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Human papillomavirus status and p16^{INK4A} expression in patients with mucosal squamous cell carcinoma of the head and neck in Queensland, Australia

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ABSTRACT

Background: The last decade has seen changes in the epidemiology of mucosal squamous cell carcinomas of the head and neck (HNSCCs), with increasing numbers of cases attributable to human papillomavirus (HPV) infection. We sought to determine the prevalence of HPV and p16^{INK4a} expression in Australian HNSCC patients and to identify predictors of HPV-positivity.
Methods: We recruited 248 HNSCC patients with histologically confirmed primary SCC of the oropharynx, oral cavity, hypopharynx or larynx diagnosed between 2004 and 2010. All patients completed a questionnaire. Clinical data were abstracted from medical records. HPV presence in paraffin-embedded tumours was determined by PCR, and expression of p16^{INK4a}, p21^{WAF1}, p53, pRB, cyclin D1, and Ki67 by immunohistochemistry.
Results: Fifty (20%) patients were HPV-positive, 63 (28%) overexpressed p16^{INK4a}, and 44 (19%) were positive for HPV and p16^{INK4a} (high concordance between HPV-positivity and p16^{INK4a} status, $\kappa = 0.72$). HPV-16 was most common (84%), followed by HPV-18 (10%), HPV-33 (4%) and HPV-69 (2%). HPV and p16^{INK4a} prevalence was highest for SCCs of the oropharynx, followed by hypopharynx, larynx and oral cavity (HPV and p16^{INK4a} $p < 0.0001$). HPV prevalence and p16^{INK4a}-overexpression were significantly higher in younger than older patients (HPV $p = 0.001$; p16 ^{INK4a} $p = 0.003$). Heavy smokers had lower HPV prevalence than non- or moderate smokers ($p = 0.017$). Gender and alcohol consumption were not associated with HPV or p16^{INK4a} status. HPV-positive tumours had significantly lower cyclin D1 and higher p21^{WAF1} expression than HPV-negative tumours.
Conclusion: HPV prevalence and p16^{INK4a}-overexpression were highest in oropharyngeal tumours, younger patients, and non-smokers.

1. Introduction

Worldwide, mucosal squamous cell carcinomas (SCC) of the head and neck (hereafter HNSCCs) are the sixth most common type of cancer with an estimated 575,000 new cases diagnosed annually [1,2]. The designation includes SCC of the oral cavity, oropharynx, larynx and hypopharynx [3]. The incidence of HNSCC, particularly of the oropharynx, has increased recently in younger patients, and many of these patients do not report a history of exposure to the classical risk factors of smoking and excessive alcohol intake [4]. While the number of patients diagnosed with other smoking-related cancers is falling in Australia, the incidence of HNSCC is increasing [5]. This recent increase in HSNCC among younger patients has been attributed to the rising prevalence of HPV infection, especially of the oropharynx, and is hypothesised to reflect changing sexual behaviours (specifically, the increasing practice of oral sex) during recent decades [6,7].

HPV prevalence in published series of HNSCC tumours ranges from 10% to 90%, depending on the site of the tumour. The highest HPV prevalence has been reported in oropharyngeal SCCs, particularly in tonsils of younger, non-smoking patients [8,9]. A recent Australian case series reported that the proportion of HPV-positive

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oropharyngeal cancers had increased from 19% in 1987–1990 to 66% in 2005–2006 [10]. A Swedish study of tonsillar SCCs observed even greater increases in HPV prevalence over nearly four decades (23% between 1970 and 1979, 29% 1980 and 1989, 57% 1990 and 1999, 68% 2000 and 2002, 77% 2003 and 2005, and 93% 2006 and 2007), with the prevalence of HPV in tonsillar cancers now approaching that of cervical cancer [9]. HPV-16 is the most commonly detected HPV type in HNSCC studies worldwide and in recent series it has been detected in more than 90% of HPV-positive oropharyngeal and oral cavity cancers [11]. Although HPV is thus strongly implicated in the aetiology of these cancers, it has also been reported that HPV-induced overexpression of $p16^{INK4a}$ in HNSCC patients confers better survival than $p16^{INK4a}$-negative patients [12].

There are plausible grounds for inferring that the observed associations between HPV and HNSCC are causal. In cervical cancer, the HPV-encoded oncoproteins E6 and E7 from the high-risk HPV types act by blocking a number of key regulatory genes in the host cell. HPV E7 forms a complex with cellular pRB, leading to over-expression of $p16^{INK4a}$. E7 also binds $p21^{WAF1}$ which results in loss of control of cyclin D1. HPV E6 binds to the p53 tumour suppressor protein and targets it for ubquitin-mediated degradation [13]. The over-expression of both E6 and E7 allows uncontrolled cell growth without checkpoint controls and this sets the cell up for further mutations, transformation, and the formation of cervical cancer. While these associations are well-established in cervical cancer, studies of HPV in HNSCCs have been heavily focused on $p16^{INK4a}$ [12,14]; little is known about its effect on other cell cycle proteins.

In this study, we sought firstly to determine the prevalence of HPV DNA and $p16^{INK4a}$ in tumours from Australian patients with HNSCC at different tumour sites and to compare risk factors for developing these molecular characteristics. We also explored whether expression of cell cycle proteins ($p21^{WAF1}$, p53, pRB, cyclin D1, and Ki67) was associated with HPV infection in HNSCC.

2. Materials and methods

2.1. Study participants

Eligible cases were patients over 18 years old with histologically confirmed primary SCC of the oropharynx, oral cavity, hypopharynx or larynx diagnosed between 1 January 2004 and 31 December 2010 and who presented to the Head and Neck Clinic at the Princess Alexandra Hospital in Brisbane, Queensland, Australia.

This study was approved by the Human Research Ethics Committees of the Metro South (HREC/11/QPAH/93) and the QIMR Berghofer Medical Research Institute (P1364).

2.2. Data collection

Eligible patients attending the Head and Neck Clinic were asked to complete a paper-based two-page questionnaire. The questionnaire requested basic demographic information about age, sex, education, height and weight. We asked participants to report their tobacco smoking and alcohol consumption. Frequency of heartburn, acid reflux, and physical activity (“strenuous physical activity for at least 20 min in leisure time”) were collected. We asked participants to report their frequency of use of aspirin, paracetamol (acetaminophen), non-steroidal anti-inflammatory drugs (NSAID) and anti-histamines during the past 5 years. Medical conditions and history of cancer as well as family history of cancer (parents and siblings) were also captured.

Clinical data were abstracted from medical records. We collected information about the disease (date and type of diagnosis, tumour differentiation, TNM score and invasion), treatment (intent, type, dose and completion) and outcome (date of last follow-up, disease status, recurrence and date where applicable, nodal involvement, metastases and date of death where applicable).

Details of histology were abstracted from pathology reports accompanying each tumour specimen. For patients who had taken part in the study, we obtained tumour specimens where possible from the relevant pathology laboratory.

2.3. HPV prevalence and type determination

We used formalin-fixed paraffin-embedded (FFPE) tissue samples as the source of tumour DNA to assess HPV prevalence and type, and for immunohistochemical (IHC) analyses of cell cycle proteins. The presence of tumour cells was confirmed in H&E sections for each specimen by an expert anatomical pathologist (GL). For the HPV analysis, 7 μm tumour section was used from FFPE blocks for DNA extraction with the QuickExtract™ FFPE DNA Extraction Kit (Epicentre® Biotechnologies) as described by the manufacturer. DNA samples were analysed by PCR for the presence of HPV with the general mucosal HPV primer GP5+/GP6+ [15]. The 25 μL of PCR solution contained 5 μL of extracted DNA, 0.5 μmol/L of the GP5+ and GP6+ primers (Geneworks), dNTPs at concentrations of 0.2 mmol/L each (Fisher), 1 U of AmpliTaq Gold DNA polymerase, 1× PCR Gold buffer, and 2.0 mmol/L $MgCl_2$ (Applied Biosystems). In each batch of tests, H_2O was used as a negative control. HeLa cells (HPV-18 – positive cervical cancer cell line) were used as a positive control in both PCR reactions. PCR-amplicons were analysed by electrophoresis (1.5% agarose gel containing ethidium bromide; SeaKem, FMC bioproducts and Sigma) and identified under UV light. To minimise the risk for contamination we used different pipettes and rooms for DNA extraction, preparing the PCR solution, adding DNA samples to PCR solution and electrophoresis analysis.

We purified 15 μL of the HPV-positive PCR products using the Agencourt® AMPure PCR purification kit (Agencourt Bioscience) in a magnetic 96-ring SPRIplate®. The sequencing reaction contained the purified PCR products together with 3.25 μM of primer and BigDye Terminator and buffer (Applied Biosystems). The sequencing reaction was performed in an Eppendorf Mastercycler Gradient PCR machine, and were purified with the Agencourt® CleanSEQ dye-terminator removal kit (Agencourt Bioscience) in a magnetic 96-ring SPRIplate®. Purified sequence reactions were analysed with an automated DNA sequencer (ABI model 3100). The DNA sequences obtained were compared with available sequences in GenBank through the BLAST server (http://blast.ncbi.nlm.nih.gov).

β-Globin PCR with the primers PCO3 and PCO4 [16] was carried out on all samples to ensure that they contained enough cells to detect human DNA and that no PCR inhibiting agents were present.

2.4. Immunohistochemistry of cellular response to HPV infection

We measured expression patterns of $p16^{INK4a}$, $p21^{WAF1}$, p53, pRB, cyclin D1, and Ki67 by immunohistochemistry (IHC). Briefly, FFPE tumour sections (4 μm) were affixed to Menzel Superfrost Plus adhesive slides and air-dried overnight at 37 °C, then dewaxed and rehydrated through descending graded ethanol to water using a Sakura DRS stainer. For all stains antigen retrieval was performed in a Biocare Medical Decloaking chamber, and then washed in TBS. The following primary antibodies were used according to the protocol recommended by the supplier: mouse anti-$p16^{INK4a}$ IgG1 (#G175-405; BD Pharmingen), mouse anti-$p21^{WAF1}$ (#EA10; Calbiochem), mouse anti-p53 (#DO7; Novocastra), rabbit anti-pRB (#9308; Cell Signalling), rabbit anti-Cyclin D1 (#CRM307; Biocare Medical) and mouse anti-Ki67 (#M7240; Dako). All IHC analyses were performed in batches with appropriate positive and negative controls.