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# Five DNA Tumor Viruses Undetectable in Human Retinoblastomas

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**Retinoblastoma (RB) is a childhood eye cancer that arises when a retinal cell lacks a functional RB gene. Recent data indicate that the transforming proteins of adenovirus, papillomavirus, and the polyomaviruses BK and JC all can bind to the product of the RB gene. Furthermore, adenovirus 12, JC virus, and simian virus 40 are able to induce RB-like tumors in rodents. In view of these findings, 50 human RBs were tested for the presence of five human DNA tumor viruses: adenovirus 12, BK virus, JC virus, and human papillomaviruses 16 and 18. Using the polymerase chain reaction, no viral sequences were detected in 50 RB DNAs. These data provide no evidence that these viruses have an etiologic role in human RB. Invest Ophthalmol Vis Sci 33:1564–1567, 1992**

Retinoblastoma (RB) is a rare ocular tumor that arises when a retinal cell lacks a functional RB gene. The tumor is thought to arise when both alleles of the RB gene are lost by mutation or deletion. In the hereditary form of RB, a child inherits one defective RB allele. The mutation rate of the second allele among the 100 million developing retinal cells is high enough to result in multifocal bilateral tumors. Approximately 70% of all RBs, however, arise spontaneously. In these cases, the tumor is thought to be caused by two consecutive somatic mutations in a single retinal cell. Because the chance of this occurring is extremely small, nonhereditary RB typically presents as a single tumor focus in only one eye.[1]

There are several indications that DNA tumor viruses may have an etiologic role in human cancer. First, human papillomaviruses (HPV) are associated with epithelial malignant lesions. Thus, HPV 16 has been detected in cervical carcinoma,[2] anal squamous cell carcinoma,[2] and dysplastic and malignant lesions of the cornea and conjunctiva.[3] The virus HPV 18 has been found in cervical carcinoma[2] and conjunctival intraepithelial neoplasia.[4] Furthermore, polyomavirus BK (BKV) has been detected in human brain tumors.[5]

Recent findings from several laboratories indicate that the transforming proteins of several DNA tumor viruses can bind to the product of the RB gene. Immunoprecipitation studies indicate that the T antigen (TAg) of simian virus 40 (SV40), BKV, and polyomavirus JC (JCV) all can bind to pRB.[6,7] Furthermore, pRB can bind the E7 protein of HPV 16[8] and HPV 18[9] and the E1a protein of adenovirus.[10] These findings suggest a model for DNA tumor virus-mediated transformation in which a genetically normal RB protein is inactivated by binding to a viral oncoprotein.

This model of tumorigenesis is supported by several in vitro studies. First, adenovirus 12 (Ad12) is able to transform human embryonic retinal cells in culture at high frequency.[11] Furthermore, it was found that mutant viruses which had lost the ability to associate with pRB had also lost transforming ability.[12] In addition, injection of Ad12 and JCV into the eyes of newborn rodents or baboons results in retinal tumors that are morphologically similar to RB.[13–15] More recently, we described a strain of transgenic mice that express SV40 TAg in the retina. These mice had hereditary ocular tumors with a striking resemblance to human RB.[16]

In view of these findings, we surveyed 50 primary human RBs for the presence of each of five human DNA tumor viruses using the polymerase chain reaction (PCR).

## Materials and Methods

### Primers

Oligonucleotides were synthesized to amplify the RB-binding oncogenes of five viruses. Primers for the

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**Table 1.** Primer sequences for polymerase chain reactions

| *Gene* | *Localization* | *Sequence* | *Product* |
|---|---|---|---|
| c-myc | | | |
| sense | 1017–1026 | 5′ GCCCC TCAAC GTTAG CTTCA | 230 bp |
| anti | 1253–1234 | 5′ AAGGG TGTGA CCGCA ACGTA | |
| HPV 16 | | | |
| sense | 645–664 | 5′ AAATG ACAGC TCAGA GGAGG | 201 bp |
| anti | 846–827 | 5′ AGAAC AGATG GGGCA CACAA | |
| HPV 18 | | | |
| sense | 590–609 | 5′ ATGCA TGGAC CTAAG GCAAC | 219 bp |
| anti | 809–790 | 5′ GCTCA ATTCT GGCTT CACAC | |
| BKV | | | |
| sense | 4392–4411 | 5′ AGTCT TTAGG GTCTT CTACC | 176 bp |
| anti | 4567–4548 | 5′ GGTGC CAACC TATGG AACAG | |
| JCV | | | |
| sense | 563–582 | 5′ ATGTG GATGC TGTCA ACCCT | 176 bp |
| anti | 739–720 | 5′ GGAAA ACCCA CAGCA ATGCA | |
| Ad 12 | | | |
| sense | 648–667 | 5′ GGAGT CTGCC GGTGA AGATA | 250 bp |
| anti | 898–879 | 5′ CAGAA ACATG CGCCA TTCCG | |
| Adeno | | | |
| sense | 456–475 | 5′ CCAGC GAGAA GAGTT TTCTC | 186 bp |
| anti | 641–622 | 5′ AGATC ATACA GTTCG TAAAG | |

human c-*myc* gene were selected as a control (Table 1).

Adenovirus has over 40 serotypes, of which only three (Ad5, Ad7, and Ad12) have been sequenced. We synthesized two sets of oligonucleotides from the E1a region of Ad12, which is known to transform retinal cells. The first set was taken from the most divergent region of E1a. Hence, these primers (Ad12) should be specific for Ad12. The second set of primers ("Adeno") was chosen from the most conserved region of E1a to recognize any novel oncogenic serotypes.

## Specimens

We extracted DNA from fresh RBs. Fifty samples were chosen randomly for PCR analysis. In addition, DNA extracted from the HeLa cell line and the LX1 cell line served as positive controls for HPV 18[2] and c-*myc*, respectively. Four plasmids containing Ad12 E1a, BKV, JCV, or HPV 16 served as controls for the remaining viruses. As a negative control, amplification reactions without any tumor DNA were run with each set of primers.

## PCR

For each set of primers, 50 ng of tumor DNA was amplified in a separate 100-$\mu$l reaction mix containing 2.5 units AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and 0.5 $\mu$g of primer. After 40 cycles of amplification (DNA Thermal Cycler, Perkin Elmer Cetus), 30 $\mu$l of the reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel. Additionally, 10 $\mu$l of each PCR reaction was analyzed by slot-blot analysis (Schleicher and Schuell, Keene, NH). The blots were probed with a $^{32}$P-deoxycytidine triphosphate-labeled fragment of the DNA tumor virus or the control. Autoradiograms were prepared by exposing the blots to X-OMAT AR film (Eastman Kodak, Rochester, NY).

## Results

To examine whether the DNA extracted from the 50 tumors could be amplified by the PCR technique, we first did PCRs on all tumor DNAs with oligonucleotides from the c-*myc* gene (Table 1). After 40 cycles of amplification, gel electrophoresis showed that a 230-base pair (BP) fragment could be amplified from the 50 tumor samples and from 9 positive control samples containing LX1 DNA. No 230-BP fragment was observed when template DNA was absent from the reaction. Slot-blot analysis (Fig. 1A) indicated that all 50 tumor samples contained a PCR product which hybridized strongly to the c-*myc* cDNA fragment. We concluded that all 50 tumor DNAs could be amplified by PCR.

Next, PCR was done on all 50 RB samples with HPV 18-specific oligonucleotides. In addition, PCRs were done on nine positive control samples containing 50 ng of HeLa DNA and nine negative control specimens containing no DNA. Agarose gel electro-

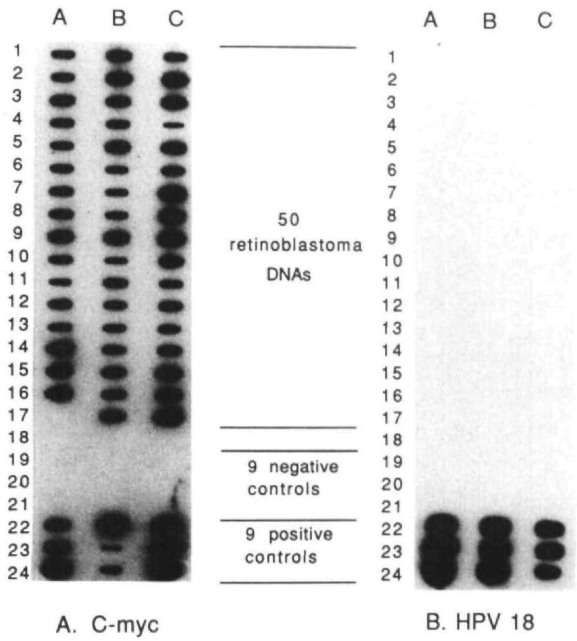


**Fig. 1.** Slot blot analysis of PCR products. DNA samples were amplified with c-myc oligonucleotides or HPV 18-specific oligonucleotides. Polymerase chain reaction products were slot-blotted onto nitrocellulose and probed with a $^{32}$P-labeled c-myc probe (left panel) or an HPV 18 probe (right panel).

phoresis of the PCRs revealed that each of the positive control samples amplified a 219-BP fragment. However, neither the tumor DNA nor the negative control specimens amplified this fragment (data not shown). Slot-blot analysis (Fig. 1B) indicated that the HPV 18 probe hybridized only to the positive control. We concluded that HPV 18 was not present in any of the 50 RB tumor samples.

We also did PCRs with HPV 16-specific oligonucleotides. As a positive control, we used 10 pg of a plasmid harboring a complete HPV 16 genome. Slot-blot hybridization showed that none of the 50 RB DNA samples showed any hybridization to the HPV 16 probe (data not shown). We concluded that all RBs were negative for HPV 16 sequences.

Next, PCR analysis was done with BKV and JCV-specific oligonucleotides. As positive control samples, we added 10 pg of a plasmid containing the genome of either BKV or JCV to nine replicate reactions. In each case, slot-blot analysis showed that BKV- or JCV-specific fragments could be amplified in the positive control reactions but not in the negative control samples or in the 50 reactions containing RB tumor DNA (data not shown). We concluded that BK and JC viral sequences were not present in any of the RB samples assayed.

Finally, we assayed the tumor samples for the presence of adenovirus DNA sequences using two sets of primers. As a positive control, we used 10 pg of a plasmid harboring the Ad12 E1a region. Slot-blot analysis of the PCR products revealed that both sets of primers could amplify Ad12 E1a fragments in the positive control reactions. Of the 50 RB tumor DNAs, only two showed weak hybridization in a slot-blot analysis using an Ad12 E1a probe. This signal, however, was not reproducible in subsequent experiments. We therefore concluded that Ad12 E1a sequences were not present in any of the tumor DNAs we analyzed.

After completion of the survey, the case histories of the patients were reviewed. Among the patients, 13 (26%) had bilateral tumors, 28 (56%) had unilateral tumors, and 9 (18%) were not documented.

## Discussion

The ability of DNA tumor viruses to transform retinal cells has been well established.[11] Recent investigations suggest that virus-induced transformation may be mediated by the binding of viral oncogenes to the product of the RB gene.[6-10] This complex may inactivate the tumor-suppressing activity of RB, even when the gene is intact genetically. This model of tumorigenesis was supported by our transgenic mouse line, in which the RB gene product was complexed with SV40 TAg in mice with RB.[16]

Because DNA tumor viruses can transform retinal cells, we surveyed 50 human RBs for the presence of five different DNA tumor viruses. We used the extremely sensitive PCR technique for our assay.

This assay, in which a target gene may be amplified $10^6$ times, is extremely sensitive to DNA contamination. The strictest precautions must be followed to avoid false-positive results. Our experiments with primers for c-*myc*, HPV 16, HPV 18, BKV, and JCV gave straightforward results. We were able to amplify c-*myc* but none of the four viruses. These results were substantiated by multiple control reactions. When the adenovirus-specific oligonucleotides were used in PCR reactions, we found initially that 2 of 50 tumor samples yielded PCR products that hybridized faintly to the adenovirus probe. However, this result could not be duplicated with two sets of adenovirus-specific oligonucleotides. It is therefore likely that the initial positive result was caused by contamination. This emphasizes the hazards of DNA contamination and the need for duplicate reactions.

Our exhaustive survey confirmed previous studies of RB. In 1982, six human RBs were tested for Ad12 using southern blot analysis.[17] Others investigated the presence of adenovirus in six additional tumors.[18] Eleven RBs were tested for adenovirus and JCV.[19] None of these surveys found viral sequences. Therefore, with the inclusion of our study, Ad12 has not been detected in 73 RBs. Sixty-one tumors were negative for JC sequences. Fifty tumors were negative for BKV, JCV, and HPV 16 and 18. We conclude that DNA tumor viruses generally do not cause human RBs.

**Key words:** retinoblastoma, DNA tumor virus, PCR, antioncogene

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