medulloblastoma	–	–	–	+
8403	Medulloblastoma	–	–	–	+
8404	Medulloblastoma	–	–	–	+
8407	sPNET	–	–	–	+
8503	Medulloblastoma	–	–	–	+
8516	Medulloblastoma	–	–	–	+
8525	Medulloblastoma	–	–	–	+
8603	sPNET	–	–	–	+
8611	Medulloblastoma	–	–	–	+
8615	sPNET	–	–	–	+
8618	Medulloblastoma	–	–	–	+
8702	sPNET	–	–	–	+
8705	sPNET	–	–	–	+
8719	Medulloblastoma	–	–	–	+
N5011	Medulloblastoma	–	–	–	+
N5017	Medulloblastoma	–	–	–	+
N5032	Medulloblastoma	–	–	–	+
N5062	Medulloblastoma	–	–	–	+
N5064	Medulloblastoma	–	–	–	+
N5072	Medulloblastoma	–	–	–	+

Middleton, Wis.). Formalin-fixed, paraffin-embedded sections of 4 primary medulloblastoma tumors were also processed similarly after deparaffinization in xylene and rehydration through ethanol series. Positive control experiments were conducted on COS cell cultures and formalin-fixed, paraffin-embedded sections of SV40-infected monkey tissues. Negative control experiments were performed by omitting the PAb419 primary antibody (secondary antibody alone) or by using unrelated primary monoclonal antibodies (data not shown).

Results

Polyomavirus Coding Sequences in Medulloblastomas and sPNETs and SV40 Noncoding Sequences in D283-Med

Genomic DNA from 15 primary medulloblastomas, 2 medulloblastoma cell lines, and 5 sPNET was amplified for polyomavirus T-ag and screened by hybridization for specific SV40, JCV, or BKV sequences. The integrity of all DNA samples was tested by amplifying a segment of the β-globin gene (Saiki et al., 1985). Only those samples with intact β-globin sequences were examined for the presence of polyomavirus T-ag sequences independently in 2 separate laboratories (S.L.P. and I.J.K.) to verify the findings. None of the primary medulloblastomas or sPNET DNA samples contained T-ag sequences from any of the polyomaviruses (Table 2).

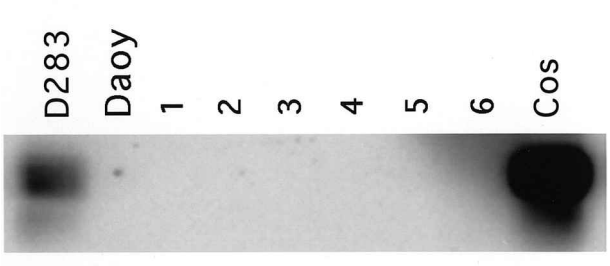

Fig. 1. Southern blot analysis of PCR-amplified DNA from primary human medulloblastomas and cell lines. DNA from medulloblastoma cell line D283-Med contains SV40-specific T-ag sequences, whereas Daoy DNA does not and neither do any primary human medulloblastoma and sPNET specimens (numbered 1-6). Positive control COS cells contain strongly positive SV40 T-ag DNA.

To control for primer selection, we performed additional experiments using different primer pairs for regions in the middle and in the C terminus of the T-ag genes, the results of which were uniformly negative. Amplification of the major capsid protein VP1 sequences for JCV has been shown to be reliable for the detection of this virus in immunosuppressed individuals (Koralnik et al., 1999). Amplification with 2 different primer pairs spanning the N terminus of VP1 was negative in all primary medulloblastomas and sPNETs. As shown in Fig. 1, the only evidence of SV40 T-ag was detected in the D283-Med cell line.

To determine whether T-ag expression was an isolated phenomenon, we sought additional polyomavirus sequences. The sequence of an upstream regulatory region of SV40 varies among different viral strains (Lednicky et al., 1995). PCR amplification with nested primer pairs specific for the regulatory region revealed that the D283-Med, but not the Daoy cell line, contains SV40 noncoding DNA sequences. Primary medulloblastomas and sPNETs were uniformly negative. These results confirm that D283-Med cells contain not only SV40 T-ag gene sequences, but also noncoding SV40 DNA sequences.

SV40 T-ag Protein Expression

Immunochemical evidence of T-ag protein was detected in the nuclei of D283-Med cells, as shown in Fig. 2. Neither the Daoy medulloblastoma cell line nor 4 primary medulloblastoma tumors revealed T-ag expression, with SV40-infected tissues being used as a positive control. These results suggest that polyomavirus T-ag may contribute to the immortalized phenotype of D283-Med.

To determine whether the positive results in D283-Med were a result of contamination within our lab, we obtained a second lot of cryopreserved D283-Med from the ATCC. Experiments were repeated in separate facilities, using separate reagents, which confirmed the presence of SV40 DNA and T-ag protein. Parallel control experiments were consistently negative.

Discussion

We did not find evidence for oncogenic polyomaviruses in primary human medulloblastomas or sPNETs. Although

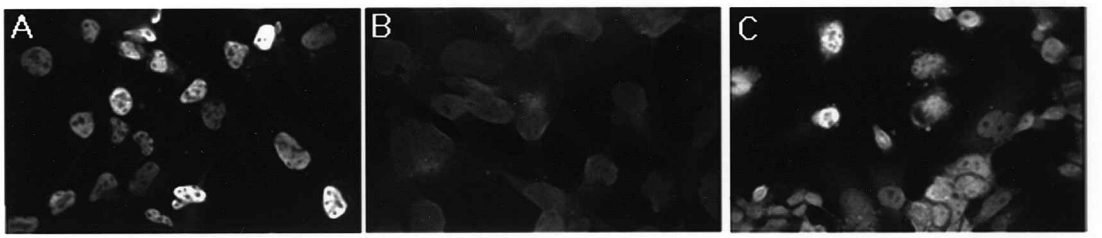

Fig. 2. Immunofluorescent staining for SV40 T-ag. A. Positive control COS cells constitutively expressing nuclear T-ag, as shown by positive immunostaining with anti-T-ag monoclonal antibody (PAb 419) and secondary fluorescein-conjugated antibody. All samples were viewed with a 40× Nikon objective, NORAN confocal microscope. B. The human medulloblastoma cell line, Daoy, does not express SV40 T-ag. C. Another human medulloblastoma cell line, D283-Med, displays nuclear SV40 T-ag.

the presence of T-ag in D283-Med is notable, possibly contributing to its phenotype, we cannot determine whether the virus was present at the time the original tumor was originally isolated or whether it subsequently was introduced during derivation of the cell line. We conclude that SV40 can be found only rarely in human medulloblastomas. Furthermore, although JCV can induce medulloblastomas in hamsters, we do not find evidence for JCV or BKV in the human disease.

Our results essentially concur with a large German report that identified SV40 DNA sequences in only 2 of 116 medulloblastomas (2%), and no JCV and BKV sequences when similar PCR methods and primers were used (Weggen et al., 2000). In another European study that included 17 medulloblastomas, SV40 DNA sequences were detected in 5 (29%) specimens, BKV sequences in 1 (6%), and JCV sequences in none with PCR amplification and Southern methods (Huang et al., 1999). A Japanese group described negative results for JCV sequences in 8 medulloblastoma specimens; 6 by PCR amplification and Southern blotting, and 2 by in situ hybridization and immunostaining (Hayashi et al. 2001). In contrast, North American investigators reported that 22 of 23 (96%) medulloblastomas contained JCV DNA sequences, and 5 of 23 (22%) also contained SV40 sequences. In their study, JCV T-ag was also detected immunohistochemically in 4 of 16 (25%) samples (Krynska et al., 1999). Our negative results in 15 North American patient specimens are significantly less than predicted by the conclusions of Krynska et al. (2-sided $P < 0.0001$ and $P = 0.07$ for JCV and SV40, respectively). One possible explanation is that geographic factors account for the striking variability of SV40, JCV, and BKV detection reported in different medulloblastoma series. Accordingly, our findings do not support a significant incidence of polyomavirus infection in medulloblastomas in the northeastern United States.

Several groups have reported the presence of SV40 T-ag in a variety of human cancers and suggested a viral role in their tumorigenesis (Bergsagel et al., 1992; Carbone et al., 1994, 1996; Lednicky et al., 1995; Martini et al., 1996). Recent epidemiological studies of the contamination of polio vaccines with SV40, which occurred in the late 1950s and early 1960s, conclude that recipients of the contaminated vaccines did not display a highly increased risk for carcinogenesis (Fraumeni et al., 1963, 1970; Mortimer et al., 1981). Several studies suggested, however, a small measurable increase in the frequency of nervous system cancers, especially among the offspring of women vaccinated during pregnancy (Innis, 1968; Heinonen et al., 1973; Farwell et al., 1979,1984). Our results indicate that evidence of infection with SV40 or other polyomaviruses is not common in human medulloblastoma or sPNET, and therefore does not appear to be a major factor in their tumorigenesis.

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