available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Expression profiles of viral responsive genes in oral and oropharyngeal cancers

Rebecca R. Laborde [a,b], *Vendula Novakova* [a,1], *Kerry D. Olsen* [b], *Jan L. Kasperbauer* [b], *Eric J. Moore* [b], *David I. Smith* [a,*]

[a] *Department of Experimental Pathology, Division of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA*

[b] *Department of Otorhinolaryngology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA*

ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 7 January 2010
Accepted 20 January 2010
Available online 19 February 2010

Keywords:
Oral cancer
Oropharyngeal cancer
Human Papillomavirus
HPV
qPCR
Gene expression
Virus

ABSTRACT

The association between Human Papillomavirus (HPV) DNA and oropharyngeal squamous cell carcinoma (SCC) has been supported by numerous studies strongly implicating HPV infection as a factor in the development of this cancer. In contrast, squamous cell carcinoma of the oral cavity has not been associated with HPV DNA, suggesting alternate aetiologic factors. The possibility that viral agents other than HPV could contribute to the development of oral cavity SCC should be given consideration, especially given the association of Epstein-Barr virus (EBV) with nasopharyngeal cancer. We used quantitative polymerase chain reaction (qPCR) studies to compare the expression levels of genes that may act as indicators of persistent stimulation by viral antigen in both oral cavity and oropharyngeal squamous cell cancers. Our results demonstrate that HPV-positive oropharyngeal tumours displayed gene expression patterns indicative of a viral signature and that HPV-negative oropharyngeal tumours do not display similar expression patterns. In contrast, we saw no evidence of either a viral or bacterial signature in the oral tumours examined. This would suggest that either an as of yet unidentified virus present in the oral tumour samples is not eliciting a typical immune response, or that there are no novel viral sequences or viruses present in the oral tumours examined.

1. Introduction

Cancers of the head and neck rank as the 6th most commonly observed cancers worldwide[1] with an average 5-year survival rate of only 50% for advanced disease.[2] Nearly 95% of head and neck cancers are squamous cell carcinomas (SSCs) and these are frequently highly invasive metastatic tumours. These cancers are often clinically grouped by their anatomical sites within the head and neck, including tumours of the nasopharynx, oropharynx and oral cavity. Exposure to tobacco and alcohol remains the most common traditional risk factors that promote the establishment and progression of tumours in the upper aerodigestive tract, with the combination of both factors resulting in a synergistic effect.

While an association of undifferentiated nasopharyngeal cancer with Epstein-Barr virus (EBV) has been established,[3] other subtypes of nasopharyngeal cancer do not have the same association suggesting heterogeneity in aetiology. In the oropharynx, recent reports find that up to 93% of

* *Corresponding author:* Tel.: +1 507 266 0309; fax: +1 507 284 1678.
E-mail address: smith.david@mayo.edu (D.I. Smith).

[1] Permanent address: Department of Dentistry, Charles University in Prague, Sokolska 581, 500 05 Hradec Kralove, Czech Republic.

0959-8049/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2010.01.026

squamous cell cancers demonstrate an association with Human Papillomavirus (HPV) DNA.[4,5] Our previous studies have indicated that most of the oropharyngeal tumours observed at the Mayo Clinic are HPV-positive.[6,7] This suggests that infection with HPV represents an additional significant risk factor for the development of oropharyngeal tumours, although the mechanisms for this interaction are not clearly understood. Corresponding studies with oral cavity tumours indicate a much lower rate of HPV infection,[8–10] even among those patients that lack exposure to tobacco and alcohol. These findings support the notion that the development of squamous cell cancers of the oral cavity is driven by a different set of factors and raises the question of whether a virus other than HPV may be involved.

Our study compared the expression levels of genes that may act as indicators of persistent stimulation by viral antigen(s) in HPV-positive oropharyngeal tumour and HPV-negative oropharyngeal normal tissues as well as HPV-negative oral cavity tumour and normal tissue to determine if the oral cavity tumours display expression patterns indicating the presence of viral antigen other than HPV.

Gene targets aimed at predicting a response to viral antigen were chosen based on the previous evidence of their involvement in the innate immune response to viral presence in epithelial cells.[11–13] The first class of gene targets includes four antimicrobial peptides human beta-defensin 1 (hBD-1), human beta-defensin 3 (hBD-3), human defensin 5 (HD5) and human defensin (HD6). These are small peptides (12–50 amino acids long), which represent an evolutionarily conserved component of the innate immune response to invading pathogens such as bacteria and viruses. These small peptides are produced by cells that play an important role in the innate immune system, primarily neutrophils, and are expressed in epithelial surfaces including the upper gastrointestinal tract.[14,15]hBD-1 was chosen as a negative control, with a suggested chemotatic role but a demonstrated lack of activity in response to HPV in oral mucosa.[14] hBD-3 is an inducible component of the innate immune response to microbial activity in epithelial cells that has been shown to be induced by exposure of oral mucosa to human immunodeficiency virus (HIV-1)[16] and implicated in blocking viral entry of HPV to oral mucosa and cultured cervical cells.[15,17]

Alpha-defensins, HD5 and HD6 are additional antimicrobial peptides found within granules of neutrophils, epithelial cells and macrophages that can be released in response to bacterial, viral or fungal presence and contribute to the induction of IL8 expression.[15] These transcripts have been shown to be viral responsive, with final peptide products playing a demonstrated role in the response of cultured cervical cells to Herpes Simplex Virus. HD6 acts by preventing viral binding and entry, and HD5 acts by inhibiting viral penetration.[15]

We then examined a marker of antigen presentation, MHC I, a key molecule for the presentation of antigen by nucleated cells to cytotoxic T cells and stimulation of the innate immune response to bacterial and viral infection.[18]

Lastly, we chose a panel of target cytokines with proven involvement in the innate immune response of epithelial cells to viral presence including Interleukin 8 (IL8), Interferon beta (IFN-β) and Interferon gamma (IFN-γ). IL8 is primarily produced by antigen presenting cells and epithelial cells and is a major mediator of the inflammatory response to presented antigen. Increased expression of IL8 has been demonstrated in HPV-positive vulvar intraepithelial neoplasia (VIN) when compared to HPV-negative cases.[19] IFN-β is a viral responsive cytokine produced by a variety of cell types in response to antigen stimulation, but produced at high rates by fibroblasts. Increased expression of IFN-β participates in the innate response to viral antigen by interfering with viral replication (reviewed).[20,21] IFN-γ is a pro-inflammatory cytokine involved with both the innate and adaptive response to bacterial and viral antigen. It has been shown to play a pivotal role in the innate immune response to HPV infection,[22] with high expression associated with effective clearance of cervical HPV infection.[23,24]

The expression levels of these target genes were measured by quantitative polymerase chain reaction (qPCR). Similar expression patterns between both anatomical sites could potentially indicate the presence of a yet unidentified virus in HPV-negative oral tumours.

2. Materials and methods

2.1. Collection and processing of tissues

All tissues used in this study were collected from patients undergoing treatment at the Mayo Clinic, Rochester, MN. Tumour samples were obtained from patients undergoing surgical treatment for oral cavity or oropharyngeal squamous cell carcinoma (SCC). Normal oropharyngeal tissues were collected from patients undergoing surgical treatment of non-cancer related conditions. Normal oral cavity tissue was collected during consented autopsy at the Mayo Clinic, Rochester, MN. All tissue samples were snap frozen in liquid nitrogen and stored according to procedure approved by the Mayo Clinic Institutional Review Board (see Supplemental Information). All tumour and normal tissues were matched for age and smoking status.

2.2. DNA extraction

Frozen slides corresponding to all samples were processed for DNA extraction by scraping tissue from the 4-μm section, using the reviewed H&E slide for reference. DNA was extracted using the QiAMP DNA Micro kit (QIAGEN, Valencia, CA). Isolated DNA was quantified by NanoDrop ND1000 (ThermoFisher Scientific, Waltham, MA).

2.3. RNA extraction

Total RNA was extracted from portions of the frozen tissue samples using the Qiagen RNAeasy Plus Kit (Valencia, CA) according to manufacturer's protocol. Extracted RNA was also quantified by NanoDrop ND1000.

2.4. Detection of HPV DNA by polymerase chain reaction (PCR)

DNA extracted from all frozen tissues was analysed for the presence of HPV specific sequences by polymerase chain reaction (PCR) with three different primer pairs: L1

consensus primers MY09 and MY11, a specific primer set to the HPV-16 E6 region and an HPV-18 specific primer set (Supplemental Table 1).

2.5. Quantitative real-time RT-PCR (qPCR)

Primers for target genes were designed using Primer3.[25] cDNA was synthesised using the Thermoscript RT-PCR System from Invitrogen using 2 μg of total RNA and oligo-dt primers. cDNA quantitation was then performed with specific primers (Supplemental Table 2) using the SYBR green method (ABI 7900HT fast real-time PCR system). Primers were optimised for qPCR with β-actin as a control gene and then with the transcript region of interest. When the optimal primer concentration produced a linear response to input cDNA concentration, RNA samples were analysed in triplicate for each tested transcript. To normalise the expression levels (ΔC_T), the threshold cycle (C_T) for each transcript was subtracted from the C_T of the more abundantly expressed control gene (β-actin). This value was then used to calculate the $2^{\wedge -\text{delta}\, C_T}$ value to compare expression patterns between various tissue samples. A significant change in expression was defined as greater than a twofold increase or decrease.

3. Results

3.1. Detection of HPV DNA

DNA extracted from frozen tissue specimens was analysed for the presence of HPV specific sequences by polymerase chain reaction (PCR). All samples were screened with both universal primers designed to detect all HPV types and HPV-16 specific primers. All 12 samples corresponding to oropharyngeal tumour tissue were found to be HPV-16 positive (Fig. 1), while 10 normal oropharyngeal tissues were negative for HPV DNA (data not shown). Interestingly, oropharyngeal tumour samples 2 and 3 have a positive PCR result with the HPV-16 specific primers, but a negative result with the universal primers (Fig. 1). This pattern is common when screening larger panels of oropharyngeal tumour samples (data not shown) and indicates that the HPV-16 primers are more sensitive for detecting HPV-16 DNA sequences that may be missed by the universal primer set. Identical analysis of 12 tumour and 10 normal tissues from the oral cavity was found to be HPV-negative with both universal and HPV-16 specific primers (data not shown).

3.2. Analysis of gene expression patterns in tumour and normal oropharyngeal and oral cavity tissues by quantitative PCR (qPCR)

Gene targets were chosen for analysis by qPCR based on their characterised involvement in the response to viral antigen in human tissue.[11–13] Gene expression levels for these gene targets were measured using cDNA prepared from oral cavity and oropharyngeal tumours. Normal tissue was collected from similar locations in patients undergoing procedures unrelated to the treatment of cancer. The gene targets fell into three distinct classes: antimicrobial peptides, markers of antigen presentation and cytokines (Table 1).

In the class of antimicrobial peptides, the first group of gene targets includes two examples of beta-defensins. Human defensin, beta 1 (hBD-1) has been shown to be constitutively expressed in epithelial tissues and is not expected to increase expression in response to virus. Gene expression levels for this target were measured and compared in tumour and normal oral cavity tissue (Fig. 2A) and oropharyngeal tissue (Fig. 2B). Similar levels of hBD-1 expression were detected in the tumour and normal tissues from both sites (Fig. 2A and B), indicating no change in constitutive hBD-1 expression in the tumour tissues. Expression patterns were examined for a second human beta-defensin (hBD-3), an inducible component of the innate immune response to microbial activity in epithelial cells. Both tumour and normal tissues from the oral cavity expressed similar levels of hBD-3 (Fig. 2A), while HPV-positive oropharyngeal tumours showed an increased expression (greater than twofold change) as compared to normal tissues (Fig. 2B). Gene expression levels were also quantified for two examples of alpha-defensins including human defensin 5 (HD5) and human defensin 6 (HD6). There was a greater than twofold increased level of expression of these genes in the HPV-positive oropharyngeal tumours (Fig. 2B) in contrast to similar levels of expression in the oral cavity tumours versus normal oral cavity tissue (Fig. 2A). The pattern of increased expression of target genes in HPV-positive oropharyngeal tumours versus normal and unchanged expression in oral cavity tumour versus normal tissue was consistent for the additional gene targets examined.

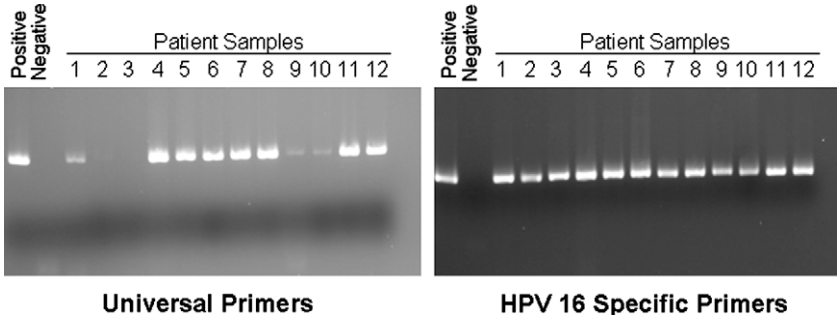

Fig. 1 – Detection of HPV DNA in oropharyngeal tumour tissue. All fresh frozen samples were screened by PCR for the presence of HPV DNA sequences, as shown for oropharyngeal tumour samples, using both universal primers for the detection of all HPV types (A) and HPV-16 E6 specific primers (B).

Table 1 – Gene targets involved in response to persistent antigen. Gene targets were chosen for analysis by qPCR based on their characterised involvement in the response to persistent antigen, such as virus or bacteria, in human tissue. These gene targets fell into three distinct classes: antimicrobial peptides, markers of antigen presentation and cytokines.

Common designation	NCBI designation	NCBI Gene ID	Full name	Function
hBD-1	DEFB1	1672	Human defensin, beta 1	Antimicrobial peptide produced by neutrophils, implicated in the resistance of epithelial cells to microbial colonisation
hBD-3	DEFC103A	55894	Human defensin, beta 3	Antimicrobial peptide produced by neutrophils, providing broad spectrum antimicrobial activity, innate immune defence
HD5	DEFA5	1670	Human defensin, alpha 5	Antimicrobial peptide produced by neutrophils, responsible for induction of IL8 in epithelial cells
HD6	DEFA6	1671	Human defensin, alpha 6	Antimicrobial peptide that contributes to innate defence of GI mucosal surfaces
MHCI	HLA-E	3133	Major histocompatibility complex, class I	Antigen presenting molecules present on all nucleated cells. Present antigen to cytotoxic T cells to promote Innate immune response in the presence of bacterial or viral infection or during tumourgenesis
IL8	IL8	3576	Interleukin 8	Secreted cytokine that acts as major mediator of the inflammatory response
IFNG	IFNG	3458	Interferon, gamma	Cytokine critical for innate and adaptive immunity against viral and intracellular bacterial infections and control of tumour development
IFNB	IFNB1	3456	Interferon, beta 1, fibroblast	Cytokines critical for antiviral response of the innate immune system

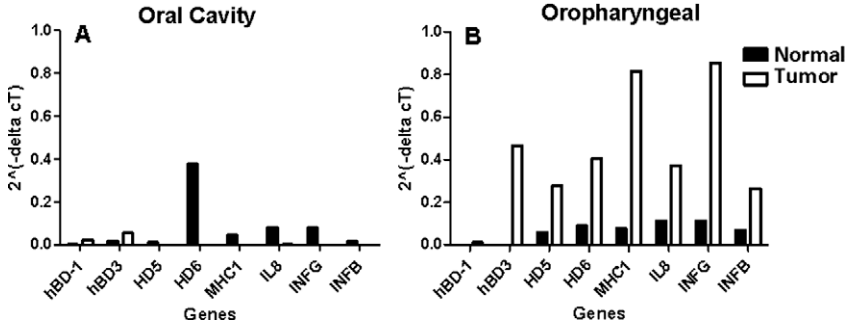

Fig. 2 – Expression patterns of antigen responsive genes. Analysis of expression patterns of target genes was measured by quantitative PCR (qPCR) in tumour tissue (white bars) and normal tissue (black bars) from two locations, (A) oral cavity and (B) oropharyngeal tissues.

The second class of markers of antigen presentation includes the expression of major histocompatibility complex class I (MHC I). MHC I followed a similar pattern of having greater than twofold higher expression in the HPV-positive oropharyngeal tumour tissue (Fig. 2B). In contrast, we observed decreased expression of MHC I in oral cavity tumour tissue as compared to normal oral cavity tissue (Fig. 2A).

The third class and remaining genes examined represent a panel of cytokines involved in the innate immune response to bacterial and viral antigen and the promotion of an inflammatory response in tissues. These genes include interleukin 8 (IL8) and interferon beta (IFN-β). An additional target, interferon gamma (IFN-γ) is involved in both the innate and adaptive response to bacterial and viral antigen. The expression of IL8, IFN-β and IFN-γ increased by greater than twofold in the oropharyngeal tumours compared to normal tissue (Fig. 2B), but similar increases were not detected in the oral cavity tumour tissue (Fig. 2A).

In an effort to validate that the increased expression of the target genes in the HPV-positive oropharyngeal tumours can be attributed to an innate response to the virus, an additional control of HPV-negative oropharyngeal tumour tissue was included for comparison. All targets displayed low levels of gene expression in the HPV-negative oropharyngeal tumour tissue (see Supplemental Fig. 1).

4. Discussion

Head and neck cancers represent a unique challenge to clinicians because of their invasive, metastatic and highly recurrent nature. While the standard risk factors of drinking and smoking apply to the majority of patients treated, the increasing numbers of patients that develop these cancers in the absence of standard risk factors suggest that additional factors may also be involved.

Another potential factor in the development of oropharyngeal cancers is persistent infection with Human Papillomavirus (HPV). HPV has been demonstrated as a key initiator of cervical cancer, leading to the development of the highly effective, multivalent HPV vaccine. Changes in sexual practices are likely contributing to increasing exposure of the oral cavity and oropharyngeal tissues to HPV virus.[26] However, studies aimed at examining rates of HPV infection of the oral cavity have detected a very low incidence of HPV sequence present in these tissues.[8–10] This is in contrast to the findings of similar studies focusing on oropharyngeal tissues, with reported frequencies up to 93%.[4,5] Our previous studies at the Mayo Clinic, using fresh frozen tissue samples, found that the majority of oropharyngeal tumours were indeed HPV (HPV-16) positive.[6,7] This supports the suggestion that tumours arising from the oral cavity and oropharyngeal tissues may represent different groups of cancers.

Despite the high degree of association between HPV and oropharyngeal cancers, there remains no clear consensus on a probable mechanistic role for this virus in cancer development and progression, alteration of resistance to adjuvant therapy or of the implications of infection to patient outcomes. In spite of this, there is a growing interest in including HPV status as a major factor in the development of treatment strategy for patients presenting with oropharyngeal cancers.[27–29] This promotes the questions regarding the role of persistent viral antigen, such as HPV, in the development of head and neck cancers to a high priority.

While the precise role of HPV in oropharyngeal tissues is unknown, it still represents a potentially important therapeutic target which can be affected with the cervical multivalent HPV vaccine. The question we addressed in this paper was whether there might be another virus that contributes to the development of oral cavity cancers. Thus we undertook examining expression of markers that might indicate the presence of novel viruses in these cancers. Utilising qPCR, we evaluated the expression levels of a variety of gene targets that would be expected to increase from constitutively low levels of expression to higher levels in the presence of antigen stimulation from viral and/or bacterial infection,[11–13] and compared the expression of these genes in normal and tumour tissue of the oral cavity and oropharynx. The expression patterns of these targets were evaluated in HPV-positive oropharyngeal tumour and HPV-negative normal oropharyngeal tissue and HPV-negative oral cavity tumour and normal oral cavity tissue.

Our results demonstrated that the HPV-positive oropharyngeal tumours displayed gene expression patterns indicative of a viral signature. Every gene, with the exception of hBD-1, displayed greater than twofold increased expression in the tumours as compared to normal samples. In contrast, we saw no evidence of either a viral or bacterial signature in the oral cavity tumours examined. This would suggest that either the presence of a currently unidentified virus in the oral tumour samples is not eliciting a typical immune response, or that there are no novel viral sequences or viruses present in the oral cavity tumours examined.

In an effort to validate that the increased expression of the target genes in the HPV-positive oropharyngeal tumours represents the innate immune response to viral antigen and not a general inflammatory response to tumour development, we compared this to the expression levels of the same targets in HPV-negative oropharyngeal tumour tissue. The comparison shows greater than twofold increased expression of all gene targets, with the exception of the hBD-1 negative control, in the HPV-positive tumour tissue and supports our assumption that this increased expression of target genes can indicate the presence of viral antigen.

Based upon our results, we demonstrate that there is no evidence of a viral signature in the oral cavity tumours examined. Our future work will involve analysing oral tumours from younger patients lacking standard risk factors utilising the new technology of Next Generation DNA sequencing, which offers the capability of more comprehensively analysing the alterations that occur during cancer formation. Next Generation DNA sequencing can probe the entire transcriptome of normal versus cancer tissue to examine changes in gene expression as well as mutations in coding transcripts and detect changes in DNA structure including deletions, insertions and balanced reciprocal translocations.[30] Finally, we will be directly analyse samples for the actual presence of bacterial and viral sequences and help delineate a potential role for persistent antigen in the development and progression of these cancers.

Conflict of interest statement

None declared.

Acknowledgements

We would like to thank Dr. Jean Lewis, MD, Department of Laboratory Medicine and Pathology, Mayo Clinic for examining all tissue slides and Nicole Tombers, study coordinator for the Department of Otorhinolaryngology, Mayo Clinic, Rochester, MN.

This work was supported by internal funding from the Mayo Clinic Research Foundation.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.01.026.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;**55**:74–108.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin* 2009;**59**:225–49.
3. Pathmanathan R, Prasad U, Chandrika G, et al. Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia. *Am J Pathol* 1995;**146**:1355–67.
4. Attner P, Du J, Hammarstedt L, et al. The role of human papilloma virus (HPV) in the increased incidence of base of tongue cancer. *Int J Cancer* 2009 [Epub ahead of print].

5. Nasman A, Attner P, Hammarstedt L, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer* 2009;**125**:362–6.
6. Ukpo OC, Pritchett CV, Lewis JE, et al. Human papillomavirus-associated oropharyngeal squamous cell carcinomas: primary tumor burden and survival in surgical patients. *Ann Otol Rhinol Laryngol* 2009;**118**:368–73.
7. Soderberg C, Perez DS, Ukpo OC, et al. Differential loss of expression of common fragile site genes between oral tongue and oropharyngeal squamous cell carcinomas. *Cytogenet Genome Res* 2008;**121**:201–10.
8. Termine N, Panzarella V, Falaschini S, et al. HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988–2007). *Ann Oncol* 2008;**19**:1681–90.
9. Miller CS, White DK. Human papillomavirus expression in oral mucosa, premalignant conditions, and squamous cell carcinoma: a retrospective review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996;**82**:57–68.
10. Liang XH, Lewis J, Foote R, Smith D, Kademani D. Prevalence and significance of human papillomavirus in oral tongue cancer: the Mayo Clinic experience. *J Oral Maxillofac Surg* 2008;**66**:1875–80.
11. Dale BA, Fredericks LP. Antimicrobial peptides in the oral environment: expression and function in health and disease. *Curr Issues Mol Biol* 2005;**7**:119–33.
12. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003;**3**:710–20.
13. Friedland JS. Chemokines in viral disease. *Res Virol* 1996;**147**:131–8.
14. Buck CB, Day PM, Thompson CD, et al. Human alpha-defensins block papillomavirus infection. *Proc Natl Acad Sci USA* 2006;**103**:1516–21.
15. Hazrati E, Galen B, Lu W, et al. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. *J Immunol* 2006;**177**:8658–66.
16. Quinones-Mateu ME, Lederman MM, Feng Z, et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *Aids* 2003;**17**:F39–48.
17. Leikina E, Delanoe-Ayari H, Melikov K, et al. Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat Immunol* 2005;**6**:995–1001.
18. Truong P, Heydari S, Garidou L, McGavern DB. Persistent viral infection elevates central nervous system MHC class I through chronic production of interferons. *J Immunol* 2009;**183**:3895–905.
19. Santegoets LA, van Seters M, Heijmans-Antonissen C, et al. Reduced local immunity in HPV-related VIN: expression of chemokines and involvement of immunocompetent cells. *Int J Cancer* 2008;**123**:616–22.
20. Biron CA. Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. *Semin Immunol* 1998;**10**:383–90.
21. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999;**17**:189–220.
22. Wu TC, Hsieh ST, Purow BW, Kurman RJ. Demonstration of human papillomavirus (HPV) genomic amplification and viral-like particles from CaSki cell line in SCID mice. *J Virol Methods* 1997;**65**:287–98.
23. Scott M, Stites DP, Moscicki AB. Th1 cytokine patterns in cervical human papillomavirus infection. *Clin Diagn Lab Immunol* 1999;**6**:751–5.
24. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. *Cancer Res* 2003;**63**:636–41.
25. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;**132**:365–86.
26. Heck JE, Berthiller J, Vaccarella S, et al. Sexual behaviours and the risk of head and neck cancers: a pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. *Int J Epidemiol* 2009 [Epub ahead of print].
27. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. *Clin Cancer Res* 2009;**15**:6758–62.
28. Vu HL, Sikora AG, Fu S, Kao J. HPV-induced oropharyngeal cancer, immune response and response to therapy. *Cancer Lett* 2009 [Epub ahead of print].
29. Lindquist D, Romanitan M, Hammarstedt L, et al. Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. *Mol Oncol* 2007;**1**:350–5.
30. Campbell PJ, Stephens PJ, Pleasance ED, et al. Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. *Nat Genet* 2008;**40**:722–9.