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**Paper:**

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Investigation of p16INK4a as a prognostic biomarker in oral epithelial dysplasia

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Abstract

Introduction
There is increasingly strong evidence that Human Papilloma Virus (HPV) is a risk factor for the development of oropharyngeal cancer. Evidence for a similar role in oral cancer (OSCC) is not as clear. Furthermore, it is also uncertain whether HPV may have an etiologic role in the development of oral epithelial dysplasia (OED) or its transformation to OSCC. Reported prevalence rates of HPV infection in oral premalignant lesions range from 0 to 100%. A recent meta-analysis estimates the prevalence of high-risk serotypes HPV16/18 in oral premalignant lesions and OSCC to be around 25%. Despite this being three times higher than was found in normal oral mucosa, it does not imply a causal relationship. Furthermore, very few studies have examined the prognostic significance of HPV positivity in the development of OSCC from oral premalignant lesions. Those that have either include cases without OED, or have very small numbers. This has led to conflicting results.

Aims
We aimed to examine the ability of p16\textsuperscript{INK4a} protein expression, a surrogate marker of HPV infection, to predict malignant progression in a large cohort of patients with OED.

Methods
A cohort of 148 cases with a range of severity of OED, as defined by the WHO grading system was compiled. As well as histological grade, other clinical factors were collated on each case. Immunohistochemical analysis was performed on 4μm sections using a well validated and reproducible mouse monoclonal antibody directed against p16\textsuperscript{INK4a}. Slides were double scored independently by two trained observers using a scoring system that considered both the intensity and proportion of cells stained. Univariate analyses using both logistic and Cox regression models (the latter also giving a time to event analysis) were performed.

Results
39 of the 148 cases progressed to cancer. 10 of the 148 cases (15%) had a p16\textsuperscript{INK4a} score, which would indicate HPV positivity. Whereas a high grade of dysplasia (p=0.0002) and lesion morphology (p=0.03) were found to be prognostic of malignant progression, sex, anatomical location, smoking and alcohol status were not. p16\textsuperscript{INK4a} score was not demonstrated to be a prognostic factor in this cohort (p=0.29). This did not change with a time to event analysis (p=0.24).

Conclusion
Very few studies have assessed the etiological role of HPV in OSCC development from dysplastic lesions. Despite the increased prevalence of HPV in OED compared to normal oral mucosa, our study, using the largest cohort of OED cases to examine this etiological role, was unable to demonstrate a prognostic ability for p16\textsuperscript{INK4a}. 
Introduction

The role of the human papilloma virus (HPV) in the pathogenesis of oropharyngeal carcinoma (OPC) is now well established\(^1-4\). However, the aetiological role of HPV infection in the development of cancers of the oral cavity is currently not as clear.

OSCC is thought to arise as a result of the accumulation of progressive genomic instability and consequent histological atypia\(^5\). This sometimes presents as oral epithelial dysplasia (OED). As only around 12% of OED lesions will undergo malignant transformation, it is termed a potentially malignant disorder\(^6\). The role of HPV in the development of OED and its transition from potentially malignant disorder to invasive disease is unclear. Several studies have examined the prevalence of HPV in oral potentially malignant lesions. A recent meta-analysis estimated HPV-16/18 to be 3 times more common in dysplastic lesions (OR, 3.29; 95% CI, 1.95–5.53) and cancers OR, 3.43; 95% CI, 2.07–5.69) than in normal mucosal biopsies\(^7\).

A second meta-analysis demonstrated an even higher association, with OED lesions 5 times more likely to be HPV positive than normal mucosa (OR = 5.10; 95% CI: 2.03–12.80)\(^8\). Yet, recent large studies show that only a small number of oral cancers are HPV positive, which is contradictory to what might be expected. Despite this, there have been few studies examining the prognostic role of HPV infection in the development of OSCC from potentially malignant lesions. These few studies report conflicting results.

The aim of this study was to examine p16\(^{\text{INK4a}}\) protein expression, a surrogate marker of HPV infection, to investigate if it was a prognostic marker of malignant progression in a large cohort of patients with OED.
Methods

This study has been reported according to the REporting recommendations for tumour MARKer prognostic studies guidelines (REMARK)⁹.

Cohort

Archived tissue specimens from patients with OED biopsied between 1996 and 2008 were identified from the pathology databases of University Hospital Coventry and Warwick, George Eliot Hospital Nuneaton, University Hospital Birmingham, Birmingham Dental Hospital and the University of Leeds. Inclusion required patients to be over 18 years of age at the time of biopsy, have a diagnosis of OED made by grading using the WHO classification and a minimum follow-up for non-transformed cases of 12 months. Any cases positive for fungi or Candida on diastase-resistant Periodic Acid Schiff (dPAS) staining were excluded from the study, as were cases diagnosed with lichenoid inflammation with atypia and proliferative verrucous leukoplakia. Further exclusions were made of cases that had previously been diagnosed with head and neck cancer, or had transformed to cancer within 3 months of diagnosis of OED. Potential risk factors such as age, gender, anatomical site, lesion morphology and smoking/alcohol history were recorded where possible and used as candidate variables in the prognostic model along with p16⁰⁰⁵⁶NK⁴⁴a.

Immunohistochemistry

4μm sections were taken from Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks and a tissue microarray (TMA) constructed from a small number of the cohort. We have previously demonstrated near perfect agreement between scoring of TMA and whole sections with this and other biomarkers in OED¹⁰. Sections were deparaffinised for 10 minutes in xylene before rehydration in graded alcohol and distilled water. All sections underwent heat-induced epitope retrieval for 20 minutes
in citrate buffers at pH 6. The Novocastra™ Polymer Detection System was used with endogenous peroxidase activity blocked for 20 minutes with 3% hydrogen peroxide, followed by 30 minutes incubation with 0.4% Casein in phosphate-buffered saline. Sections were incubated overnight at 4°C in 1:5 concentration primary antibody (CINtec® monoclonal mouse antibody clone E6H4T directed to human p16^{INK4a} protein, mtm laboratories AG, Germany). Sections were then exposed to 30 minutes in post primary block and 30 minutes in NovoLink™ Polymer. Sections were finally incubated with the substrate/chromogen, 3,3’- diaminobenzidine (DAB), before counterstaining with Haematoxylin. A case of oropharyngeal carcinoma with diffuse strong p16^{INK4a} staining throughout was used as a positive control.

**Histological assessment of immunocytochemistry**

Two trained observers from the same institution scored slides independently, with consensus scoring in all cases where there was disagreement. A scoring system that considered both the intensity and proportion of cells stained was used. Intensity of staining was scored on a 4 point scale: 0, negative (No staining); 1, weakly positive staining; 2, moderately positive staining; and 3, strongly positive staining. The proportion of cells stained was also scored on a 4-point scale: 1 (<25% of cells stained); 2 (25-50% of cells stained); 3 (51-75% of cells stained); 4 (>75% cells stained). These two scores were multiplied to give an overall score of 0 – 12 for each case. Only cases scoring >9 were considered positive.

**Statistical analysis**

The strength of agreement for immunohistochemical scoring between the two raters was assessed using both kappa (κ) and intraclass correlation coefficients (ICCC). ICCC has been suggested as superior in assessing agreement in this setting, however kappa scores were also calculated to allow comparison with other studies. Agreement was interpreted using well accepted standards. The ability of p16^{INK4a}...
or clinical factors to predict progression was calculated initially using a univariate logistic regression analysis. The primary outcome was progression to cancer. 

\( p16^{\text{INK4a}} \) was handled as a binary variable, with cases scoring <12 considered negative for the marker. Clinical factors were analysed as categorical variables in the following way: gender (male, female), Age (≤50, >50), smoker (Never, ex and current), alcohol (None, <21 units, >21 units) morphology (white patch, speckled red/white, red patch, ulcer and mass), site (tongue, floor of mouth, palate, buccal, retromolar) and histological grade (mild, moderate, severe, carcinoma in situ).

Listwise deletion was used to handle missing data, whereby any cases with missing data relevant to that particular analysis were excluded. A Kaplan Meier survival analysis was subsequently performed to assess the effect of time on this prognostic ability, expressed as oral cancer free survival. Differences between oral cancer free survival curves were calculated using a Log-rank (Mantel-Cox) test with significance defined as \( p<0.05 \). Consensus scores were used for the \( p16^{\text{INK4a}} \) analyses.

Calculations were performed using SPSS version 19.0 for Mac; SPSS Inc., Chicago, IL, USA and SAS version 9.2.
Results

Cohort characteristics

148 cases of OED were included in the analysis. The cohort comprised 72 females (mean age 62, SD=14) and 76 males (mean age 60, SD=13). 69 cases (47%) were graded as mild, 50 (24%) as moderate, 27 (18%) as severe and 2 (1%) as carcinoma in situ. Median follow-up time was 42 months, (3 – 156 months) with 39 cases undergoing malignant transformation (26%). Further clinical characteristics are summarized in Table 1.

Immunohistochemistry

Excellent agreement was demonstrated between the two raters when scoring p16\(^{\text{INK4a}}\). This was consistent for both the intensity of staining and proportion of cells scored (κ=0.85, 0.86; ICC= 0.93,0.94 respectively). 10 cases (7%) demonstrated positivity for p16\(^{\text{INK4a}}\) protein on immunohistochemical staining. 4 of these 10 cases with positive p16\(^{\text{INK4a}}\) staining progressed to cancer. 86 cases (58%) demonstrated no immunohistochemical staining for p16\(^{\text{INK4a}}\) protein. A further 52 cases (35%) exhibited a variable pattern of staining for p16\(^{\text{INK4a}}\), with none of these cases meeting the criteria for p16\(^{\text{INK4a}}\) positivity. Examples of each staining pattern are shown in figure 1.

Prognostic markers of malignant progression

p16\(^{\text{INK4a}}\) score was not demonstrated to be a prognostic factor for malignant transformation in this cohort (p=0.29). This did not change even when performing a time to event analysis (hazard ratio 1.86; 95% CI 0.66, 5.3, p=0.24 figure 2). While a high grade of dysplasia (p= 0.0002) and lesion morphology (p=0.03) were found to be prognostic of malignant progression on univariate analysis, gender, anatomical location, smoking and alcohol status were not. On multivariate analysis, only
histological grade remained an independent predictor of malignant progression (HR 1.64; 95% CI 1.12, 2.40, p=0.01).
Discussion

Our study, the largest multi-center cohort to examine the prognostic role of a surrogate marker of HPV oncoprotein expression in OED, has revealed positivity to occur in only a small proportion of OED lesions. Furthermore, p16\textsuperscript{INK4a} positivity demonstrated no evidence of a prognostic ability to predict malignant progression in OED.

The relatively low rate of p16\textsuperscript{INK4a} positivity in this cohort may be explained by our use of high intensity and proportion scores before assigning a case this status. This is in keeping with previously proposed classifications by Weinberger and supported by diagnostic algorithms proposed by Robinson et al\textsuperscript{14,15}. In our study, other known risk factors such as smoking and alcohol intake were not prognostic, with only a high histopathological grade of dysplasia significantly predicted progression in this cohort, (p= 0.0002).

Several studies have examined the prevalence rates of HPV infection in oral premalignant lesions. These have been summarized in a recent systematic review\textsuperscript{8}. 956 cases of oral potentially malignant disorders (OPMD) and 675 controls were included in the analysis. In all 19 cross sectional studies, HPV was seen in a higher proportion of the OPMD groups than in the controls (OR 3.87 (95% CI: 2.87–5.21). This association was even more significant with a subgroup analysis of cases of OED (OR 5.10; 95% CI: 2.03–12.80). These findings were confirmed in a second systematic review\textsuperscript{7}. While most studies have used polymerase chain reaction (PCR) or in-situ hybridization (ISH), for the detection of HPV DNA, detection of p16\textsuperscript{INK4a} protein overexpression by immunohistochemistry has also been shown to be a surrogate marker of HPV infection\textsuperscript{16–19}.

Detection of HPV DNA does not necessarily confirm the presence of active infection
however\textsuperscript{20}. Furthermore, cross sectional studies do not allow the assumption to be made that detection of HPV DNA has any prognostic significance. To date, few longitudinal studies have examined the prognostic significance of the increased incidence of HPV infection in OPMD, and those that have report conflicting results. Nielsen et al reported HPV to be a likely cofactor in OSCC development, as 100% of the patients developing cancers were HPV positive\textsuperscript{21}. However, this was only in 3 patients, and the cohort of 49 OPMD contained a mixture of cases with and without dysplasia. Montebugnoli et al demonstrated p16\textsuperscript{\textit{INK4a}} positivity in 9/20 OPMD cases progressing to cancer\textsuperscript{22}. Once again, the numbers were small and not all the cases progressing to cancer had preceding dysplasia. Furthermore, only 5% of cells were required to be stained for cases to be considered positive. No prognostic role for HPV was found in a large cohort of oral leukoplakias by Yang et al\textsuperscript{23}. 11/167 cases progressed to malignancy, with 5 of these 11 cases being positive for HPV DNA. Yet only 45% of this cohort had dysplasia, and p16\textsuperscript{\textit{INK4a}} status was not examined.

\textit{Limitations of the study}

This was a retrospectively collected cohort. Prospective collection, with more cases and longer follow-up would be favorable for validation of these findings. This study has also not correlated the detection of p16\textsuperscript{\textit{INK4a}} protein with HPV DNA and so a proportion of cases with p16\textsuperscript{\textit{INK4a}} positivity may not be due to HPV infection. However, this would further confirm our findings that p16\textsuperscript{\textit{INK4a}} positivity appears to have no prognostic role in this situation. Despite being one of the largest cohorts reported in the literature, a small chance of a type II error exists, due to small subgroup sizes.
Conclusion

We have found no evidence that p16\textsuperscript{\textit{INK4a}} expression is able to predict malignant progression in cases of OED. Its use as a biomarker in helping to stratify the malignant potential of patients with OED cannot therefore be justified.
**Table 1:** Clinical characteristics of patient cohort and univariate analysis of risk factors for malignant transformation

<table>
<thead>
<tr>
<th>Smoking status at biopsy</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>69 (47)</td>
<td>0.29</td>
</tr>
<tr>
<td>Ex</td>
<td>9 (6)</td>
<td></td>
</tr>
<tr>
<td>Non</td>
<td>47 (32)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>23 (15)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alcohol consumption at biopsy</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;28 Units/week</td>
<td>23 (15)</td>
<td>0.61</td>
</tr>
<tr>
<td>&lt;28 Units/week</td>
<td>58 (40)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>44 (30)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>23 (15)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morphology of lesion</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White patch</td>
<td>94 (63)</td>
<td>0.03</td>
</tr>
<tr>
<td>Red patch</td>
<td>15 (10)</td>
<td></td>
</tr>
<tr>
<td>Speckled patch</td>
<td>13 (9)</td>
<td></td>
</tr>
<tr>
<td>Ulcer</td>
<td>22 (15)</td>
<td></td>
</tr>
<tr>
<td>Lump</td>
<td>4 (3)</td>
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</table>

<table>
<thead>
<tr>
<th>Site of lesion</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue</td>
<td>69 (47)</td>
<td>0.73</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>20 (13)</td>
<td></td>
</tr>
<tr>
<td>Palate</td>
<td>18 (12)</td>
<td></td>
</tr>
<tr>
<td>Buccal</td>
<td>38 (26)</td>
<td></td>
</tr>
<tr>
<td>Retromolar</td>
<td>3 (2)</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>76 (51%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Female</td>
<td>72 (49%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p16^{INK4a}</th>
<th>Number (%)</th>
<th>Logistic regression (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10 (7%)</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Figure 1: Variability in p16$^{\text{INK4a}}$ expression in oral epithelial dysplasia. a) No staining; b) staining score of 2; c) Staining score of 6; d) Positive staining with a score of 12
Figure 2: Kaplan-Meier survival analysis curve demonstrating differences in oral cancer free survival between p16<sup>INK4a</sup> positive and negative groups.
References


