Epstein-Barr Virus Detected in a Head and Neck Squamous Cell Carcinoma Cell Line Derived From an Immunocompromised Patient

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Background: DNA tumor viruses potentially play a role in the development of squamous cell carcinoma of the head and neck (SCCHN). Human papillomavirus is found in up to 50% of SCCHN specimens, and Epstein-Barr virus (EBV) has been detected in nasopharyngeal carcinoma, Burkitt lymphoma, and other lymphomas. However, the role of EBV in nonnasopharyngeal SCCHN has not been thoroughly investigated.

Methods: Twenty-one SCCHN cell lines derived from tumors of various subsites were used to screen for EBV DNA as well as latent viral protein expression. The method of EBV DNA detection was polymerase chain reaction with 3 independent primer sets from distinct regions of the genome. Expression of the viral protein EBNA-1, critical for the maintenance of the viral episome, was monitored by immunofluorescence using an antibody specific for EBNA-1. In addition, 12 paraffin-embedded tumor specimens and adjacent normal tissues were analyzed by polymerase chain reaction. These tumor specimens were further characterized by immunohistochemistry with the use of a mouse monoclonal antibody that recognizes EBNA-1.

Results: Little or no EBV DNA was detected in 20 of 21 cell lines or in any of the tumor specimens, while detecting approximately 40 genome copies in a control reaction. Accordingly, these cell lines and specimens were negative for EBNA-1 expression. In 1 cell line derived from an immunosuppressed patient, EBV DNA was detected, and on further examination a small percentage of cells expressed EBNA-1 as shown by immunofluorescence.

Conclusions: Although EBV may not be a major cofactor contributing to the proliferation of SCCHN, the limited initial evidence suggests that EBV may be involved in development of SCCHN in immunosuppressed patients.

gens, including 6 nuclear proteins (EBNA1, 2, 3A, 3B, 3C, and LP), 3 membrane proteins (LMP1, 2A, and 2B), and small RNA molecules (EBERs). Molecular genetic analysis of these latent genes has demonstrated that EBNA2, EBNA3A, EBNA3C, and LMP1 are essential for transformation of B lymphocytes. The EBNA1 binds to the “oriP” domain of the EBV episome and allows the episome to replicate and persist in latently infected cells. The EBV oncogene LMP1 is capable of transforming rodent fibroblasts and is shown to down-regulate p53-mediated apoptosis. In addition, there is a body of data supporting a link between LMP1 mutations and EBV-associated malignancies. The EBNA1 is expressed in all predominant forms of EBV-infected human cancers. The LMP1 is expressed in latency types 2 and 3, associated with NPC and lymphoproliferative disorders, respectively. Hence, these latent proteins are important for investigating the expression of EBV in NPC and carcinomas of other sites in the head and neck region.

Histologically, NPC is classified by the World Health Organization (WHO) into type 1 (squamous cell carcinoma [SCC]), type 2 (nonkeratinizing carcinomas), and type 3 (undifferentiated or lymphoepitheliomas and anaplastic carcinomas) tumors. Measuring serum antibody titers to capsid protein, Neel and colleagues found EBV more strongly associated with WHO type 2 and 3 tumors (elevated titers in 85% of 114 patients) than type 1 tumors (elevated titers in 16% of 37 patients). They also found elevated titers in individuals with other malignant tumors of the head and neck, although titers in patients in the NPC group as a whole were more significantly elevated. This study did not look for the presence of EBV DNA in actual tumor specimens, and given that there are known immunodeficiencies in patients with SCCHN, it is possible that measurement of antibodies in patient serum could underestimate the role of EBV, especially in WHO type 1 and other SCCHN tumors.

A limited number of studies resulted in conflicting conclusions regarding EBV detection in nonnasopharyngeal carcinomas of the head and neck. It has been postulated that other tissues of Waldeyer tonsillar ring including the palatine tonsil and base of tongue have histopathological characteristics similar to those of the nasopharynx and thus may also harbor EBV. Although SCCs account for the majority of tumors at these sites, nonkeratinizing, undifferentiated, and lymphocytic type cancers are also frequently seen. Klijanienko and colleagues evaluated 425 undifferentiated SCCs of the tonsil and determined that 18 had histologic characteristics of undifferentiated carcinomas of nasopharyngeal type. Of the 7 with serum samples available for analysis, 4 had strongly elevated anti-EBV titers, leading to the conclusion that EBV-associated tumors may arise outside of the nasopharynx. Similarly, another study reported 23 nonnasopharyngeal EBV-associated carcinomas arising from various head and neck regions, including salivary glands, sinonasal regions, the middle ear, the lacrimal gland, and the tonsil. Using polymerase chain reaction (PCR), were able to detect EBV in 30 (68%) of 44 nonnasopharyngeal head and neck carcinomas. Finally, a Japanese study found 15% of oral SCCs to be positive for EBV by PCR and Southern blot.

Studies from other groups resulted in contrasting results. One study using in situ hybridization with sulfur S 35–labeled probes could not detect EBV in 26 SCCs of the tonsil. Similarly, Atula and colleagues, using Southern blot analysis, could not detect EBV in any of 79 head and neck carcinoma specimens. Nicholls and colleagues compared 5 undifferentiated carcinomas of the tonsil with 5 SCCs of the tonsil for the presence of EBV by in situ hybridization with probes for early RNA (EBERs). All cases of SCC and 4 of 5 cases of undifferentiated carcinomas were negative for EBV. One study found 35% of oral SCCs to test positive for EBV DNA by PCR, but concluded that EBV infection was not directly associated with the pathogenesis, since 25% of healthy oral mucosa also tested positive for EBV DNA. Finally, a PCR-based study also failed to detect EBV DNA in 52 of 53 cases of SCC of the tonsil.

After reviewing these studies, it was difficult to draw a conclusion regarding whether EBV is present in nonnasopharyngeal SCCHN. First, PCR was the most sensitive method of detection used, and the few studies that used PCR yielded conflicting results. Khabie and colleagues did not detect EBV in SCCHN, but they only looked at tonsil carcinomas. Another study found EBV in 68% of specimens, with the hypopharynx being the most common subsite. Thus, while it may be intuitive that EBV is more likely present in tissues of Waldeyer tonsillar ring, it may actually play a role in SCCHN of other subsites. Second, studies looking for EBV by only one method may be problematic, as genomic deletions or rearrangements may exist in the targeted area of DNA and herpesviruses are known to have defective genomes. Likewise, methods looking for EBV protein expression may give false-negative results, since the EBV latent antigens may be expressed temporarily or have specific expression patterns in distinct types of human tumors.

Detection of EBV can be greatly increased by selecting primers and probes from multiple genomic regions to maximize detection if deletions are present, as well as measuring the expression of essential genes like EBNA1 that are more likely to be a predominant viral antigen expressed in the majority of latently infected cells. Therefore, we analyzed SCCHN cell lines derived from tumor biopsy specimens as well as tumor specimens from various subsites for the presence of EBV DNA with multiple PCR primer sets. In addition, we analyzed these samples for the expression of essential latent antigens. Our results from these initial studies suggest that EBV may have a role in the development of SCCHN, primarily in immunocompromised patients.

**METHODS**

**CELL LINES AND ANTIBODIES**

Twenty-one established University of Michigan SCC (UMSCC) cell lines derived from various head and neck subsites were obtained from the University of Michigan Head and Neck Cancer Core (Ann Arbor). Cell lines were grown under standard...
tissue culture conditions in Dulbecco modified Eagle medium (Invitrogen/GIBCO, Bethesda, Md) with 10% fetal bovine serum supplemented with penicillin and streptomycin. Cells were harvested with trypsin at about 70% confluency and passed or used for DNA extraction or plated for immunofluorescence. The EBV-positive lymphoblastoid cell lines, P3HR-1 and Namalwa, were used as positive controls, and the B-cell line, BJAB, was used as a negative control. These suspension cells were grown in RPMI-1640 medium (Invitrogen/GIBCO) with 10% fetal bovine serum supplemented as above. Cells were passaged regularly and harvested when needed for DNA extraction or plated for immunofluorescence.

Antibodies against EBNA-1 were obtained from human polyclonal serum adsorbed against B-cell antigens, which were then tested for EBNA-1 specificity. The EBNA-1 monoclonal antibody (13-156-10) was purchased from Advanced Biotechnologies Inc, Columbia, Md.

DNA EXTRACTION

The UMSCC cell lines were harvested by trypsinization with the use of 0.25% trypsin-EDTA (Invitrogen/GIBCO), washed with phosphate-buffered saline (PBS), and counted with a hemocytometer. Approximately 1 × 106 cells were resuspended in 500 mL of 0.2× PBS. Cells were lysed at 95°C for 30 minutes, incubated with 500 µg of proteinase K at 55°C for 1 hour, and heated at 95°C for 30 minutes to inactivate the proteinase K.

PCR AND SOUTHERN BLOT HYBRIDIZATION

All extracted samples were first amplified with β-actin primers to ensure that the integrity of extracted DNA was consistent for each sample. The 25-µL reactions consisted of 5 µL of DNA, 1× PCR buffer, 200µM deoxyribonucleotides triphosphates, 2mM magnesium chloride, 500nM each of forward and reverse primers, and 2.5 U of Taq polymerase. Amplification was carried out in a thermocycler (MJ Research, Inc, Boston, Mass) as follows: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 40 cycles followed by a final extension at 72°C for 7 minutes. The P3HR1 cell line was used as a positive control, and water (no DNA added to the PCR) as the negative control. Samples were run on a 3% agarose gel stained with ethidium bromide to confirm a 353-base pair amplified product. Precautions were taken in all PCRs, including UV irradiation of reaction mixtures, and all work areas and instruments were cleaned before every analysis to avoid cross-contamination with viral DNA.

Three specific PCRs were performed for EBV detection for each series of analysis. All sets included the P3HR-1 cell line as a positive control, the BJAB cell line as a negative control, and a no-DNA-added (water) control. In addition, serial dilutions were made of Namalwa cells, containing 2 copies of EBV genome per cell, and run with each PCR to estimate the sensitivity of the reactions. All PCR products were then run on a 3% agarose gel stained with ethidium bromide and photographed. The BamHI-122 primers and the PO primers and reaction conditions have been previously described. The 25-µL reactions consisted of 5 µL of DNA, 1× PCR buffer, 200µM dNTPs, 1.5mM magnesium chloride, 400mM each of forward and reverse primers, and 2.5 U of Taq polymerase. Cycling consisted of 40 cycles of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, followed by a 10-minute extension at 72°C.

The PCR products from the reaction with BamHIW primers were transferred from the agarose gel to a nylon membrane (NEN Life Science Products, Boston, Mass). The membrane was prehybridized in 0.8× SSAC (sodium saline citrate), 5× Denhardt solution, 0.3% sodium dodecyl sulfate, and 100-µg/mL salmon sperm DNA at 60°C for 3 to 4 hours, and then hybridized with a γ-phosphorilus P32-labeled EBV-specific probe from the BamHIW region. The membrane was hybridized overnight at 60°C, washed at moderate stringency (melting temperature, 20°C), and exposed to film at −80°C for 24 to 72 hours. In a separate experiment, the membrane was also hybridized with a digoxigenin-labeled oligonucleotide probe (Roche Diagnostics Inc, Mannheim, Germany) specific for a sequence internal to the PCR primers (previously described by Gan et al46). Prehybridization and hybridization were performed in digoxigenin hybridization granules (DIG Easy Hyb Granules; Roche Diagnostics Inc) according to the manufacturer’s protocol. After hybridization overnight at 32°C, the membrane was washed and the color detection was performed with DIG Nucleic Acid Detection Kit (Roche Diagnostics Inc). The membrane was photographed after various time intervals up to 24 hours.

ANALYSIS OF CELL LINES BY IMMUNOFLUORESCENCE

The cells of the UMSSC cell line were harvested, plated onto 8-well chamber slides, and incubated overnight in 500 µL of Dulbecco modified Eagle medium with 10% fetal bovine serum as described above (“Cell Lines and Antibodies” subsection of the “Methods” section). When cells reached confluency, they were washed with PBS, fixed in a 1:1 ratio of methanol to acetone at −20°C for 10 minutes, and air dried. Control slides using P3HR-1 (positive) and BJAB (negative) cells were prepared as previously described. The fixed samples were blocked in 20% goat serum for 1 hour at room temperature in a moist chamber and washed with PBS 3 times. The primary antibody is a human polyclonal serum that detects the EBNA-1 antigen, used at a 1:25 dilution in 1× PBS, and was confirmed by Western blot for specific EBV nuclear antigens. Samples were then incubated in primary antibody at room temperature in a moist chamber for 2 hours and washed with PBS 4 times at 5-minute intervals. The samples were then incubated in the secondary antibody, goat anti–human IgG Alexa-Fluor at 1:1000 dilution (Molecular Probes, Inc, Eugene, Ore), for 1 hour in a dark, moist chamber at room temperature. After washing with PBS 5 times at 5-minute intervals, antifade was applied and slides were sealed with a coverslip and nail polish. Slides were visualized by standard fluorescence microscopy. Photographs were collected with a digital camera (Pixel Fly; Cooke Co, Auburn Hills, Mich).

PROCUREMENT OF TUMOR SPECIMENS

Approval for the use and analysis of archived paraffin-embedded tumor specimens was obtained from the institutional review board at the University of Michigan (institutional review board No. 002-0253). All patients presenting with previously untreated SCCHN who underwent primary surgical therapy for their disease between April 1999 and June 1999 were chosen for analysis. Twelve paraffin-embedded specimens were obtained from the surgical pathology archives. The first slide was cut for hematoxylin-eosin staining and analyzed by a staff pathologist (A.P.) for the presence of SCC and adjacent normal tissue.
All extracted samples were first amplified with sterile, distilled water. Samples were then extracted with phenol–chloroform–proteinase K was inactivated by heating at 95°C for 15 minutes. K, 0.75 μg/mL proteinase

Each of forward and reverse primers, and 2.5 U of Taq polymerase. Cycling consisted of 40 cycles of 95°C for 1 minute, 58°C for 1 minute, and 72°C for 75 seconds followed by a 10-minute extension at 72°C.

**RESULTS**

**ABSENCE OF EBV DNA IN MOST SCCHN CELL LINES**

The PCR analyses of UMSCC cell lines indicated that there was little or no specific EBV DNA in the majority of cell lines. Twenty-one UMSCC cell lines were analyzed for the presence of EBV. The cell lines were derived from carcinomas of varying head and neck subsites (Table 1). Cell lines designated “A” and “B” are derived from the same patient and indicate different stages in disease progression or treatment after diagnosis. After adequate DNA extraction was confirmed by performing PCR with β-actin primers on all samples, PCR for specific EBV sequences was performed. The BamHI-122 and the PO primers are derived from conserved sequences on the EBV genome and have been previously shown to be sensitive primers for detection of viral DNA41,42 (Table 2). In these sets of analyses, little or no detectable amplification of EBV was seen in any of the 21 samples by PCR using the BamHI-122 primers as well as the PO primers. However, both reactions were able to detect relatively low copy numbers of EBV genome in the Namalwa cell line controls (400 copies for BamHI-122 primers and 40 copies for the PO primers), indicating that the limits of detection for these primer sets are likely not the explanation Table 3).

The PCR analysis with BamHIW primers, which amplify a portion of the EBV large internal repeat region, was then performed so as to maximize sensitivity due to copy number. This region consists of an approximately
A 3-kilobase sequence that is repeated up to 11 times in the EBV genome, hence increasing sensitivity. Again, this reaction showed adequate sensitivity, with the ability to detect about 40 copies of EBV DNA in the Namalwa control lanes, but showed little or no definitive detection of EBV DNA in any of the samples with the exception of UMSCC 81B (lane 25, Figure 1A). After Southern blot was performed on these PCR products, EBV signal was confirmed in the UMSCC 81B cell line, and possibly in the UMSCC 17A and 53 cell lines (lanes 14 and 23), but was undetectable in any of the other samples (Figure 1A, lower panel). The signal from UMSCC 81B, 17A, and 53 was low, being less than the signal in lane 31, which corresponded to 40 copies of Namalwa EBV DNA (Figure 1A; compare lanes in lower and upper panels).

POSSIBLE PRESENCE OF EBV LATENT GENES IN ONE SCCHN CELL LINE

Immunofluorescence analysis confirmed a potential positive signal for EBV in UMSCC cell line 81B (Figure 2). Next, the samples were analyzed for the presence of EBV latent gene expression by immunofluorescence with human polyclonal serum that strongly expresses EBV EBNA-1 antibodies. An EBNA-1 antibody was chosen because it is expressed as one of the predominant latent antigens in the types of EBV latency states described. All samples were analyzed along with both positive (P3HR-1) and negative (BJAB) controls. A strong signal was derived from the positive cells and a faint green background was seen in the negative cells (Figure 2). Only UMSCC 81B showed a specific signal similar to the positive control above the background level. Note that this signal seen was low compared with the positive control cell line, which showed signal in nearly all cells, indicating that only a small percentage of cells were expressing EBNA-1 and may have been positive for EBV genome. The other 20 cell lines analyzed showed no signal above the background level, as shown in a similar analysis of UMSCC cell line 11B (Figure 2).

NO DETECTABLE EBV DNA IN SCCHN TUMOR SAMPLES

The EBV DNA was not specifically amplified by PCR analysis of SCCHN tumor specimens. Twelve primary

<table>
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<tr>
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<th>Product Size, bp</th>
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<td>EBV ORF for major capsid protein</td>
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<td>PO</td>
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<td>EBV ORF expressed in late viral replication</td>
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<td>BamHIW</td>
<td>5'-GGTCCGATGCACAGG</td>
<td>110</td>
<td>Region of EBV large internal repeat</td>
<td>Sixbey and Yao, 1992</td>
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Abbreviations: bp, base pairs; EBV, Epstein-Barr virus; ORF, opening reading frame; PCR, polymerase chain reaction.

<table>
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<tr>
<th>Sample</th>
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<tr>
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<td>+</td>
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<td>UMSCC 14A</td>
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<tr>
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<tr>
<td>Namalwa—4</td>
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Abbreviations: EBV, Epstein-Barr virus; PCR, polymerase chain reaction; UMSCC, University of Michigan squamous cell carcinoma.

SCCHN specimens were obtained from previously untreated patients. After the hematoxylin-eosin staining of the specimen was analyzed, 7 specimens had normal tissue adjacent to the tumor tissue present in the sample. This normal tissue was extracted as a control along with the tumor for comparison. The tumor specimens were labeled “A,” and the corresponding adjacent normal tissue, if present, was labeled “B.” The specimens were derived from various sites and were mostly of higher stages (Table 4).
As with the UMSCC cell lines, the presence of amplifiable DNA was confirmed in all 19 specimens (12 tumor specimens and 7 adjacent normal specimens) by PCR with \( \beta \)-actin primers (Figure 3B). The PCR was then performed with both the BamHI–122 and the PO primers. The EBV was not detected in any of the 19 specimens by either the Bam HI–122 primer set (data not shown) or the PO primer set (Figure 3A). The PCR with BamHI–122 primers was able to detect as few as 400 copies of EBV DNA, and the reaction with PO primers was able to detect 40 copies. Thus, both reactions were relatively sensitive despite the inability to detect EBV in any of the 19 specimens. Estimating a lower limit of 10 copies per cell, a minimum of approximately 4 cells positive for EBV would be detected if 10,000 cells were used in each reaction.

Further immunohistochemistry analysis of SCCHN tumor specimens did not indicate any specific EBV signal for EBNA-1 expression. The EBV EBNA-1 expression was then analyzed by immunohistochemistry with the use of a mouse monoclonal EBNA-1 antibody (Figure 4). An EBNA-1 antibody was chosen because it is the predominant viral antigen expressed in the known types of latency.\(^3\) There was distinctive brown nuclear staining in the positive control, indicating EBNA-1 expression, in approximately 30% of the lymphoma cells. However, no nuclear staining was appreciated in either of the negative controls, indicating that the staining in the positive control is specific and not background. Analysis was performed on all 12 tumor specimens and all 7 of the adjacent normal tissues. These tumor samples failed to show specific nuclear staining consistent with EBNA-1 antigen expression. There was no difference in staining between the tumor specimens and the corresponding adjacent normal tissues from the 7 control regions.

### COMMENT

Accurate determination of viral prevalence and gene expression in head and neck carcinomas will help us understand whether these tumors represent a biologically distinct subset of SCCHN with different implications for treatment and prognosis. For example, our group previously demonstrated that survival is statistically longer in patients whose SCCHN contained HPV DNA sequences than in those without,\(^7\) and Gillison et al\(^1\) had similar findings, with HPV-positive tumors having a 59% reduction in risk of death from cancer compared with HPV-negative tumors. Head and neck tumors associated with EBV also seem to represent a unique subset. It is well known that NPCs are highly radiosensitive, especially when compared with other SCCHNs. It has not been determined whether this radiosensitivity is due to the effects of EBV. However, Neel and colleagues\(^26\) found a definite trend toward worse prognosis in WHO type 1 (less associated with EBV) vs WHO types 2 and 3 (commonly associated with EBV). The WHO types 2 and 3 had 3- and 5-year survival rates of 65% and 52%, respectively, and WHO type 1 had rates of only 37%. Furthermore, Klijanienko and colleagues\(^32\) found that in 18 cases of undifferentiated carcinoma of nasopharyngeal type of the tonsil, 10-year survival was 77%, which is significantly better than for other histologic types of tonsillar carcinoma. Seven of these patients had serum samples available for analysis, and 4 of the 7 had EBV serologic levels similar to those seen in NPC. Thus, the difference
in biologic behavior of the specific tumors may depend on the presence or activity of associated biologic agents such as EBV and HPV.

Given the conflicting and limited data regarding the presence of EBV in nonnasopharyngeal SCCHN, we set out to determine whether EBV was present in SCCHN from various subsites. First, 21 established SCCHN cell lines were analyzed by PCR and immunofluorescence. Our data showed that, with the exception of 1 cell line (UMSCC 81B), little or no EBV was detected by PCR analysis with the use of 3 independent primer sets in the majority of cell lines. These primers were determined to be sensitive enough to detect as few as 40 copies of EBV per reaction, as demonstrated with control DNA. A positive signal for EBV in UMSCC 81B samples was detected in 1 of 3 primer sets used in our analysis. This indicates that the remaining viral genome may have deletions or rearrangements so that it would not be detectable by the other 2 primers. The results from immunofluorescence corroborate the PCR results, namely, EBNA-1 expression was detected in the UMSCC 81B sample and not in the other cell lines.

There is evidence indicating that EBV DNA may be lost in cell lines passaged over time in culture. The EBV is generally maintained as an episome in EBV-infected cells, and the maintenance is dependent on binding of EBNA-1 to the replication origin of the plasmid (oriP). However, up to 4% of infected cells per generation may lose EBV plasmids. A substantial decrease in the level of EBV plasmids was shown in Akata and Mutu lines of Burkitt lymphoma when transfected with EBERs but not in other Burkitt lymphoma lines, suggesting that the EBERs are critical components of the EBV proliferation scheme. Spontaneous loss of episomal DNA during long-term culture of the Mutu cell line has also been observed. Others found low concentrations of hydroxyurea to completely eliminate EBV episomes from latently infected Burkitt lymphoma cell lines. Nasopharyngeal carcinoma cell lines infected with EBV also experienced loss of the viral episome, with EBV detected at passage 35 but not at passage 45, correlating with a progressive decrease in level of EBNA-1 expression.

Thus, while many EBV-transformed cell lines do maintain episomal DNA, the evidence that many do not, led us to also investigate paraffin-embedded tumor specimens. Twelve tumor specimens and 7 adjacent normal tissues from patients with previously untreated SCCHN were analyzed. The EBV DNA was not detected in our experimental systems in any of the tumor specimens or the adjacent normal tissue specimens, and the sensitivity of the PCR minimized the chance of false negatives. Thus, our results are comparable with those of another PCR-based study that found that EBV DNA is not increased in tonsillar carcinoma. Our results suggest that EBV DNA is not present in other subsites of the head and neck, which is in contrast to the other PCR-based studies that detected EBV sequences in 30 (68%) of 44 non-NPC SCCHN specimens and in 7 (15%) of 46 oral SCC specimens. These studies may be a consequence of genetic and environmental differences between Asia and North America, which may explain the difference in EBV detection.

Interestingly, the UMSCC 81B cell line was positive for EBV as detected by PCR and Southern blot with the BamHIW primers, as well as showing an EBNA-1–
Figure 4. Immunohistochemistry for detection of EBNA-1 by means of EBNA-1 monoclonal antibody and biotin-streptavidin-peroxidase detection system. A, Positive control (Epstein-Barr virus–positive lymphoma tissue) demonstrating brown nuclear staining representing expression of EBNA-1 in approximately 20% to 30% of cells. B, Negative control (normal mouse serum as antibody instead of EBNA-1 antibody) showing no nonspecific staining. C, Negative control (no antibody added) demonstrating no nonspecific staining from detection reagents. D, Tumor specimen 1A demonstrating no cells positive for expression of EBNA-1.

Table 3. Analysis of tumor specimens and adjacent normal tissues.

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<td>M + W</td>
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Figure 3. Analysis of tumor specimens and adjacent normal tissues. A, Polymerase chain reaction (PCR) with PO primers demonstrating a 178–base pair (bp) product. Lane 1, φX 174 DNA marker. Lane 2, P3HR1 cell line (Epstein-Barr virus [EBV]–positive control). Lane 3, BJAB cell line (EBV-negative control). Lane 4, no DNA added (water control). Lanes 5 to 23, tumor specimens and adjacent normal tissue (see Table 3). Lane 24, blank. Lanes 25 to 29, serial dilutions of Namalwa cell line (contains 2 copies of EBV genome per cell) indicating the number of copies of EBV genome per reaction. B, PCR with β-actin primers showing a 353-bp amplified product. Lane 1 represents φX 174 DNA marker. Lanes 2 and 3 represent the positive control (P3HR1 cell line) and water control (no DNA added), respectively. Lanes 4 to 22 represent the tumor specimens and adjacent normal tissues.

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positive signal in a small percentage of cells. This cell line was derived from an SCC of the larynx in a patient who had received immunosuppressive therapy for 20 months for a heart transplant before his cancer diagnosis. We did not have access to other cell lines or specimens that were derived from immunosuppressed patients. There is a well-known association between EBV-induced disorders and immunosuppression. Oral hairy leukoplakia, a nonmalignant lesion of the tongue, is associated with multiple strains of EBV and is seen almost exclusively in patients infected with human immunodeficiency virus. In addition, EBV-induced posttransplant lymphoproliferative disorders ranging from plasmacytic hyperplasias to malignant B-cell lymphomas arise after bone marrow transplantation or immunosuppression for transplant surgery and can be particularly aggressive. Posttransplant lymphoproliferative disorders occur in 1% to 20% of solid-organ transplant recipients, and EBV infection is the greatest risk factor for development of this proliferative disease. Thus, EBV may play a role in the development of SCCHN in the immunosuppressed population, as seen in this initial study in which UMSCC 81B was identified as positive for EBV. The facts that EBV was detected only after Southern blot of PCR products and that only a small percentage of cells were expressing EBNA-1 supports the hypothesis that EBV may have played an early role in oncogenesis but was lost in the majority of tumor cells on passage in culture.

In conclusion, this study suggests that EBV may not be present at significantly high levels in SCCHN, with the possible exception of SCCHN in the immunosuppressed population. This is in contrast to HPV, which can be detected in up to 40% of SCCHN, and in contrast to NPCs, which have a well-documented association with EBV. The association is much less clear for WHO type 1 NPC (squamous histologic features) than WHO type 3 (undifferentiated), and the differing histologic features may account for the predilection of EBV for undifferentiated tissues over squamous tissues. Knowledge of this difference may lead to future studies investigating the mechanisms of EBV-related oncogenesis in NPC. In addition, the possibility of antiviral therapy for patients with SCCHN arising in the setting of immunosuppression clearly deserves further investigation.

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