Presence of Human Papilloma Virus in Tumor Tissue from Children with Retinoblastoma: An Alternative Mechanism for Tumor Development

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INTRODUCTION

Retinoblastoma is a malignancy that arises in primitive neuroectodermal cells of the retina. Risk factors for the development of the nonfamilial form of the disease are poorly understood. The incidence of retinoblastoma is not equally distributed throughout the world and is higher in less industrialized countries and in less affluent populations relative to other malignancies of early childhood (1). This variation in incidence may be due to differential exposure to infections or other environmental factors causing mutations in utero. The incidence of retinoblastoma has been increasing among some populations severely affected by the HIV epidemic (2), suggesting a possible role for infectious agents. An increased incidence of retinoblastoma is associated with low levels of maternal education, lack of prenatal vitamin supplementation, lack of prenatal care, and other conditions of poverty during pregnancy (3). In the United States, maternal use of barrier methods of contraception (with or without spermicide) periconceptionally significantly decreases the risk of having a child with retinoblastoma (3). The purpose of this study was to attempt to explain the geographic variation in incidence and risk factors through characteristics evident in the tumor tissue of patients affected by sporadic retinoblastoma.

There is considerable overlap in the epidemiological risk factors for the development of retinoblastoma and HPV infection. The use of barrier methods of contraception is associated with a reduced incidence of both retinoblastoma (3) and HPV16/HPV18 infection (4, 5). As with the likelihood of having a child with retinoblastoma, the likelihood of infection with HPV is inversely correlated with a woman’s educational level and socioeconomic status (6). Diaphragms and condoms are significantly protective for the development of cervical carcinoma (7–10), although some studies have not found them to prevent HPV infection (11). Most cervical carcinoma is caused by infection with HPV (12). In addition, there is considerable overlap between those countries in which the relative incidence of retinoblastoma is greatest and those in which the incidence of cervical carcinoma is highest (1, 13, 14).

Retinoblastoma cells lack functional pRB (15). pRB binds and inactivates transcription factors, such as E2F members, thereby regulating cell cycle progression (16). Mutations in the retinoblastoma gene (RB1) result in the absence of functional pRB and a concomitant increase of unbound E2F proteins. Although most cases of retinoblastoma have been found to have RB1 mutations, it has been reported that between 17% and 80% of nonfamilial cases have an intact RB1 gene (17–19), suggest-
ing the existence of an alternative mechanism for pRB inactivation. The HPV E7 protein binds to and inactivates pRB (20). Transgenic HPV16 mice overexpressing E6 and E7 oncoproteins possess an intact retinoblastoma gene, but the ectopic expression of E6 and E7 in the retina leads to pRB inactivation and the development of retinoblastoma-like lesions (21). These epidemiological and molecular data suggest that HPV may play a role in the development of sporadic retinoblastoma. Therefore, we examined tumor DNA from children with nonfamilial retinoblastoma for the presence of HPV sequences and pRB expression status, correlating laboratory results with clinicopathological features.

MATERIALS AND METHODS

Patient Population and Tissue Procurement. Tissue was obtained as part of a larger collaborative study at the INP (Mexico City, Mexico). Fresh tissue was collected prospectively between August 1994 and August 1995 from 40 children less than 5 years of age without a family history of retinoblastoma who were undergoing enucleation for presumed retinoblastoma. The parents of the children were interviewed as part of a larger questionnaire-based case-control study in which data were collected on pre- and postnatal exposures to potential carcinogens. Informed consent was obtained from all participating parents.

Clinical stage, as defined by the St. Jude’s staging system (22), was determined from the clinical record of the patient and from the histopathological report given for the enucleated eye. H&E stains of the enucleated eye were reviewed independently for histopathological staging by the INP study pathologist (C. R.). One child was found to have a benign retinal defect, and this child’s tissue was then used as a negative control. The specimens from the remaining 39 children were diagnosed as retinoblastoma.

Extreme precautions were taken to avoid contamination of tissue samples. The enucleated eye was handled under sterile conditions. The sterile portion of the tumor was embedded in OCT and frozen immediately in liquid nitrogen. Samples were collected on pre- and postnatal exposures to potential carcinogens. Informed consent was obtained from all participating parents.

DNA was extracted from 39 primary retinoblastoma tissues using a nonorganic method (Oncor, Gaithersburg, MD) and filter-tipped pipettes. Amplifiable DNA was verified in all samples using β-globin 2 gene primers PC04 and GH20 (Perkin-Elmer, Foster City, CA). PCR conditions consisted of: (a) preincubation at 97°C for 4 min; (b) 50 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and (c) elongation at 72°C for 3 min. Tumor DNA (100 ng) was examined for the presence of HPV-like sequences using PCR with HPV L1 (MY09/MY11; Ref. 23) degenerate consensus primers (Perkin-Elmer). One nonneoplastic enucleated eye and DNA blank samples were used as negative controls. DNA extracted from SiHa and HeLa cell lines was used as a positive control. To avoid contamination, the following precautions were taken: (a) SiHa and HeLa DNA was handled in a separate room; (b) tumor DNA was added as the final reagent before adding Taq polymerase (Amplitaq; Perkin-Elmer); (c) SiHa and HeLa DNA was added after the Taq polymerase; and (d) filtered pipette tips were used. Conditions for the HPV-specific PCR reactions consisted of preincubation at 94°C for 5 min; 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and elongation for 5 min at 72°C. PCR products were analyzed on 2% agarose gels visualized with ethidium bromide. PCR-amplified fragments containing the L1 sequence were expected to appear between 448 and 454 bp in length. Southern blotting was carried out with [α-32P]dCTP-labeled probes that were specific for either HPV18 or HPV16. These probes were generated by using DNA from the SiHa (HPV16) and HeLa (HPV18) cell line cell line in PCR reactions with species-specific oligonucleotide primers that were internal to the degenerate primers in L1. The primers used were checked using GenBank and control tissues to ensure that they would not amplify any human or other non-HPV viral sequences. No other HPV subtypes, besides HPV16 and HPV18, were recognized with either of these primer sets. For HPV16, the internal primer sequences were 5'-TACCTACGACATGGGGAGGA-3' and 5'-TGACAAAGCATTGCCTGGTTG-3'. The sequences for the HPV18 internal primers were 5'-CCTGGGCAATATGATGCTAD-3' and 5'-CCTTTTTCAGCCGGTTGCA-3'. Conditions for the PCR reactions consisted of preincubation at 95°C for 5 min; 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and elongation for 5 min at 72°C. Membranes were hybridized overnight with the HPV16 internal probes and then washed to a final stringency of 0.1% SSC/0.1% SDS at 52°C for 30 min and exposed to X-ray films (Kodak, Rochester, NY) at −80°C with intensifying screens for approximately 40 h. The membranes were then stripped and rehybridized with the HPV18 internal probe, using the same procedure.

Immunohistochemical Analysis. IHC with avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, CA) was performed using Mabs to pRB (clone Ab-5; detected underphosphorylated and hyperphosphorylated pRB proteins; 1 μg/ml dilution; Oncogene/Calbiochem, Cambridge, MA), clone G99-549 (detected only underphosphorylated pRB proteins; 5 μg/ml dilution; PharMingen, San Diego, CA), Ki67 (MIB-1; 1 μg/ml dilution; Immunotech Co., Marseille, France), and E2F-1 (1 μg/ml dilution; Santa Cruz Biotechnology, Santa Cruz, CA).
Tumors were reported to have undetectable pRB if no tumor cell nuclei were stained with Ab-5 antibody, whereas endothelial cells and other normal cells were stained. Tumors were scored as having detectable pRB if $>1\%$ of tumor cell nuclei stained with Ab-5. Detection of underphosphorylated pRB was defined as tumors having $>10\%$ detectable nuclear staining with G99-549 antibody. Slides were examined independently by two pathologists who were blinded to the HPV status of the tumor tissues and to the clinical stage of the patient. For Ki67 and E2F1, tumors were scored according to the percentage of cells stained over five high-power fields. Cases were excluded from analysis if the slides were not interpretable because of necrotic tissue.

**Statistical Analysis.** Statistical analysis was done using SPSS version 8.0 software (SPSS, Inc. Chicago, IL). HPV status was classified as present or absent. Ki67 and E2F1 were analyzed as continuous variables for the percentage of tumor cells stained with antibody. Clinical stage was analyzed as an ordinal variable (four stages) or was grouped into two categories, tumor confined to the globe or retina (stage $<3$) and extracocular or metastatic disease (stage $\geq3$). The relationships between HPV status and the presence of detectable pRB and between HPV status and clinical stage were examined with $\chi^2$ analysis and Fisher’s exact test. The relationship between the percentage of Ki67 expression and HPV status was examined using Student’s $t$ test. The relationship between Ki67 expression and clinical stage was examined using linear regression. The significance level of $\alpha = 0.05$ was chosen. Data on prenatal exposures were obtained from parental interviews. These were compared with data on the presence of HPV DNA in tumor DNA using a case-series design (24). Demographic characteristics were compared between cases with and without HPV DNA using $\chi^2$ analysis for categorical variables and Student’s $t$ test for continuous variables. Those variables such as monthly income, which were not normally distributed, were taken to the square root, and statistical analysis was then done on the square root values. Those variables significantly associated with the presence of HPV DNA in univariate analysis were analyzed in logistic regression for multivariate analysis using an $\alpha$ level of 0.05 for significance. All tests of statistical significance were two-sided.

**RESULTS**

**HPV DNA Detection in Retinoblastoma.** Using the L1 consensus primers for PCR amplification, we found HPV sequences in 14 of 39 (36.0\%) tumors. HPV16 DNA sequences were detected in 4 of 39 (10.3\%) tumors (see Fig. 1), and HPV18 sequences were detected in 11 of 39 (28.2\%) cases. In one case, we identified sequences of both HPV16 and HPV18.

Negative controls, DNA blank specimens, and benign retinal disease did not demonstrate amplified DNA in the PCR experiments using L1 primers. PCR reactions were carried out in duplicate to verify reproducibility. PCR-amplified fragments created with L1 primers and DNA extracted from HeLa and SiHa cells were hybridized with the HPV probes, confirming their specificity (Fig. 1).

**pRB Protein Expression.** We investigated pRB expression by IHC in all 39 tumors. The use of a Mab, G99-549, detecting specifically the underphosphorylated pRB products allowed us to further evaluate the functional status of pRB proteins present in the tumor cells. One case could not be evaluated because the tissue was too necrotic. In all other cases, endothelial cells stained strongly with the anti-pRB antibodies, serving as internal positive controls. We observed that tumor cells in 5 of 38 evaluable sporadic retinoblastoma cases were immunoreactive for pRB, suggesting that pRB was present in a subset of these tumors. Moreover, all of these five cases were found to be unreactive with antibody G99-549, suggesting that the identified pRB was hyperphosphorylated. The presence of HPV DNA was associated with the presence of pRB by IHC (Fig. 2), although this association did not achieve significance (OR, 3.0; $P = 0.2$). Three of five (60\%) tumors with detectable pRB were HPV positive, whereas only 11 of those 34 (32\%) tumors without detectable pRB had HPV DNA. Three of 14 (21\%) HPV-positive tumors had detectable pRB, whereas only 2 of 24 (8\%) HPV-negative tumors had detectable pRB.

**E2F1 Expression and Proliferation Index.** Proliferation rate in tumors was determined by Ki67 IHC with MIB-1. Six cases were not informative with MIB-1 because of necrosis. The presence of pRB was significantly associated with a decreased percentage of cells staining with MIB-1 ($P = 0.04$), and the percentage of cells staining with MIB-1 was significantly higher in tumors without HPV DNA ($P = 0.015$). This relationship remained significant after adjusting for the presence of detectable pRB ($P = 0.04$). There was no difference in the percentage of cells staining positive for E2F1 between HPV-positive (72\%) and HPV-negative (75\%) tumors. There was no difference in the percentage of cells staining positive for E2F1 antibody between tumors with and without detectable pRB (64\% versus 72\%; $P = 0.3$).
Clinical and Demographic Associations with the Presence of HPV. Of the 39 children, 25 (64%) had unilateral disease, and 14 (36%) had bilateral disease. Three children (7.7%) had metastatic disease (stage 4), 14 (36%) children had extraocular disease (stage 3), 21 (56%) had ocular disease (stage 2), and 1 (2.6%) had retinal disease (stage 1). The Ki67 proliferative index of tumors increased significantly with increasing clinical stage ($b = 0.39; P = 0.025$). Children with HPV-positive tumors were significantly less likely to have advanced-stage disease (Table 1). The relative risk of having extraocular disease (with or without metastatic disease) if the tumor did not have HPV DNA was 9.0 (95% CI, 1.7–49.1; $P = 0.008$).

Among the 14 cases positive for HPV, no cases were stage 4 (metastatic), and only 2 cases were stage 3 (orbital), whereas the majority were localized disease (ocular [11 cases] or retinal [1 case] disease). Among the 25 cases without HPV DNA, 3 cases had metastatic disease (stage 4), 12 cases had orbital disease (stage 3), and 10 cases had ocular disease (stage II). The proportion of cases with bilateral disease was equal for tumors with HPV DNA and without HPV DNA (OR, 1.2; 95% CI, 0.30–4.69; $P = 0.81$). Mortality was not significantly increased among the HPV-negative cases (OR, 1.7; 95% CI, 0.16–18.0; $P = 0.7$). However, there were only four known deaths among the 39 children under study.

There was no significant difference in age at diagnosis between those children whose tumors contained HPV and those whose tumors did not contain HPV. Because some reports have found that an increased age at diagnosis correlates with an increased stage at presentation, we examined the relationship between HPV status and stage at diagnosis, adjusting for age at diagnosis. We found that HPV status remained significantly associated with stage at diagnosis after adjusting for age at diagnosis ($P = 0.03$).

Results of case-series analysis of demographic variables associated with the presence of HPV DNA in the tumors are shown in Table 1. Demographic data from the questionnaire interviews demonstrated that children with HPV DNA in their tumors had lower birth weight and were breastfed for fewer months than children whose tumors did not contain HPV. Mothers of children with HPV DNA in their tumors were more likely to have been diagnosed with a vaginal infection, although this difference was not statistically significant. There was also no significant difference in mean per capita monthly income (360 versus 220 N. pesos), mean number of years of maternal schooling (6.0 years versus 5.5 years), mean maternal age at delivery, or birth order of the index child between the two groups. Fathers of children with HPV DNA in their tumors were older than fathers of children whose tumors did not contain HPV DNA. In multivariate analysis using logistic regression, only birth weight remained significantly associated with the presence of HPV DNA after adjustment for paternal age and maternal history of vaginal infection.

**DISCUSSION**

Certain viral proteins regulate host cell proliferation by binding to cell cycle regulators such as pRB and p53. The oncoproteins HPV E7, adenovirus E1A antigen (25), and the SV-40 T antigen bind to and inactivate pRB. This binding also contributes to oncogenic transformation in certain neoplasms such as cervical carcinoma (26). Results from this study suggest that pRB inactivation by HPV may also play a role in the development of some retinoblastomas.

A role for HPV in retinoblastoma could explain the overlap in risk factors for retinoblastoma and cervical carcinoma. Cervical cancer is the major cause of mortality among Mexican children. Further studies are needed to determine the role of HPV in the development of retinoblastoma.
Table 1  Clinical and phenotypic characteristics of cases examined for the presence of HPV DNA

<table>
<thead>
<tr>
<th>Variable (measurement units)</th>
<th>HPV positive</th>
<th>HPV negative</th>
<th>P (ORa; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of pRB-positive cases (n = 38)</td>
<td>3/14</td>
<td>2/24</td>
<td>0.2 (3.0; 0.4–20.7)</td>
</tr>
<tr>
<td>Proportion with bilateral disease</td>
<td>4/14</td>
<td>10/25</td>
<td>0.81 (1.2; 0.30–4.69)</td>
</tr>
<tr>
<td>Mean % Ki67 (SD; n = 33)</td>
<td>68 (11.1)</td>
<td>78 (10.7)</td>
<td>0.015</td>
</tr>
<tr>
<td>Proportion with stage ≥ 3</td>
<td>2/14</td>
<td>15/25</td>
<td>0.008 (9.0; 1.6–49)b</td>
</tr>
<tr>
<td>Mean % E2F (SD; n = 38)</td>
<td>72 (18.1)</td>
<td>75 (19.2)</td>
<td>0.65</td>
</tr>
<tr>
<td>Total no. of deaths</td>
<td>1/14</td>
<td>3/25</td>
<td>0.7 (1.7; 0.2–18)</td>
</tr>
<tr>
<td>Mean birth weight (kg; SD; n = 29)</td>
<td>2.9 (0.71)</td>
<td>3.5 (0.61)</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean length of breast feeding (months; SD; n = 31)</td>
<td>6.3 (6.2)</td>
<td>11.8 (10.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Proportion with mothers with history of vaginal infections</td>
<td>4/9</td>
<td>5/18</td>
<td>0.4 (2.1; 0.4–11)</td>
</tr>
<tr>
<td>Mean age of father at birth of patient (years; SD; n = 35)</td>
<td>32.6 (8.3)</td>
<td>29.0 (7.1)</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean monthly per capita income (Mexican pesos; SD; n = 36)</td>
<td>360 (707)</td>
<td>220 (204)</td>
<td>0.36c</td>
</tr>
<tr>
<td>Mean no. of years of schooling, mother (n = 34)</td>
<td>6 (3.1)</td>
<td>5.2 (3.4)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

a ORs (except where noted) are calculated comparing HPV-positive tumors to HPV-negative tumors.
b OR comparing HPV-positive cases to HPV-negative cases.
c Statistical analysis was performed on square root data that were normally distributed. Nontransformed means and SDs are shown for reference.

women of child-bearing age (27), and the prevalence of asymptomatic HPV infection in Mexican women is higher than that reported in other countries in the Americas (28). A low level of education among women and a lack of use of barrier methods of contraception are both associated with an increased risk of cervical HPV infection (6, 29), as well as with a significantly increased risk of having a child with retinoblastoma (3). Poverty among urban Latin American women has been associated with increased incidence of persistent (asymptomatic) cervical HPV infection (30) and invasive cervical carcinoma (31). Poverty has also been associated with incidence of retinoblastoma in New York City,4 in Latin America (32), and in the geographic distribution of worldwide incidence of retinoblastoma (1). Additionally, the association of an increasing incidence of retinoblastoma in countries severely affected by the HIV epidemic is also consistent with the possibility of HPV infection because women with HIV have higher rates of persistent HPV infection (33). Therefore, the presence of HPV DNA in retinoblastoma tissues is consistent with the overlap in epidemiologically determined risk factors for both the development of this disease and the detection of HPV infection.

In our series, children whose tumors contained HPV had significantly lower birth weights than children whose tumors did not contain HPV. This finding may suggest that these children may have been nutritionally deprived during pregnancy when compared with children with retinoblastoma without HPV, or it may be consistent with perinatal infection. The shorter duration of breast feeding in the children with HPV DNA in their tumors suggests that children with HPV DNA may have been living in less rural living conditions because breast feeding is known to be prolonged in more rural communities in Mexico. It is possible that those parents living in less rural communities might have had an increased number of sexual contacts. The older age of the fathers of children with HPV might be a proxy for an increased number of lifetime sexual contacts and an increased probability of infection (34). The fact that there was no difference in maternal age at delivery and no difference in the birth order of the index child suggests that there was no difference in the age at which the mothers began sexual activity. This is consistent with the study by Hernandez-Avila et al. (28), who found that the age at which Mexican women began sexual activity was not related to their risk of developing cervical cancer. The nonsignificant increase in self-reported vaginal infections in the mothers does suggest the possibility that this population may be more at risk for sexually transmitted disease. The incidence of urethral infections in fathers and information on paternal sexual histories were not available. Because of the transient nature of most HPV cervical infections, we would not be able to document maternal prenatal infection with HPV retrospectively, although serological testing of these women for the presence of anti-HPV VLP antibodies might be helpful. Unfortunately, we do not have sera available for either the mothers or the children.

The proportion of HPV16 to HPV18 in these tumors is the inverse of what would be expected given the distribution of high-risk HPV species among cervical cancer specimens from Mexican women. Among Mexican women with invasive cervical carcinoma, HPV16 has been found in 30–48% of cases, whereas HPV18 has been found in only 7–10% of cases (28, 35). However, the presence of HPV18 DNA in cervical cancer is associated with a higher incidence of adenocarcinoma and with significantly poorer survival than the presence of HPV16 DNA (36). The apparent excess of HPV18 in our study samples may be due to an increased oncogenic potential of HPV18 in retinal tissue. In addition, initial perinatal transmission rates for mothers infected with HPV18 are higher than those for mothers infected with HPV16 (37).

The presence of DNA from the high-risk HPV subtypes suggests that oncogenesis in retinoblastoma could occur in part as a result of E7 inactivation of pRB. There is evidence that osteogenic sarcoma, another pediatric tumor associated with RB alterations, may develop as a result of viral inactivation of pRB. Recent studies have demonstrated the presence of the pRB pocket-binding domain of the SV40 virus in 32% of osteogenic sarcomas examined (38). Detection of HPV DNA in retinoblastoma tissue appears to be correlated with pRB expression;
however, these pRB products appear to be hyperphosphorylated and are probably deficient in their activity, as reflected by the phenotypes observed using phosphorylation and E2F1-specific antibodies. Similarly, the finding of an equivalent number of cells staining with the anti-E2F1 antibody, regardless of HPV status, is consistent with the finding that HPV E7 activates E1A-responsive promoters on the E2F1 gene, thereby causing its release from the macromolecular complex that binds it (39, 40).

The Ki67 proliferative index of the retinoblastoma lesions studied increased significantly with increasing stage and was significantly higher in HPV-negative cases than in HPV-positive cases. It may be possible that in some tumors, E7 binds and inactivates most but not all pRB products, such that partial pRB activity remains. This functional pRB would therefore be able to keep some of the tumor cells from entering into the cycle.

The fact that HPV-positive tumors were less advanced in stage is particularly interesting in light of recent findings by Andl et al. (41). In their study examining the presence of HPV DNA in squamous cell carcinoma tumors of the head and neck region, tumors containing HPV DNA had a more favorable prognosis than expected, given the clinical staging (41). Additionally, some studies have demonstrated that HPV-negative cervical carcinomas appear to progress more rapidly, have a higher metastatic potential (12), and have a higher mortality rate (42) than HPV-positive tumors, although others have not found this association (43).

Four cases of nonfamilial bilateral disease included in the present study displayed HPV sequences. The ratio of bilateral to unilateral disease among the tumors containing HPV DNA was the same as the ratio found in the general population of patients with retinoblastoma. Although bilateral disease is usually caused by germ-line mutations in RB1, between 17% and 77% of cases of bilateral disease that have been sequenced have not been found to contain germ-line mutations in RB1 or its promoter (17, 19, 44). Again, the proportion of bilateral tumors found to have germ-line RB1 mutations appears to vary by geographic origin, suggesting that the proportion of bilateral disease caused by RB1 mutations may also vary geographically. Our findings suggest that some bilateral disease in tumors with intact RB1 may be caused by viral inactivation of pRB.

Sequences of HPV DNA are detectable by PCR-based assays in some retinoblastomas. The presence of viral DNA from the high-risk HPV types suggests that oncogenesis in certain retinoblastomas may occur as a result of E7 inactivation of pRB. This is consistent with the overlap in epidemiological risk factors between HPV infection (29, 30) and retinoblastoma (1). Detection of HPV DNA in retinoblastoma tissue appears to be associated with the presence of pRB expression, lower proliferative indices, and lower clinical stage. Additional studies are necessary to investigate the relationship of HPV and the presence of its E6 and E7 oncogenes to the development and progression of retinoblastoma.

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