Published in final edited form as:

Oral Oncol. 2012 September ; 48(9): 836–841. doi:10.1016/j.oraloncology.2012.03.017.

Oral dysplasia and squamous cell carcinoma: correlation between increased expression of CD21, Epstein-Barr virus and CK19

Ru Jiang^{a,b}, Xin Gu^c, Tara N. Moore-Medlin^d, Cherie-Ann Nathan^{b,d}, and Lindsey M. Hutt-Fletcher^{a,b}

Ru Jiang: rjiang@lsuhsc.edu; Xin Gu: xgu@lsuhsc.edu; Tara N. Moore-Medlin: tmoor5@lsuhsc.edu; Cherie-Ann Nathan: cnatha@lsuhsc.edu; Lindsey M. Hutt-Fletcher: lhutf@lsu.edu

^aDepartment of Microbiology and Immunology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA

^bCenter for Molecular and Tumor Virology and Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA

^cDepartment of Pathology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA

^dDepartment of Otolaryngology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA

Abstract

OBJECTIVES—Epstein-Barr virus is an orally transmitted human gammaherpesvirus that infects B lymphocytes and epithelial cells. Although most primary infections are asymptomatic, long term carriage of the virus can be associated with either lymphoid or epithelial malignancies. The association of EBV with oral squamous cell carcinomas is sporadic and it is uncertain if the virus is involved in initiation of the tumor or, possibly, in its progression. Complement receptor type 2, CR2 or CD21, is a receptor for the major attachment protein of EBV, which significantly enhances epithelial cell infection, but its expression on normal tissues is restricted to tonsil and adenoid epithelium. As cells become dysplastic they are reported to express higher levels of CK19. We sought to evaluate whether CD21 and CK19 expression change as oral epithelial cells outside Waldeyer's ring become dysplastic.

MATERIALS AND METHODS—Epithelial cells were isolated by laser capture microdissection and levels of CD21, CK19 and EBV RNA were measured by quantitative reverse transcriptase PCR.

RESULTS—We report that expression of CD21 increases in frequency and intensity as oral epithelial cells become more dysplastic and that expression correlates with an increase in infection

© 2012 Elsevier Ltd. All rights reserved.

All correspondence to: Lindsey Hutt-Fletcher, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA, Tel: 318 675 4948, Fax: 318 675 5764, lhutf@lsuhsc.edu.

CONFLICTS OF INTEREST

None declared.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

by EBV. Tumors or dysplastic lesions that carry EBV also generally express higher levels of CK19 than those that do not.

CONCLUSION—The findings suggest that dysplasia may make cells more susceptible to infection by EBV and that infection by the virus may alter the phenotype of the infected cell in a manner which could affect prognosis.

Keywords

Epstein-Barr virus; CD21; CK19; oral squamous cell carcinoma

INTRODUCTION

Epstein-Barr virus (EBV) is an orally transmitted human gammaherpesvirus carried by more than 90% of the world's population (reviewed in¹). Although primary infections are typically asymptomatic or accompanied by the benign, if temporarily debilitating, syndrome infectious mononucleosis, long term carriage of the virus can be associated with both epithelial and B cell malignancies. These reflect the predominant tropisms of the virus. The latent reservoir of virus is harbored in long lived memory B cells and amplification of virus by replication in epithelial cells is thought to contribute to replenishment of the reservoir and spread of virus to new hosts.^{2, 3, 4}

The principal cancer of the head and neck associated with EBV is nasopharyngeal carcinoma. All non-keratinizing forms of nasopharyngeal carcinoma carry EBV⁵ and typically express the most abundant non-coding EBER RNAs, EBNA1 transcripts and, to varying degrees, transcripts encoding the LMP proteins.⁶ EBV has also been associated with, though not causally linked to, oral squamous cell carcinoma (OSCC). Studies conflict with respect to the percent of cases that show evidence of EBV, only some have convincingly demonstrated that the virus is present in tumor cells rather than in infiltrating lymphocytes and, as EBV is part of the normal oral flora, it is not clear whether its presence simply reflects baseline levels that might be expected in cells in the oral cavity (reviewed in⁷). Several issues are nevertheless raised by the finding of EBV in OSCC at all. First, whether it is possible that there are phenotypes expressed by dysplastic or malignant cells that make them more vulnerable to infection and second, irrespective of whether the virus is causally related to the initiation of a malignancy, whether its presence potentially influences the subsequent behavior of an infected cell.

Infection of any cell by an enveloped virus such as EBV requires attachment of virus and fusion of its envelope with the plasma membrane or with the membrane of an endocytic vesicle. The EBV envelope contains eleven glycoprotein as many as five of which are potentially involved in epithelial cell entry. Attachment may occur as a result of interactions of at least two of them with cellular integrins,^{8, 9, 10, 11} but entry and infection is disproportionately enhanced if a cell expresses the complement receptor type 2, CR2 or CD21^{12, 13}, to which a third and most abundant virus glycoprotein, gp350, can bind.^{14, 15} CD21 is expressed on all B lymphocytes, where it is possibly the exclusive attachment receptor for virus, but it is not typically expressed in epithelial cells in the oral cavity, with the exception of epithelial cells in tonsils,¹⁶ and adenoids (data not shown) which are thought to be among the primary sites of EBV replication. To explore whether dysplastic or malignant cells in the oral cavity might have any increased susceptibility to EBV infection we therefore used laser capture microdissection of archived tissues to determine if mRNA transcripts corresponding to the domain of CD21 to which EBV attaches are expressed in dysplastic or malignant cells. The identity of epithelial cells was confirmed morphologically and by expression of mRNA encoding the cytokeratin CK19. The exclusion of contaminating B cells was determined by lack of expression of mRNA for CD20. We report

here not only that expression of CD21 is increased in dysplastic cells, but also that the presence of EBV is associated with increased levels of expression of CK19.

MATERIALS AND METHODS

Tissue specimens

Tissue blocks collected between 1999 and 2011 were obtained from patients treated at Louisiana State University Health Sciences Center-Shreveport after receiving approval from the institutional review board. Seventy-eight mucosal biopsies from the oral cavity and oropharynx were examined. They included 23 samples of normal oral squamous epithelium (NOSE), including 5 from tonsil, 12 from base of tongue and 6 from normal margins of dysplasia. The remaining biopsies were either squamous dysplasia (29 cases) or oral squamous cell carcinoma (OSCC, 26 cases). The cases with squamous dysplasia were further classified as mild dysplasia (MLD, 8 cases), moderate dysplasia (MOD, 8 cases) and severe dysplasia (SED, 13 cases). The cases with OSCC were further classified as: well differentiated (6 cases), moderately differentiated (12 cases) and poorly differentiated (8 cases). All pathology specimens were routinely fixed in 10% neutral formalin and embedded in paraffin. Three biopsies of oral hairy leukoplakia (OHL) embedded in OCT were obtained from the AIDS and Cancer Specimen resource of the NCI and two were a gift of Dr. Sharof Tugizov (University of California, San Francisco). Specimens were sectioned to a thickness of 4 μ m for laser capture microdissection and immunohistochemistry.

Tissue processing and Laser capture microdissection (LCM)

Sections were stained with hematoxylin and eosin (H&E), dewaxed in xylene, dehydrated in ethanol, air-dried and stored in desiccant until used for LCM. As previously described,¹⁶ 3,000 to 4,000 epithelial cells, identified by morphology, were captured by a PixCell Iie LCM system (Arcturus Engineering, Inc., Mountain View, CA, USA). CapSure HS LCM caps (Arcturus) were placed over the sections, laser spot size, power, and pulse duration were adjusted 7.5 μ m in diameter, 55–60 mW, and 1.5–1.8 ms, respectively. Epithelial cells were captured from whole layers of epithelium in the normal margin of dysplasia, all dysplasia, OHL and OSCC sections. In some tonsil and base of tongue sections epithelial cells were captured above or close to the basal layer to avoid lymphocyte contamination. Each section analyzed was sampled independently two to four times.

Reverse transcription and Quantitative real-time PCR (QPCR)

Total RNA from captured cells was extracted, purified, DNase treated, and reverse transcribed using the Paradise^{Plus} Whole Transcript Reverse Transcription (WT-RT) Reagent System (MDS, Analytical Technologies) according to the manufacturer's instructions. A human formalin-fixed universal reference RNA, included in the Reagent System, was reverse transcribed in parallel as a positive control. Quantitative real-time QPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with conditions as previously specified.¹⁶ The target gene probes were labeled by FAM (6-carboxy-fluorescein) (Integrated DNA Technologies, Coralville, IA, USA) and a house keeping gene, GAPDH probe was labeled by VIC (glyceraldehyde- 3-phosphate dehydrogenase) (Applied Biosystems). The sequences of primers and probes of target and housekeeping genes were as previously reported.¹⁶ Primers were optimized for QPCR with GAPDH as a control gene. When the optimal primer concentration produced a linear response to input cDNA concentration, RNA samples were analyzed in triplicate for each tested transcript. To normalize the expression levels (Δ Ct), the threshold cycle (Ct) for each transcript was subtracted from the Ct of the more abundantly expressed control gene (GAPDH). This value was then used to calculate the

$2^{-\Delta\Delta C_t}$ to compare expression patterns between various tissue samples. A significant change in expression was defined as greater than a fold increase or decrease.

Cell culture and RNA extraction

Akata Burkitt's lymphoma B cells, which have lost the EBV genome,¹⁷ were grown in RPMI (Sigma), AGS cells (ATCC), CD21-negative gastric carcinoma cells were grown in Ham's F12 medium (Invitrogen). All media were supplemented with 10% heat inactivated fetal bovine serum (Gibco / BRL). For QPCR analysis, total RNA from cells was isolated and purified using RNA STAT-60 reagent according to the manufacturer instructions (TEL-TEST, INC.). For cDNA synthesis, 5 µg RNA was reversed transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) using random hexamer primers.

Comparison of relative amounts of CK19 mRNA

The relative amounts of target gene CK19 mRNA were calculated by the comparative threshold cycles (Ct) method.^{16, 18} For the $\Delta\Delta C_t$ calculation to be valid, amplification efficiencies of the target and reference GAPDH must be approximately equal.¹⁹ ΔC_t represents the difference in Ct values between target genes and the GAPDH reference gene from the lesion at the same condition points for each sample. $\Delta\Delta C_t$ is described as target gene expression in different tissues and cell lines minus target gene expression in the arbitrary constant. As previously reported,¹⁶ to assess if target and reference amplicons had the same efficiency, serial dilutions of cDNA from AGS cells were amplified by QPCR using CK19 and GAPDH primers. The ΔC_t numbers of CK19 and GAPDH were calculated for each cDNA dilution and the absolute value of the slope of the plot of the log cDNA dilution vs. ΔC_t was close to zero indicating that the efficiencies of the target and reference genes were similar (Figure 1). The numerical value for $\Delta\Delta C_t$ was then used in the $2^{-\Delta\Delta C_t}$ calculation. The final result represents the differential expression of target CK19 gene in different tissues relative to that in the tissues that were, in each case, used to establish an arbitrary constant of 1.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue blocks. Four-micrometer-thick sections were dewaxed in xylene, rehydrated in ethanol and the immunohistochemical staining done in a Ventana Banchmaker XT autostainer (Ventana ULTRA system, Tucson, AZ, USA) using standard peroxidase immunohistochemistry techniques, heat-induced epitope retrieval buffer and primary antibody against CK19 (pre diluted A53-B/A2.26, Ventana). Detection of the staining was achieved by an enzyme-conjugated polymer complex (HRP multimer kit, Ventana). Appropriate positive and negative controls were included.

Statistical Analysis

Statistical analysis was done with Student's t-test. Results were expressed as means $\pm$ SD. A difference of $p < 0.05$ was considered statistically significant.

RESULTS

Compared with normal margin epithelial tissue, which was negative for CD21 transcripts, we found that CD21 mRNA was expressed in 12.5% of mild grade, 37.5% of moderate grade and 69.2% of severe grade dysplastic epithelial specimens (Table 1). Although Ct values in the formalin-fixed paraffin-embedded tissues were high, analysis of the melting curves revealed identical melting temperatures as for those of tissues, such as fresh Akata B cells, which had much lower Ct values. Relative to the levels of CD21 in the Akata B cell

line, which were given the arbitrary value of 1, CD21 transcript levels in mild dysplasia averaged 0.35, in moderate dysplasia 1.77, and in severe dysplasia 4.99. The frequency and levels of expression in OSCC epithelial tissues did not differ from those in severe grade dysplastic epithelium (data not shown).

Since CD21 is a major attachment receptor for EBV, expressed in mature B cells and normal tonsil epithelial cells but not in normal oral tongue, uvula, or buccal mucosa epithelium,¹⁶ we assessed the prevalence of EBV in dysplastic cells by measuring expression of EBER1, one of the EBV small nuclear RNAs. EBER1 transcripts were not detected in normal margin epithelium, but were found in 5/8 mild, 6/8 moderate and 5/13 severe grade dysplasia tissues. There was a strong correlation between the presence of CD21 and EBER1 transcripts (Figure 2). As the expression levels of CD21 increased in those dysplastic cells that expressed any detectable levels of CD21, the likelihood of infection with EBV increased (Figure 3), although the correlation was not as strong.

Expression of CK19 was used as a marker of epithelial cells. CK19 transcripts were detected in all samples of normal tonsil epithelium or normal base of tongue epithelium from patients with lymph node metastases of unknown origin, although expression levels were low. Relative to the CK19 expression in these tissues, set at an arbitrary value of 1, CK19 transcript levels (Table 2) were increased in all grades of dysplasia, in all but well differentiated OSCC and in oral hairy leukoplakia, an epithelial lesion caused by lytic replication of EBV which carries a very high virus load.²⁰ The high expression of CK19 in oral precancerous lesions and OSCC was consistent with other studies, which have reported upregulation of CK19 in head and neck dysplasias and cancers^{21, 22}, but the highest levels were clearly seen in the EBV infected OHL tissue.

To evaluate further whether EBV infection overall was associated with increased expression of CK19, EBV positive and EBV negative cases were separated and CK19 transcript levels were re-calculated with the EBV negative normal epithelium (normal tonsil, normal margins of dysplasia and normal base of tongue) set at the arbitrary value of 1 (Table 3). Compared with the EBV negative normal epithelium, CK19 expression was almost twice as high in EBV positive normal epithelium, but the difference did not reach statistical significance. In contrast, CK19 transcript levels were significantly higher in EBV positive, but not in EBV negative dysplastic epithelium. Both EBV positive and negative OSCC expressed significantly higher levels of CK19 transcripts than that of normal epithelium, but again, expression levels in EBV positive tissues were higher than in EBV negative tissues.

We also compared CK19 transcript levels in EBV positive and EBV negative cases within the same groups of dysplasia and OSCC. In each group, CK19 mRNA expression in EBV negative cases was now set as an arbitrary value of 1. CK19 levels were 1.96 times higher in EBV positive normal epithelium than in EBV negative normal epithelium, 2.45 times higher in EBV positive dysplasia and 2.66 times higher in EBV positive OSCC (Figure 4).

To confirm that increased CK19 mRNA expression levels were reflected in protein expression levels, CK19 was examined by immunohistochemistry in samples of normal mucosal epithelium, dysplasia and OSCC. CK19 staining was weak in the basal layer of normal epithelium (Figure 5, a and b). There was an increase in staining intensity in dysplasia and OSCC (Figure 5, c–f), which was greatest in tissues that were EBV positive (Figure 5, d and f).

DISCUSSION

The finding that CD21 mRNA was expressed in mild, moderate and severe grade dysplasia with a frequency that increased with grade, was unexpected. Confirmation of simultaneous

expression of protein was not possible since at least one monoclonal antibody to CD21 cross-reacts with an unrelated epithelial cell protein,²³ only a subset of antibodies that recognize CD21 on B cells is reactive with all epithelial cells and reports from different groups using the antibodies are not consistent.^{24, 25, 26, 27} Even subclones of a single epithelial cell line were found to be recognized differently.¹⁶ However, the good correlation between the expression of CD21 mRNA and infection with EBV suggests that CD21 protein itself is expressed. Such findings are consistent with those *in vitro* which demonstrated that infection can occur in the absence of CD21, but is more likely in its presence.^{9, 13} They raise the interesting possibility that, relative to normal tissue, tissues that have taken the first potential steps towards malignancy become more susceptible to infection with a virus which is known to express proteins that can impact cell growth and behavior.

The observation that CK19 expression at both the mRNA and protein level increases in all grades of dysplasia was not unexpected. Several studies have noted that expression of CK19, which has been proposed to be a stem cell marker,^{28, 29, 30} in suprabasal layers of epithelium is associated both with premalignant changes and OSCC. Moderately and poorly differentiated OSCC express more CK19 than well-differentiated OSCC which in turn correlates with poorer prognosis.^{21, 22, 31} However, the finding that those lesions that carried EBV expressed even higher levels of CK19 relative to the EBV negative tissues with the same grade of dysplasia or malignant differentiation was of new interest.

The variable presence of EBV in head and neck tumors complicates a single cohesive interpretation of its role in tumor growth. However, one potential threat of infection is that it provides an additional growth advantage to a premalignant cell. This could perhaps be a transient event, instigating epigenetic changes, or one mediated by the facilitation of persistence of latent virus in the cell. There is a rich and abundant literature documenting the potential roles that EBV latent proteins and transcripts may play in altering cell behavior^{1, 32} and a recent report has implicated the EBV latency protein LMP1 in increased expression of CK19 in nasopharyngeal epithelial cells and a concomitant increase in proliferation rates.³³ The fact that EBV-positive tumors carry episomes with identical numbers of terminal repeats has provided strong support for the contention that, in cancers consistently associated with EBV, such as nasopharyngeal carcinoma, virus infection provides the critical, initiating, oncogenic insult.³⁴ Infection of a cell is followed by circularization of the virus genome as a result of random recombination of variably reiterated terminal repeat sequences. The number of terminal repeats remaining in the retained episome is thus particular to each infecting event. Infection of a preexisting tumor would produce cells carrying episomes with variable and not identical numbers of repeats. However, there is also evidence for evolution towards a predominant episomal form as one cell gains a growth advantage over its peers.³⁵ Pre-invasive NPC has been shown to contain monoclonal EBV termini, supporting an early role for EBV in tumorigenesis,³⁶ but other studies find the genome in only a small portion of tumor cells in early NPC and progressively increased numbers of cells at more advanced stages.^{37, 38} EBV infection has been detected in all high grade dysplastic lesions and NPC, but not in low grade lesions or nasopharyngeal tissue of normal adults.³⁹

The finding of a virus in a malignancy may have some prognostic value. In recent years there has been a dramatic increase in the number of carcinomas of the tonsil and base of tongue associated with infection with human papillomavirus.^{40, 41} The tumors do, however, have a better prognosis than those more typically seen in heavy smokers and drinkers which are virus negative. Whether this reflects an increased immunogenicity or less profound abnormality is not clear. In view of the sporadic, but significant association of EBV with tumors of the oral cavity it may be equally appropriate to evaluate EBV status and its impact on long term outcome.

Acknowledgments

This work was supported by Public Health Service grant DE016669 (to LMH-F) from the National Institute of Dental and Craniofacial Research.

References

1. Rickinson, AB.; Kieff, E. Epstein-Barr virus. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. 5. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2655-2700.
2. Thorley-Lawson DA, Gross A. Persistence of Epstein-Barr virus and the origins of associated lymphomas. *New Engl J Med*. 2004; 350:1328–1337. [PubMed: 15044644]
3. Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson DA. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. *PLoS Pathog*. 2009; 7:e10000496.
4. Jiang R, Scott RS, Hutt-Fletcher LM. Epstein-Barr virus shed in saliva is high in B cell tropic gp42. *J Virol*. 2006; 80:7281–7283. [PubMed: 16809335]
5. IARC Working Group on the Evaluation of Carcinogenic Risk to Humans. Epstein-Barr virus and Kaposi Sarcoma Herpesvirus/Human Herpesvirus 8. Lyon, France: International Agency for Research on Cancer; 1997.
6. Raab-Traub N. Epstein-Barr virus in the pathogenesis of NPC. *Semin Cancer Biol*. 2002; 12:431–441. [PubMed: 12450729]
7. Scott, RS.; Moody, CA.; Sixbey, JW. Epstein-Barr virus and oral malignancies. In: Robertson, ES., editor. *Epstein-Barr Virus*. Norfolk, England: Caister Academic Press; 2005. p. 55-70.
8. Xiao J, Palefsky JM, Herrera R, Berline J, Tugizov SM. The Epstein-Barr virus BMRF-2 protein facilitates attachment to oral epithelial cells. *Virology*. 2008; 370:430–442. [PubMed: 17945327]
9. Molesworth SJ, Lake CM, Borza CM, Turk SM, Hutt-Fletcher LM. Epstein-Barr virus gH is essential for penetration of B cell but also plays a role in attachment of virus to epithelial cells. *J Virol*. 2000; 74:6324–6332. [PubMed: 10864642]
10. Chesnokova LS, Nishimura S, Hutt-Fletcher L. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral proteins gHgL to integrins $\alpha 6$ or $\alpha 8$. *Proc Natl Acad Sci USA*. 2009; 106:20464–20469. [PubMed: 19920174]
11. Chesnokova LS, Hutt-Fletcher LM. Fusion of EBV with epithelial cells can be triggered by $\alpha \nu \beta 5$ in addition to $\alpha \nu \beta 6$ and $\alpha \nu \beta 8$ and integrin binding triggers a conformational change in gHgL. *J Virol*. 2011; 85 In Press.
12. Valencia SM, Hutt-Fletcher LM. Important but differential roles for actin in trafficking of Epstein-Barr virus in B cells and epithelial cells. *J Virol*. 2012; 86 In Press.
13. Borza CM, Morgan AJ, Turk SM, Hutt-Fletcher LM. Use of gHgL for attachment of Epstein-Barr virus to epithelial cells compromises infection. *J Virol*. 2004; 78:5007–5014. [PubMed: 15113881]
14. Nemerow GR, Mold C, Schwend VK, Tollefson V, Cooper NR. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J Virol*. 1987; 61:1416–1420. [PubMed: 3033269]
15. Tanner J, Weis J, Fearon D, Whang Y, Kieff E. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping and endocytosis. *Cell*. 1987; 50:203–213. [PubMed: 3036369]
16. Jiang R, Gu X, Nathan C, Hutt-Fletcher L. Laser-capture microdissection of oropharyngeal epithelium indicates restriction of Epstein-Barr virus receptor/CD21 mRNA to tonsil epithelial cells. *J Oral Path Med*. 2008; 37:626–633. [PubMed: 18710421]
17. Shimizu NA, Tanabe-Tochikura A, Kuroiwa Y, Takada K. Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. *J Virol*. 1994; 68:6069–6073. [PubMed: 8057484]
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- Ct method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]

19. Jiang, R.; Scott, RS.; Hutt-Fletcher, LM. Laser capture microdissection for analysis of gene expression in formalin-fixed paraffin-embedded tissue. In: Murray, GL., editor. *Laser Microdissection: Methods and Protocols*. 2. New York: Humana Press; 2011. p. 77-84.
20. Webster-Cyriaque J, Middeldorp JM, Raab-Traub N. Hairy leukoplakia, an unusual combination of transforming and permissive Epstein-Barr virus infections. *J Virol*. 2000; 74:7610–7618. [PubMed: 10906215]
21. Xu X-C, Lee JS, Lippman SM, Roy JY, Hong WK, Lotan R. Increased expression of cytokeratins CK8 and CK19 is associated with head and neck carcinogenesis. *Cancer Epidemiol Biomarkers Prev*. 1995; 4:871–876. [PubMed: 8634660]
22. Safadi RA, Musleh AS, Al-Khateeb TH, Hamasha AA. Analysis of immunohistochemical expression of K19 in oral epithelial dysplasia and oral squamous cell carcinoma using color deconvolution-image analysis method. *Head Neck Pathol*. 2010; 4:282–289. [PubMed: 20882374]
23. Young LS, Dawson CW, Brown KW, Rickinson AB. Identification of a human epithelial cell surface protein sharing an epitope with the C3d/Epstein-Barr virus receptor molecule of B lymphocytes. *Int J Cancer*. 1989; 43:786–794. [PubMed: 2469656]
24. Levine J, Pflugfelder SC, Yen M, Crouse CA, Atherton SS. Detection of the complement (CD21)/Epstein-Barr virus receptor in human lacrimal gland and ocular surface epithelia. *Reg Immunol*. 1990–1991; 3:164–170.
25. Niedobitek G, Herbst H, Stein H. Epstein-Barr virus/complement receptor and epithelial cells. *Lancet*. 1989; 2:110. [PubMed: 2567860]
26. Talacko AA, Teo CG, Griffin BE, Johnson NW. Epstein-Barr virus receptors but not viral DNA are present in normal and malignant oral epithelium. *J Oral Path Med*. 1991; 20:20–25. [PubMed: 1848290]
27. Thomas JA, Crawford DH. Epstein-Barr virus/complement receptor and epithelial cells. *Lancet*. 1989; 2:449–450. [PubMed: 4124935]
28. Michel M, Torok N, Godbout M-J, Lussier M, Gaudreau P, Royal A, et al. Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. *J Cell Sci*. 1996; 109:1017–1028. [PubMed: 8743949]
29. Haruna Y, Saito K, Spaulding S, Nalesnik MA, Gerber MA. Identification of bipotential progenitor cells in human liver development. *Hepatology*. 1996; 23:471–481. [PubMed: 8617426]
30. Chen S, Takahara M, Kido M, Takeuchi S, Uchi H, YT, et al. Increased expression of an epidermal stem cell marker, CK19, in cutaneous squamous cell carcinoma. *Br J Dermatol*. 2008; 159:952–955. [PubMed: 18647309]
31. Zhong L-P, Chen W-T, Zhang C-P, Zhang Z-Y. Increased CK19 expression correlated with pathologic differentiation grade and prognosis in oral squamous cell carcinoma patients. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics*. 2006; 104:377–384.
32. Kieff, ED.; Rickinson, AB. Epstein-Barr virus and its replication. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. 5. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2603-2654.
33. Kondo S, Wakisaka N, Muramatsu M, Zen Y, Endo K, Murono S, et al. Epstein-Barr virus latent membrane protein 1 induces cancer stem/progenitor-like cells in nasopharyngeal epithelial cell lines. *J Virol*. 2011; 85:11255–11264. [PubMed: 21849440]
34. Raab-Traub N, Flynn K. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell*. 1986; 47:883–889. [PubMed: 3022942]
35. Moody CA, Scott RS, Su T, Sixbey JW. Length of Epstein-Barr virus termini as a determinant of epithelial cell clonal emergence. *J Virol*. 2003; 77:8555–8561. [PubMed: 12857925]
36. Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N. Clonal proliferation of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. *New Engl J Med*. 1995; 333:693–698. [PubMed: 7637746]
37. Yeung WM, Zong YS, Chiu CT, Sham JS, Choy DT, Ng MH. Epstein-Barr virus carriage by nasopharyngeal carcinoma in situ. *Int J Cancer*. 1993; 53:746–750. [PubMed: 8383629]
38. Wu HC, Lin YJ, Lee JJ, Liu YJ, Liang ST, Peng Y, et al. Functional analysis of EBV in nasopharyngeal carcinoma cells. *Lab Invest*. 2003; 83:797–812. [PubMed: 12808115]

39. Lo KW, Huang DP. Genetic and epigenetic changes in nasopharyngeal carcinoma. *Semin Cancer Biol.* 2002; 12:451–462. [PubMed: 12450731]
40. Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer.* 2009; 125:362–366. [PubMed: 19330833]
41. Attner P, Du J, Nasman A, Hammarstedt L, Ramqvist T, Lindholm J, et al. Human papillomavirus and survival in patients with base of tongue cancer. *Int J Cancer.* 2011; 128:2892–2897. [PubMed: 20725995]

Figure 1. Validation of the $2^{-\Delta\Delta C_t}$ method for determination of relative levels of expression of CK19. The target gene, CK19, and the internal control, GAPDH, were amplified from serial dilutions of cDNA of AGS epithelial cells and the ΔC_t ($C_{tCK19} - C_{tGAPDH}$) was calculated for each. The slope of the curve fitted using least squares linear regression analysis was 0.0006.

Figure 2.
Percent of normal margins or epithelial tissues with different grades of dysplasia that express both CD21 and EBER1 transcripts.

Figure 3. Levels of expression of CD21 mRNA in EBER1 positive epithelial tissues with different grades of dysplasia.

Figure 4. CK19 mRNA expression levels in normal epithelium, dysplastic epithelium and OSCC samples grouped according to whether tissues do or do not express EBV EBER1 mRNA.

Figure 5. Immunohistochemical staining for CK19 expression in normal oral tissues, dysplastic epithelium and OSCC that are positive (EBV+) or negative (EBV-) for expression of EBV EBER1 mRNA.

Table 1

Expression of CD21 and EBER1 mRNA in oral epithelial dysplasia

Tissue source	GAPDH (Ct)	CD21 (Ct)	Δ Ct (CR2)	2 ^{-$\Delta\Delta$Ct}	CD21 expression (%)	EBER positive (%)
Akata B cell line	18.88±0.18	27.47±0.14	8.59	1	100%	-
Normal margin (n=6)	29.26±1.05	-	-	-	0/6 (0%)	0/6 (0%)
Mild dysplasia (n=8)	27.57±0.12	37.76±0.51	10.09	0.35	1/8 (12.5%)	5/8 (62.5%)
Moderate dysplasia (n=8)	29.92±1.70	37.69±0.19	7.77	1.77	3/8 (37.5%)	6/8 (75%)
Severe dysplasia (n=13)	29.53±1.57	35.80±1.51	6.27	4.99	9/13 (69.2%)	5/13 (38.5%)

Table 2
Comparison of CK19 mRNA in normal oral epithelium with expression in OHL, all grades of dysplasia and OSCC

Tissue source	Case (n)	GAPDH (Ct)	CK19 (Ct)	ΔCt (CK19-GAPDH)	P-value	2 ^{-$\Delta\Delta Ct$}
Normal epithelium	23	28.85±1.93	36.38±1.40	7.91±1.64		1
OHL	5	28.31±3.12	33.62±2.34	5.31±1.26	0.0001	6.06
Mild dysplasia	7	28.99±2.68	36.30±2.46	7.28±1.20	0.355	1.55
Moderate dysplasia	6	30.09±1.81	36.97±1.50	6.93±1.29	0.186	1.97
Severe dysplasia	7	29.45±1.08	36.95±2.04	7.50±1.80	0.579	1.33
Well differentiated OSCC	6	27.70±3.38	36.70±1.96	8.83±3.92	0.598	0.53
Moderately differentiated OSCC	12	26.15±2.57	33.04±2.18	6.10±2.03	0.007	3.51
Poorly differentiated OSCC	8	29.14±3.71	35.27±3.24	6.13±1.64	0.013	3.43

Table 3

Comparison of CK19 mRNA expression in EBV^{-ve} and EBV⁺ve tissues.

Tissue source	GAPDH (Ct)	CK19 (Ct)	ΔCt (CK19-GAPDH)	P-value	$2^{-\Delta\Delta Ct}$
Normal epithelium					
EBV ^{-ve} (n=16)	29.33±1.75	37.53±1.49	8.20±1.15		1
EBV ⁺ ve (n=7)	28.36±2.10	35.23±1.31	7.23±2.41	0.343	1.96
Dysplasia					
EBV ^{-ve} (n=9)	29.54±1.20	37.43±1.22	7.96±1.00	0.593	1.18
EBV ⁺ ve (n=11)	29.47±2.24	36.11±2.46	6.67±1.46	0.005	2.89
OSCC					
EBV ^{-ve} (n=10)	27.55±3.07	34.57±2.89	6.83±1.70	0.021	2.58
EBV ⁺ ve (n=11)	27.81±3.06	33.23±2.14	5.42±1.72	0.0003	6.87