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Association between HPV/EBV co-infection and oral carcinogenesis

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Abstract

Background—The recent epidemic of head and neck squamous cell carcinomas (HNSCC) associated with human papilloma virus (HPV) has not addressed its association with lymphoid tissue in the oropharynx or the potential role of Epstein-Barr virus (EBV)/HPV co-infection.

Methods—The prevalence of HPV and EBV infection/co-infection and CD21 mRNA expression were determined in normal and cancerous tissues from the oropharynx using in situ hybridization (ISH), p16 and quantitative reverse transcriptase PCR (qRT-PCR). The effects of co-infection on tumorigenicity were evaluated using proliferation and invasion assays.

Results—Normal oropharynx, tonsil, non-cancer base of tongue (BOT) and BOT from sleep apnea patients, demonstrated EBV positivity ranging from 7-36% depending on the site and methods of detection used (qRT-PCR or ISH). Among non-malignant BOT samples HPV positivity was noted only in 20%. The percent of tonsil and BOT cancers positive for HPV (up to 63% and 80%, respectively) or co-infected with HPV/EBV (up to 25% and 70%, respectively) were both significantly associated with cancer status. Notably HPV/EBV co-infection was observed only in malignant tissue originating in lymphoid-rich oropharynx sites (tonsil, BOT). CD21 mRNA (the major EBV attachment receptor) was detected in tonsil and BOT epithelium, but not in soft palate epithelium. Co-infected cell lines showed a significant increase in invasiveness ($p < 0.01$).

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Conclusions—There is a high prevalence of HPV/EBV infection and co-infection in BOT and tonsil cancers, possibly reflecting their origins in lymphoid-rich tissue. In vitro, cells modeling co-infection have an increased invasive potential.

Keywords

oral carcinogenesis; human papilloma virus; Epstein-Barr virus; squamous cell carcinoma; co-infection; tumorigenesis

Introduction

In the last few years, there has been a dramatic and alarming epidemic of human papilloma virus (HPV)-associated head and neck squamous cell carcinomas (HNSCC). Importantly the incidence of oral cavity cancers has decreased due to decrease in tobacco use, while oropharyngeal tonsil and BOT cancers have increased, especially in the younger population (1). HPV has been detected in 25% of all head and neck cancers and 50-70% of oropharyngeal carcinomas (2). Ninety percent of the HPV-associated tumors are positive for the high risk HPV type 16; less frequently oropharyngeal tumors are associated with HPV type 18. Unlike most other head and neck cancers, they are often seen in men in their 30s or 40s who are non-smokers and non-drinkers. Although cure rates are high, these young patients are left with long lasting functional morbidity and difficulty swallowing. Many are feeding tube and some are tracheostomy-dependent. Case-control epidemiologic studies have shown that the HPV-associated malignancies are sexually transmitted with oral sex being the major predisposing factor. Their incidence is directly related to the number of oral sex partners (3-5).

Many studies indicate improved survival in patients with HPV-positive tumors (6), but none have evaluated the reason for an association between HPV-related malignancies and the oropharynx. It is not clear why HPV-induced carcinomas are only noted in the tonsil and base of tongue (BOT) and not in the other areas of the oral cavity such as the anterior oral tongue, floor of mouth buccal mucosa, or even the third site of the oropharynx the soft palate. The tonsils and BOT differ from all the other sites in the oral cavity in their histologic make up of lymphoid tissue. The only other region of the head and neck where lymphoid tissue is abundant is the nasopharynx. The lymphoid tissues of these sites of oropharynx and nasopharynx are referred to as Waldeyer's Ring and the nasopharynx is also the site of tumors caused by the oncogenic Epstein-Barr virus (EBV).

EBV is an orally transmitted herpesvirus, carried by more than 90% of the adult population that is known to have tropism for lymphoid and epithelial cells (7). B lymphocytes are the main reservoir of latency in the tonsil (8) and virus is amplified in tonsil epithelium (9, 10). Most individuals are asymptotically infected, but it was recognized early on that the virus has tumorigenic potential and is associated with both lymphoid and epithelial malignancies (11, 12). It is associated with close to 100% of undifferentiated nasopharyngeal carcinomas and, although controversial, with a small percentage of oral squamous carcinomas (13). EBV and HPV associated tumors differ from smoking related HNSCC in many ways. Both NPC and oropharyngeal tumors have a high incidence of nodal metastasis but prognosis is

not adversely affected by metastasis, unlike at other sites where nodal metastasis decreases survival by 50% (14). Virally associated tumors are also radiosensitive and have a much better prognosis than other HNSCC (15). These similarities led us to hypothesize that oropharyngeal malignancies might be associated with both viruses rather than just HPV alone.

Exposure to EBV and HPV is much more common than the incidence of malignancy. Hence, identifying biomarkers that can potentially predict which individuals exposed to the viruses are likely to develop tumors is crucial to prevention of disease. Susceptibility of cells to infection with EBV is tightly controlled by cell surface proteins with which virus envelope glycoproteins interact. They mediate virus attachment and trigger fusion of the virus envelope with the cell membrane to facilitate entry of the infectious tegument and nucleocapsid. The major attachment receptor for EBV is the complement receptor type 2 or CD21. It is found on all mature B cells, but its expression on epithelial cells *in vivo* is less frequent. Previously we demonstrated that of all the epithelial tissues in the oral cavity and oropharynx, which included oral tongue, buccal mucosa, soft palate, and the uvula, only tonsil epithelium expressed CD21, a major factor in terms of cell susceptibility (16). Hence we wanted to determine if CD21 is expressed in the base of tongue potentially predisposing epithelium in this oropharynx site to EBV infection and if CD21 expression could be a potential biomarker.

In this study, we test our hypothesis that it is the co-infection of HPV with EBV and not HPV alone that may be required for malignant transformation of HPV associated malignancies and that co-infection increases the tumorigenic potential of the cells. We sought to determine the occurrence of EBV and HPV co-infection in patients with tonsil and BOT tumors, to compare them to non-lymphoid soft palate tumors, the third site of the oropharynx, and assess the expression of CD21. We also compared various techniques for virus detection: *in situ* hybridization (ISH) or reverse transcriptase quantitative polymerase chain reaction (qRT-PCR) of RNA from cells obtained by laser capture microdissection for EBV, and ISH and p16 expression for HPV virus detection. To determine whether co-infection of EBV and HPV further increases cellular tumorigenic potential compared to either virus alone, we assessed cell proliferation and invasiveness of normal and malignant cell lines expressing various combinations of these viruses.

Methods

Tissue Samples

Tissue sections on positively charged slides from archived paraffin embedded blocks were obtained for each of the following categories: 1) Tonsil SCC ($n=16$); 2) base of tongue (BOT) SCC ($n=15$); 3) soft palate SCC ($n=10$) as negative controls; 4) normal tonsils from patients with obstructive sleep apnea ($n=13$); 5) non-cancerous BOT samples from random biopsies of patients with unknown primaries ($n=10$); 6) normal BOT samples from the patients undergoing base of tongue reduction for obstructive sleep apnea ($n=15$; obtained from Dr. Eben L. Rosenthal from the University of Alabama at Birmingham); 7) soft uvula from patients who underwent uvulopalatopharyngoplasty for sleep apnea ($n=12$). Study samples were obtained from newly diagnosed HNSCC patients treated at Louisiana State

University Health Science Center, Shreveport. The study was approved by the Institutional Review Board.

Laser Capture Microdissection

Paraffin-embedded specimens of all tissue groups were cut to a thickness of 4 μ m and stained. Epithelial cells were identified by morphology and captured using Laser Capture Microdissection (LCM) (16). Only cells in the keratogenous zone were captured. Total RNA from captured cells was extracted, purified, DNase treated, and reverse transcribed. A human formalin-fixed universal reference RNA was reverse transcribed in parallel for use as a positive control.

Detection of EBV and HPV

EBV status was determined on tissue slides by ISH using EBER1 DNP Probe (Ventana, Tucson, Arizona). Also EBV status was determined in the epithelial cell samples captured by LCM via analysis of EBV-encoded RNA1 (EBER1) expression using qRT-PCR. Tissue samples were examined for their HPV status using in-situ hybridization (performed by The Delta Pathology Group, LLC; Shreveport, LA) and our study pathologist then scored the samples as either positive or negative for staining of epithelial cells. The INFORM HPV III Family 16 (B) probe from Ventana (Tucson, Arizona), was used to detect HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. Tissue samples were also evaluated by immunohistochemistry for p16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma (CINtec® p16 Histology, Ventana, Tuscon, Arizona).

Reverse Transcriptase Real-Time Quantitative PCR (qRT-PCR)

qRT-PCR was performed as previously described (16) to detect the presence of the EBV EBER 1, CD21, GAPDH and CD20 transcripts. EBER 1 was detected with primers 5'-CGTCCCGGGTACAAGTCC-3' and 5'-AAGACGGCAGAAAGCAGAGTCT-3' and the probe 5'-/56-FAM/TGAGGACGGTGTCTGTGGTTGTCTTCC/36-TAMSp/-3'. Primers and probes for CD21, CD20 and GAPDH were as described previously (16). The level of CD21 mRNA expression relative to GAPDH was calculated by the comparative Ct method (17). CD20 was amplified to control for potential inclusion of EBV infected B cells. Control experiments showed that the sensitivity of the assay was such that 1 B cell could be detected in a background of 1,000 epithelial cells (data not shown). qRT-PCR to detect HPV16 E7 was done with primers 5'-atgagcaattaaatgacagctcagag-3', (nt 660-635) and 5'-cacactgcaacaaaagggtacaat-3' (nt 732-745) and amplification was detected with Power SYBR green (Applied Biosystems). The specificity of amplifications was confirmed by melting curve analysis.

Cell Lines

FaDu, a cancer cell line established from hypopharyngeal SCC, was procured from ATCC. FaDu cultures were maintained in MEM media supplemented with 10% FBS and non-essential amino acids. Normal oral keratinocytes (NOK), a gift from Dr. Carl Munger at Harvard Medical School/Brigham and Women's Hospital, Boston, MA, was cultured in

Keratinocyte Serum Free Medium (KSFM) supplemented with bovine pituitary extract and Human Epidermal Growth Factor (hEGF).

The cell clones of FaDu and NOK with four different combinations of EBV/HPV expression were generated (HPV-/EBV-, HPV-/EBV+, HPV+/EBV-, and HPV+/EBV+) by stably transfecting the cells with a plasmid carrying HPV16 E6 and E7 ORFs under the control of the immediate early cytomegalovirus promoter in plasmids carrying hygromycin resistance (FaDu) or Zeocin (NOK) for selection. Cognate control vector lacking E6 and E7 were transfected as controls. Then these cells were infected with a recombinant EBV strain carrying a neomycin resistance cassette or transfected with a vector control carrying the neomycin resistance cassette. EBV-positivity and ectopic expression of E6 and E7 were confirmed by using reverse-transcription PCR with gene specific primers (HPV16 E6F – 5' CTG CAA TGT TTC AGG ACC C 3'; HPV16 E6R – 5' TCA GGA CAC AGT GGC TTT T 3'; HPV16 E7F – 5' CCC AGC TGT AAT CAT GCA TG 3'; HPV16 E7R – 5' TGC CCA TTA ACA GGT CTT CC 3'; EBV LMP2F – 5' CGACCCCATATCGCAACACT 3'; EBV LMP2R – 5' CGTGCCATTGCTGTGGAAG 3').

Cell Proliferation Assay—In order to determine whether HPV/EBV co-infection enhances cell growth, an *in vitro* cell proliferation assay was conducted on both FaDu and NOK by either cell counting or using MTS tetrazolium compound/phenazine methosulfate system according to the manufacturer's instructions (CellTiter 96 AQueous cell proliferation assay; Promega Corp., Madison, WI). Each assay was conducted at least 3 times for statistical significance.

Cell Invasion Assay—The same four HPV/EBV cell clones of FaDu and NOK were used for cell invasion assay using 24-well plates and transwell inserts with an 8-μm pore size. The interiors of the transwells were collagenized using 100 μL of 15 μg/mL collagen solution (Sigma-Aldrich, from human fibroblasts) and incubated at 4°C overnight. The next day, wells in 24-well plates were loaded with 600 uL of serum-free (for FaDu cells) or pituitary extract/EGF-free (for NOK cells) media. In one set of wells, medium with no lysophosphatidic acid (LPA; Sigma-Aldrich) was used to evaluate baseline cell invasiveness and compared to LPA induced invasiveness. Collagenized transwell inserts were washed, and placed into the wells with culture media. 20,000 cells were plated per well into each insert and incubated for 24 hours. Then the inside of each transwell was swabbed to remove any cells that had not migrated through the 8-μm pores. Cells were then fixed with methanol and stained with 0.2% Crystal Violet. The number of cells that had invaded was determined using light microscopy at x200 magnification. This experiment was repeated six times for statistical significance.

Statistics—Proliferating cell percentages were compared using one-way analysis of variance (ANOVA). Student's *t* test was used to determine significant differences between non-cancer and cancer groups for expression of each biomarker. One way ANOVA was also used to determine significant differences in CD21 levels, and the differences between single and co-infected tissues. Tukey's multiple comparison *post-hoc* test was performed to evaluate differences between groups. Chi-square test of independence was performed to examine the relation between cancer and virus combinations.

Results

Number of HPV, EBV, and Co-infected Cases

Detection of EBV and HPV infection in SCC of BOT tissue using EBER1 and HPV ISH and p16 immunohistochemistry is demonstrated in Figure 1. As determined by EBER1 and HPV ISH analyses in the normal tonsils, none of the specimens were positive for HPV, while 2/13 (15%) were infected with EBV only (Table 1). In tonsil cancer, 2/16 (12%) were only EBV positive, 6/16 (38%) only HPV positive, 4/16 (25%) were positive for both viruses, and 4/16 (25%) were negative for both viruses. HPV positivity was significantly associated with cancer status in tonsil specimens ($p = 0.0004$). While only fifteen percent of normal tonsils were positive for EBV and 37% of tonsil cancers were positive for EBV, there was no statistically significant difference between normal and malignant specimens. In contrast, HPV positivity was detected only in malignant tonsils in this cohort and importantly the prevalence of HPV infection was very high (63%) in tonsil SCC.

Two out of ten (20%) non-cancer BOT tissue specimens collected from random biopsies of patients with unknown primaries were positive for EBV only and the rest were negative for both viruses. These non-cancer BOT samples were obtained from patients with metastatic nodes from unknown primaries and the primary is often found to be in the BOT. Therefore we sought to confirm these results using true normal BOT tissue from patients undergoing base of tongue reduction for obstructive sleep apnea. 1/15 (7%) normal BOT specimens was positive for EBV only and 3/15 (20%) normal BOT specimens were positive for HPV only. In the cancer samples of the BOT 1/15 (7%) was positive for EBV alone, 9/15 (60%) were positive for HPV alone and 3/15 (20%) were co-infected with both viruses and 2 samples were not infected with any virus. The presence of EBV in BOT cancer has not previously been reported. There were significantly more HPV-positive specimens ($p < 0.0001$ vs. combined normal BOT) in BOT cancers than among either non-cancer or normal BOT samples. Importantly, HPV/EBV co-infection was observed only in cancerous tissue originating in two oropharynx sites (tonsil, BOT) rich in lymphoid tissue and it was significantly associated with cancer status in these two sites ($p=0.0024$).

Although EBER ISH is the gold standard for determining EBV-positivity, EBER expression has been noted to be less abundant in WHO type I well differentiated squamous cell carcinoma (18). Thus, we also assessed prevalence of EBV and HPV using an EBER1 qRT-PCR approach to increase the sensitivity for detecting EBV. EBER ISH (Figure 1) detected EBV both in tonsil tumor tissue and in surrounding lymphocytes. Only cells from tumor tissue were captured for qRT-PCR and the absence of contaminating EBV infected B cells was confirmed by the failure to detect CD20 transcripts. Furthermore, direct evaluation of HPV prevalence assessed by ISH was compared to the surrogate HPV marker, p16. In normal tonsils, none of the specimens were positive for HPV, while 4/11 (36%) were infected with EBV only (see Table 2). In tonsil cancer, 2/12 (17%) were only EBV positive, 6/12 (50%) only HPV positive, 3/12 (25%) were positive for both viruses, and only 1/12 (8%) were negative for both viruses. HPV positivity was significantly associated with cancer status in tonsil specimens ($p = 0.0003$). A substantial number of normal tonsils and tonsil cancers were positive for EBV and the prevalence of EBV infection was similar in normal

and malignant tonsils. In contrast, HPV positivity was detected only in malignant tonsils in this cohort and importantly the prevalence of HPV infection was very high (75%) in tonsil SCC. In the non-cancer BOT tissue collected from random biopsies of patients with unknown primaries the results were very surprising as 4/12 specimens (33%) were positive for EBV only and the rest were negative for both viruses. We also examined two true normal BOT tissue from patients undergoing base of tongue reduction for obstructive sleep apnea. Neither was positive for either HPV or EBV. In the cancer samples of the BOT 1/10 (10%) were positive for EBV alone, 2/10 (20%) were positive for HPV alone and a majority - 7/10 (70%) were co-infected with both viruses. There were no samples free of any virus. There were significantly more HPV-positive specimens ($p < 0.0001$) in BOT cancers than among non-cancer BOT samples. HPV/EBV co-infection as determined by EBER1 qRT-PCR and HPV ISH was again observed only in malignant tissue originating in two oropharynx sites (tonsil, BOT) rich in lymphoid tissue and it was significantly associated with cancer status in these two sites ($p = 0.0002$).

Next we tested the incidence of EBV and HPV infection in cancerous soft palate tissue and normal uvula, sites of the oropharynx that have a different histologic make up compared to tonsil and BOT sites since it lacks lymphoid tissue. In SCC soft palate and normal uvula, no HPV infected cells were found. EBV was found in ~20% of normal uvula samples by EBER1 qRT-PCR.

Nine of the HPV positive tumors were analyzed by qRT-PCR using HPV 16 specific E7 primers. Eight of the 9 (88%) amplified, a number consistent with the reported prevalence of HPV16 in HNSCC (19). Four of the 8 were EBV positive and 4 were negative for EBV. There was no significant difference in the Ct values between the groups. The integration status of 14 HPV positive tumors was determined by APOT analysis (20; data not shown). All contained integrated HPV; 1 contained both episomal and integrated DNA.

Comparison of virus detection techniques

p16 immunohistochemistry is commonly used to diagnose HPV infection in a clinical setting with p16 protein overexpression being used as a surrogate marker for HPV infection in oropharyngeal squamous cell carcinoma. Therefore we sought to determine how well HPV ISH and p16 IHC correlated in our dataset that includes 120 normal and malignant oropharynx cases. We found a highly significant correlation between HPV status using these two techniques ($r = 0.765$; $p < 0.0001$). 71 cases determined to be HPV negative by ISH were also p16 negative by IHC, while 8 cases that were negative by ISH were p16 positive by IHC ("false-positive" p16 cases). In 2/8 p16 positive cases the presence of EBV infection was noted by EBER1 ISH. Out of 41 cases determined to be HPV positive by ISH, 36 were p16 positive, while 5 were false negatives (p16 negative; HPV ISH positive).

Next we compared the concordance between EBV detection by EBER1 ISH and EBER qRT-PCR. 64 cases were evaluated by both techniques. There was a weak, but significant correlation between the results obtained by these two techniques ($r = 0.255$; $p = 0.0403$), and discordant results were initially observed in 21 out of 65 cases (32%). In 16 cases, samples determined to be EBV negative by ISH were positive by qRT-PCR whereas 5 EBER1 ISH positive cases were negative by qRT-PCR. Since qRT-PCR methodologies are considered

more sensitive than ISH and are capable of detecting very few EBV infected cells (21) we researched the reason for this discrepancy. Three available EBER1 ISH positive and qRT-PCR negative tumors demonstrated a heterogeneous (patchy) pattern of EBER1 ISH staining with some areas that showed EBV positive staining and some that were EBV negative in areas where HPV was clearly positive (Figure 1). When we collected epithelial cells using LCM from those areas that showed positive EBER1 ISH staining we found them to be positive by EBER1 qRT-PCR as well.

Expression of CD21 in normal and cancerous oropharynx tissue

We also determined the expression of CD21 mRNA in various subsites of the oropharynx to better understand the predisposition of different subsites to EBV infection. The levels of CD21 expression for normal and malignant tissue samples are shown in Table 3. EBV and HPV infection status were determined using EBER1 qRT-PCR and HPV ISH.

CD21 mRNA expression was detected only in the specimens from tonsil and BOT epithelium, but not in soft palate epithelium. In non-cancer BOT specimens CD21 expression levels were significantly higher in HPV-/EBV+ specimens than in HPV-/EBV- specimens ($p=0.0205$; Table 3). Although there was no statistically significant difference in CD21 expression between normal and cancer tissues ($p = 0.28$) there were significantly higher levels of CD21 expression in the specimens positive for EBV ($p = 0.0021$), for HPV ($p = 0.0295$) or positive for both viruses ($p = 0.0194$) as compared to the specimens not infected with either virus. The highest level of CD21 expression was observed in co-infected specimens (8.97 ± 22.17), although there was no significant difference in CD21 levels between co-infected and individually infected samples.

The Effects of HPV and EBV Infection on Proliferation and Invasiveness of FaDu and NOK Cells

After generating stable HPV/EBV clones of FaDu and NOK cells we confirmed their status by analysis of HPV E6, HPV E7 and EBV LMP2 mRNA expression using RT-PCR with gene specific primers (Figure 2). Next, we analyzed if the growth rate was altered in the various HPV/EBV cell lines derived from NOK and FaDu. We found that EBV and/or HPV infection had no significant effect on the growth of FaDu and NOK cells (Figure 3A-C).

As HPV associated tumors are known to be highly invasive, we next evaluated the effect of either HPV 16 E6 and E7 or EBV alone and in combination on *in vitro* cell invasion assays. EBV and HPV infection of NOK cells had no significant effect on baseline cell invasiveness evaluated in the absence of LPA (Figure 3D). However, there was a significant difference between HPV/EBV cell clones of NOK in the presence of LPA, a small glycerophospholipid that is present in all eukaryotic tissues and is known to stimulate cell migration. As shown in Figure 3D there was more than a three-fold increase in the invasiveness of co-infected NOK cells in the presence of LPA compared to HPV-/EBV- and HPV-/EBV+ NOK cells ($p < 0.01$). It also can be noted that HPV-infected NOK cells exhibited a trend toward increased invasiveness (Figure 3D). Similarly FaDu co-infected cells showed a significant increase in invasiveness compared to the other combinations in the presence of LPA ($p < 0.05$) (Figure 3E).

Discussion

HPV is associated with almost 70% of tonsil cancers and with 15-20% of head and neck squamous cell carcinomas of all sites. EBV is present in almost all undifferentiated nasopharyngeal carcinomas and a small percentage of oral squamous carcinomas. A small number of studies have shown co-infection by EBV and HPV in WHO type 1 and WHO type III nasopharyngeal carcinomas (22, 23), but co-infection with the two viruses in the oropharynx has not been extensively explored (2). Our results indicate that a significant proportion of cancers of the tonsil and base of tongue carry both viruses. The rate of co-infection was 25% in tonsil SCC and 70% in SCC BOT (as assessed by EBER1 RT-PCR and HPV ISH). In tumors of the soft palate, where no lymphoid tissue exists, none of the samples was infected with either virus. All HPV positive tumors analyzed for integration status carried integrated HPV DNA; since it is assumed that under such circumstances all cells in the tumor contain HPV, this provides support for the conclusion that those in which EBV was also detected carried two viruses in the same cell.

EBV infection of normal tonsil epithelium was not unexpected as the virus has been shown in many studies to be present at this site. Infection in the base of tongue has not previously been reported, but this may simply reflect the difficulty of accessing normal BOT. Although our sample numbers are small, they are consistent with a more widespread distribution of virus than formerly appreciated.

The detection of HPV DNA by ISH provides the more definitive evidence of infection, but p16 expression has been used as a surrogate marker for the presence of virus. Like others (24), we found a highly significant correlation between the results of HPV detection by ISH and p16 IHC with 4% of the cases determined to be false-negative and 6.6% of the cases evaluated to be false-positive by p16 analysis. Overexpression of p16 in some of the false-positive cases could potentially be a result of altered gene expression by other agents. In support of this, EBV too was detected in 2/8 of the p16 positive cases.

Both techniques that we used to evaluate the presence of EBV, ISH and qRT-PCR, were based on detection of EBER1 transcripts. There was only a weak correlation between EBV status as determined by these two techniques with a significant percentage of the cases tested by both methods (20/64; 31%) demonstrating discordant results. For 15 of those samples this represented cases that were positive by qRT-PCR and negative by ISH, probably reflecting the fact that qRT-PCR is the more sensitive method. It cannot be attributed to B cell contamination since we were able to detect 1 B cell in 1000 epithelial cells by amplification of CD20 transcripts and none of our samples was positive for CD20. Of the remaining 5 that were positive by EBER ISH and negative by qRT-PCR, two were normal tissues where random sampling by LCM might easily pick up only uninfected cells. The three tumor specimens were reevaluated visually for EBER ISH distribution. Staining was not uniform throughout the tumor and resampling by LCM of areas of the tumor where ISH was positive confirmed the presence of virus by qRT-PCR. It is of course impossible to know whether areas lacking EBV had lost virus or had never been infected. However, in this regard, it is worth noting that EBV can stably alter gene expression following transient

infection (25) and that absence of virus does not necessarily mean that it has made no contribution to tumor development.

Biomarkers that could predict which patients infected by these ubiquitous viruses are likely to develop a malignancy will be key to screening for this devastating disease. EBV uses CD21 to attach to B cells, where it is constitutively expressed. Expression of CD21 on an epithelial cell greatly increases the ease with which infection occurs, but not all epithelial cells are CD21-positive. We previously demonstrated CD21 mRNA in normal tonsil epithelium but not in any of the other normal oral epithelia that were then available for examination (16). In this study we were able to add BOT, where we found its expression in both normal and cancer tissues. The average CD21 mRNA expression levels in normal tonsil and BOT were similar. Higher levels of CD21 were noted in EBV-positive normal specimens and the difference reached statistical significance for non-cancer BOT. This might be explained by the fact that the non-cancer samples were obtained from patients with metastatic nodes from unknown primaries which could have been in the BOT. In a recent study we found that CD21 expression in oral epithelial cells increases in frequency and intensity as tissues becomes dysplastic (26). We also detected significantly higher levels of CD21 in specimens infected with EBV, HPV or both viruses as compared to HPV-/EBV-specimens (Table 3), perhaps reflecting both the increased likelihood that EBV would infect tissue expressing CD21 and an increase in dysplasia resulting from infection with HPV. Expression of CD21, which might be detected in brush biopsies, does not however seem to be a robust biomarker of increased risk for tumor development.

So, why might co-infection with HPV and EBV have a high tumorigenic potential? It has already been shown that the oncogenic properties of HPV are dependent on expression of E6 and E7. HPV E6 and E7 proteins promote upregulation of intracellular anti-apoptotic signaling leading to the avoidance of normal physiological programmed cell death. Increased survival can lead to the accumulation of DNA damage and mutations over a number of cell replications resulting in malignant transformation and development of invasive carcinomas (27). Here, we show that HPV/EBV co-infection and to some extent HPV infection also increases cell invasiveness. Similar results have been observed in other studies. HPV type 16 E6/E7 proteins have been shown to not only increase cell invasion, but also cell metastasis (28).

The oncogenic potential of EBV is related to expression of latent genes (8). LMP1 and 2 are considered the viral oncogenes in EBV-associated nasopharyngeal cancer, an aggressive and highly metastatic tumor (29, 30). Ectopic LMP1 or LMP2 expression has been shown to increase cellular invasion (31). In our study using an in vitro model that mimics the setting of co-infection (overexpression of E6 and E7 simulating HPV integration followed by EBV infection), the cancerous cell line FaDu and the normal cell line NOK demonstrated a significant increase in invasion in the absence of any effect on proliferation. Clinically, HPV positive tumors often have small primary tumors but significant metastasis, which could explain why the co-infection of these viruses may have a more profound effect on invasion than proliferation.

HPV-associated oropharyngeal cancer is a growing epidemic of oral carcinomas, specifically in tonsil and BOT tumors. We have shown that there is a significant increase of co-infection of EBV with HPV especially in BOT and tonsil but not in the soft palate where there is no lymphoid tissue. Thus, association with lymphoid tissue may explain the propensity for HPV and EBV associated tumors to occur in the BOT and tonsil. Furthermore using an invasion assay we demonstrated that the co-infected cells have much higher tumorigenic potential as compared to normal cells. Future studies evaluating the mechanisms of tumorigenicity induced by HPV/EBV co-infection are warranted.

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Figure 1.

Detection of EBV and HPV infection in SCC of BOT and Tonsil tissue using EBER1 and HPV in situ hybridization (ISH) and p16 immunohistochemistry (IHC). (100x magnification).

H&E - Hematoxylin and eosin stain.

Figure 2.

Expression of HPV16 E6, HPV16 E7 or EBV latent membrane 2 (LMP2) mRNA in various HPV/EBV clones derived from NOK and FaDu cells. Shown are ethidium stained amplicons following reverse transcription PCR using specific primers for HPV16 E6, HPV16 E7 and EBV LMP2.

Figure 3.

Effect of EBV and HPV infection on proliferation and invasion of FaDu and NOK cells. **A.** Growth of various HPV/EBV clones of FaDu in 1% serum. Shown are the average cell number and standard deviation of the mean of 3-4 independent experiments. **B.** Growth of various HPV/EBV clones of FaDu in 10% serum. Equal cell numbers were plated and counted at 24 hour intervals. Shown are the average cell number and standard deviation of the mean of 3 independent experiments. **C.** Growth of various HPV/EBV clones of NOK. The average absorbance and standard deviation of the mean from 4-5 independent experiment are shown. Proliferation was measured at 24 hour intervals using a tetrazolium based colorimetric assay. EBV and/or HPV infection had no significant effect on the growth of FaDu and NOK cells. **D, E.** Baseline and LPA-induced invasion of HPV/EBV cell clones of NOK (**D**) and FaDu (**E**).

* indicates $p < 0.05$ relative to HPV-/EBV- cells;

** indicates $p < 0.01$ relative to HPV-/EBV- cells.

Table 1
The prevalence of HPV, EBV infection and HPV/EBV co-infection in normal and malignant oropharynx specimens as determined by EBER1 and HPV ISH

	HPV-/EBV-	HPV-/EBV+	HPV+/EBV-	HPV+/EBV+	Total HPV+	Total EBV+
Normal Tonsil	11/13 (85%)	2/13 (15%)	0/13 (0%)	0/13 (0%)	0/13 (0%)	2/13 (15%)
Non-Cancer BOT Unknown primary	8/10 (80%)	2/10 (20%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	2/10 (20%)
Normal BOT Sleep apnea	14/15 (93%)	1/15 (7%)	3/15 (20%)	0/15 (0%)	3/15 (20%)	1/15 (7%)
Normal Uvula	12/12 (100%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)
SCC Tonsil	4/16 (25%)	2/16 (12%)	6/16 (38%)	4/16 (25%)	10/16 (63%)	6/16 (37%)
SCC BOT	2/15 (13%)	1/15 (7%)	9/15 (60%)	3/15 (20%)	12/15 (80%)	4/15 (27%)
SCC Soft Palate	10/10 (100%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

Table 2

The prevalence of HPV, EBV infection and HPV/EBV co-infection in normal and malignant oropharynx specimens as determined by EBER1 RT-PCR and HPV ISH.

	HPV-/EBV-	HPV-/EBV+	HPV+/EBV-	HPV+/EBV+	Total HPV+	Total EBV+
Normal Tonsil	7/11 (64%)	4/11 (36%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	4/11 (36%)
Non-Cancer BOT Unknown primary	8/12 (67%)	4/12 (33%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	4/12 (33%)
Normal BOT Sleep apnea	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Normal Uvula	7/9 (78%)	2/9 (22%)	0/9 (0%)	0/9 (0%)	0/9 (0%)	2/9 (22%)
SCC Tonsil	1/12 (8%)	2/12 (17%)	6/12 (50%)	3/12 (25%)	9/12 (75%)	5/12 (42%)
SCC BOT	0/10 (0%)	1/10 (10%)	2/10 (20%)	7/10 (70%)	9/10 (90%)	8/10 (80%)
SCC Soft Palate	10/10 (100%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

Table 3
Expression of CD21 (mean value of $2^{-Ct} \pm SD$) in oropharynx tissues ^a

	HPV-/EBV-	HPV-/EBV+	HPV+/EBV-	HPV+/EBV+
Normal Tonsil	1.06±1.61 (n=6/7)	3.77±5.12 (n=4/4)	-	-
Non-Cancer BOT	0.20±0.56 (n=7/8)	6.85±7.16 ^b (n=3/4)	-	-
Normal BOT (True)	0.0±0.0 (n=0/2)	-	-	-
Normal Uvula	0.0±0.0 (n=0/7)	0.0±0.0 (n=0/2)	-	-
SCC Tonsil	0.0 (n=0/1)	0.10±0.13 (n=1/2)	10.41±21.68 (n=3/6)	3.49±2.91 (n=3/3)
SCC BOT	0.0 (n=0/1)	0.3±0.42 (n=1/2)	1.08±1.98 (n=2/5)	11.31±26.70 (n=2/7)
SCC Soft Palate	0.0±0.0 (n=0/10)	-	-	-
All specimens	0.25±0.82 (n=7/36)	3.09±5.14 ^c (n=9/14)	6.17±16.13 ^c (n=5/11)	8.97±22.17 ^c (n=5/10)

^a In parenthesis are the numbers of specimens demonstrating positive CD21 expression out of total number of specimens analysed.

^b - p=0.0205, compared to HPV-/EBV- specimens

^c - p<0.05, compared to HPV-/EBV- specimens.