Association of Keratinization With 5-Year Disease-Specific Survival in Oropharyngeal Squamous Cell Carcinoma

Timothy Cooper, MD; Vincent L. Biron, MD, PhD, FRCSC; Ben Adam, MD; Alexander C. Klimowicz, PhD; Lakshmi Puttagunta, MD, FRCP; Hadi Seikaly, MD, FRCSC

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OBJECTIVE To quantify the prognostic value of keratinization in a large cohort of patients with OPSCC with subgroup analysis based on p16 status, basaloid differentiation, and smoking status.

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INTERVENTIONS Patients were treated with curative intent with surgery, radiation, and/or chemotherapy.

MAIN OUTCOMES AND MEASURES The primary outcome measure was 5-year disease-specific survival (DSS) in OPSCC according to keratinization. Univariate and multivariate survival analyses were performed to estimate survival according to histopathologic profile and smoking status.

RESULTS In the 208 samples, 96 were keratinizing and 112 were nonkeratinizing. Patients with keratinizing tumors were more likely to have advanced-stage disease and be p16 negative. Keratinization was independently associated with adverse outcomes. The 5-year DSS was significantly higher for nonkeratinizing tumors (63.3%) compared with keratinizing tumors (44.8%; P = .007). In subgroup analysis, nonkeratinization was associated with improved DSS in those with nonbasaloid and p16-negative tumors and in patients who were smokers. When stratifying patients based on keratinization, p16-status, and smoking status, patients with p16-negative keratinizing tumors who were smokers had the lowest 5-year DSS (26.7%).

CONCLUSIONS AND RELEVANCE Patients with nonkeratinized OPSCC have improved survival compared with those with keratinizing tumors. Information on keratinization is most useful prognostically in those who have p16-negative and nonbasaloid tumors and in patients who are smokers. Survival can be stratified using keratinization, p16 status, and smoking status.

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Keratinization is a histologic feature observable from hematoxylin-eosin (H&E) staining of tissues. It is routinely reported on histopathology reports and can be used to dichotomously describe squamous cell carcinomas (SCCs) as either keratinizing or nonkeratinizing.1 In patients with oropharyngeal squamous cell carcinoma (OPSCC), nonkeratinizing tumors have been shown to have a strong association with human papillomavirus (HPV) positivity.2,3

The proportion of OPSCCs that is HPV-associated has increased dramatically in recent decades.4-5 p16 Immunohistochemical analysis is routinely used as a surrogate marker for oncogenic HPV infection6 and has been shown to be a positive prognostic factor in OPSCC.2,6-8 HPV-positive OPSCC has been described as nonkeratinizing, basaloid, and poorly differentiated and presenting more commonly in younger non-smoking patients.3,9 Conversely, HPV-negative OPSCC, which is associated with the traditional head and neck cancer risk factors of alcohol and tobacco, has been associated with keratinization.1,10

Several authors11-13 have examined the significance of keratinization in head and neck cancer, with many focusing on oral cavity SCC. A number of these studies have shown keratinization to be associated with increased mortality. These findings, in combination with the association of HPV with nonkeratinization, suggest that patients with nonkeratinizing OPSCC should have improved outcomes. To date, 2 studies12,13 have examined the prognostic role of keratinization in OPSCC and neither found a statistically significant effect on survival. However, a number of covariates that may influence survival were not included in these reports.

The aim of this study was to quantify the prognostic value of the H&E feature of keratinization in a large series of patients with OPSCC with subgroup analysis based on p16 status, basaloid differentiation, and smoking status. To address our study goal, we used an objective study design that included blinded scoring of histologic features of tissue microarrays.

Methods

This is a retrospective cross-sectional study using data collected in a prospective fashion in a regional head and neck cancer treatment center. Institutional ethics review board approval was obtained prior to the commencement of the study. Patients were identified through the Alberta Cancer Registry from 2002 to 2009 for inclusion in the study. Patient demographics, smoking status, staging, treatment, and survival data were collected. Patients with a smoking history of 10 or more pack-years were defined as smokers.8

All patients diagnosed and treated with OPSCC in Edmonton, Alberta, Canada, between 2002 and 2009 were eligible for inclusion. Each patient required a core or tissue biopsy to be performed for use in a tissue microarray (TMA). Included patients were treated with curative intent with any combination of cancer treatment modalities including surgery, chemotherapy, and radiation. Patients and their associated TMAs were excluded if their cancer was treated with palliative intent or if inadequate tissue was obtained for assessment of the H&E staining features of basaloid differentiation and keratinization.

TMA Construction

The TMAs were constructed with formalin-fixed, paraffin-embedded (FFPE) tumor tissue from either pretreatment biopsy specimens or primary surgery as recently described.14,15 A pathologist (L.P.) reviewed the blocks and excluded cases with inadequate tissue for future diagnosis. The FFPE blocks were marked by a pathologist for TMA construction. The TMAs were constructed with duplicate or triplicate cores of FFPE blocks as per the TMA protocol previously described.6 These TMAs had been used in our previous studies.14-16

Histologic Analysis

The H&E features of each TMA, including basaloid differentiation and keratinization, were scored by a pathology resident (B.A.) and confirmed by a staff head and neck pathologist (L.P.) who were blinded to p16 immunohistochemical analysis results and patient identity. Both keratinization and basaloid differentiation were scored as either present or absent in each of the TMAs. The standard definition used for basaloid differentiation was the presence of 2 or 3 features used to describe basaloid differentiation interpretable from a microarray slide, including peripheral palisading, high nuclear-cytoplasmic ratio, and solid growth pattern.

Immunohistochemical Analysis

Immunohistochemical analysis for p16 was performed using the diaminobenzidine staining method as previously reported by Lau et al.6 The p16 antibody used was clone JC8 (Santa Cruz Biotechnology). In accordance with previously established standards in the literature, p16 positivity was defined as high-intensity staining in greater than 70% of cells.

Statistical Analysis

Kaplan-Meier survival analysis was performed to calculate disease-specific survival (DSS), using the log-rank statistic for statistical comparisons between strata.17 Multivariate analysis was performed using the Cox proportional hazards model18 for the variables age, sex, nodal status, basaloid differentiation, keratinization, p16 status, and smoking. Comparison of proportions was performed using χ² and continuous data using f test. Statistical significance was accepted as P < .05 in all cases. All statistical analyses were performed using SPSS software (version 21; SPSS Inc.).

Results

A total of 208 patients were included in the study. The mean age was 58.4 years (range, 32-95 years), with a male to female ratio of 3:4:1:0. One hundred forty-six patients were smokers, and 59 were nonsmokers. Three patients did not have smoking data available. Most patients (189) presented with clinically advanced-stage disease (stage III/IV) with only 19 patients with early-stage disease (stage I/II). Nodal disease was...
present in 172 patients on presentation. Ninety-six tumor specimens (46%) were keratinizing, and 112 (54%) were non-keratinizing. Of the 208 patients, 111 were p16 positive and 97 were p16 negative. Eighty-four patients had basaloid differentiated tumors compared with 124 with nonbasaloid differentiated tumors. Surgery followed by chemotherapy and radiation was the most common treatment modality (Table 1).

Subgroup analysis was performed comparing the features of patients with keratinizing and nonkeratinizing SCC (Table 1). There were no statistically significant differences with regard to age, sex, use of surgical or nonsurgical treatment modalities, or smoking status. Although most patients in both subgroups had advanced-stage disease, more patients with keratinizing tumors had advanced-stage disease. Of the keratinizing SCCs, 33 also demonstrated basaloid differentiation, whereas 63 were nonbasaloid differentiated. In the 112 nonkeratinizing OPSCC specimens, 51 were basaloid differentiated whereas 61 were not. There was no statistically significant difference in the presence of basaloid differentiation in keratinizing and nonkeratinizing OPSCC. Patients with nonkeratinizing OPSCC were more likely to be p16 positive. Seventy-two patients with tumors with nonkeratinizing histologic characteristics were p16-positive compared with only 39 of those with keratinization ($P = .001, \chi^2 = 11.6$).

Cox proportional hazards regression by univariate analysis showed that the risk of death was significantly influenced by age, nodal status, smoking status, p16 positivity, basaloid differentiation, and keratinization (Table 2). Multivariate analysis incorporating all these features showed that age, smoking status, p16 status, and keratinization were independent predictors of the risk of death (Table 2).

Five-year DSS was calculated using Kaplan-Meier analysis for patients with keratinizing and nonkeratinizing OPSCC treated both surgically and nonsurgically (Figure 1). Patients with nonkeratinizing tumors had a statistically significant survival advantage with a 63.3% 5-year DSS compared with 44.8% in those with keratinizing tumors ($P = .007$).

In comparing DSS based on keratinization in patients with nonbasaloid differentiated tumors, this survival advantage in nonkeratinizing cancers remained, with a 5-year DSS of 55.8% in those with nonkeratinizing disease compared with only 26.6% in those with keratinizing tumors ($P = .002$) (Figure 2A). However, in those patients with basaloid differentiated tumors, there was no statistically significant difference in DSS (Figure 2A). In this group, patients with keratinizing tumors had a 76.5% 5-year DSS compared with 71.7% in those with nonkeratinizing tumors ($P = .74$).

In p16-positive patients, there was no statistically significant difference in 5-year DSS in patients with keratinizing OPSCC compared with those with nonkeratinizing tumors (Figure 2B). Five-year DSSs were 69.0% and 74.9%, respectively ($P = .88$). However, in p16-negative patients, the 5-year DSS was 47.7% in patients with nonkeratinizing tumors compared with 26.8% in those with keratinizing tumors ($P = .01$) (Figure 2B).

For patients who had a smoking history of 10 or more pack-years, there was a statistically significant difference in 5-year DSS in those with nonkeratinizing OPSCC (Figure 2C). The 5-year DSS was 53.3% in those with nonkeratinizing OPSCC compared with 41.1% in those with keratinizing OPSCC ($P = .03$). However, in patients who were defined as nonsmokers (history of < 10 pack-years), there was no statistically significant difference in 5-year DSS based on the keratinization ($P = .24$). Nonsmokers with nonkeratinizing tumors had a 5-year DSS of 84.4% compared with 57.6% in those with keratinizing tumors.

### Table 1. Clinicopathologic Characteristics, Staging, and Treatment of Patients According to Keratinization

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>Keratinizing</th>
<th>Nonkeratinizing</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, No.</td>
<td>208</td>
<td>96</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>Age, mean, y</td>
<td>58.4</td>
<td>59.4</td>
<td>57.6</td>
<td>.24</td>
</tr>
<tr>
<td>Male sex</td>
<td>161</td>
<td>69</td>
<td>92</td>
<td>.08</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>146</td>
<td>68</td>
<td>78</td>
<td>.92</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>59</td>
<td>27</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Staging</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced</td>
<td>189</td>
<td>83</td>
<td>106</td>
<td>.04</td>
</tr>
<tr>
<td>Early</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>p16 Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>111</td>
<td>39</td>
<td>72</td>
<td>.001</td>
</tr>
<tr>
<td>Negative</td>
<td>97</td>
<td>57</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Basaloid differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>84</td>
<td>33</td>
<td>51</td>
<td>.10</td>
</tr>
<tr>
<td>Absent</td>
<td>124</td>
<td>63</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Treatment, No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary surgery</td>
<td>155</td>
<td>66</td>
<td>89</td>
<td>.07</td>
</tr>
<tr>
<td>Primary chemo-RT</td>
<td>53</td>
<td>30</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: chemo-RT, chemotherapy and radiation therapy.
By adding keratinization to the classification scheme used by Ang et al,8 which used HPV status and smoking history, 3 levels of risk can be delineated. The high-risk group includes p16-negative smokers with keratinizing tumors, p16-negative smokers with nonkeratinizing tumors, and p16-negative nonsmokers with keratinizing tumors with DSSs of 26.7%, 34.7%, and 37.4%, respectively. An intermediate-risk group would include p16-positive smokers with keratinizing tumors, p16-positive smokers with nonkeratinizing tumors, and p16-positive nonsmokers with keratinizing tumors. The 5-year DSSs within the intermediate-risk group were 64.5%, 68.3%, and 72.2%, respectively (Figure 3).

Discussion

To our knowledge, this study represents the largest cohort describing survival based on keratinization in OPSCC. As hypothesized, there was a significant survival advantage in patients with nonkeratinizing OPSCC, with a difference in DSS of 18.5% compared with those with keratinizing OPSCC. Two previous studies specific to the oropharynx have examined survival and keratinization. Chernock et al2 did not find a statistically significant survival advantage in patients with nonkeratinizing SCC after adjusting for age and treatment in 118 cases of OPSCC. Crissman et al11 also looked at keratinization and OPSCC survival, again finding no effect on survival based on keratinization in 77 patients. It should be noted that this study was performed in the early 1980s prior to the rise of HPV-associated OPSCC. In a later study by Crissman et al,19 a statistically significant survival advantage was found in patients with nonkeratinizing SCC. However, this study included patients with head and neck cancers of all sites undergoing chemotherapy and radiation, with those with OPSCC comprising about 40% of patients. Our study demonstrates that keratinization status is a significant prognostic factor in OPSCC.

Table 2. Cox Proportional Hazard Model of Survival in Patients With Oropharyngeal Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate, HR (95% CI)</th>
<th>P Value</th>
<th>Multivariate, HR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>1 [Reference]</td>
<td>.001</td>
<td>1 [Reference]</td>
<td>.005†</td>
</tr>
<tr>
<td>≥50</td>
<td>3.89 (1.69-8.96)</td>
<td></td>
<td>3.31 (1.42-7.72)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>.36</td>
<td></td>
<td>.50</td>
</tr>
<tr>
<td>Male</td>
<td>1 [Reference]</td>
<td></td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.78 (0.47-1.32)</td>
<td></td>
<td>1.20 (0.70-2.05)</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>1 [Reference]</td>
<td>.01</td>
<td>1 [Reference]</td>
<td>.07</td>
</tr>
<tr>
<td>N1-N3</td>
<td>1.14 (1.03-1.26)</td>
<td></td>
<td>1.09 (0.99-1.21)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>.03</td>
<td></td>
<td>.007†</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>1 [Reference]</td>
<td></td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>2.58 (1.39-4.80)</td>
<td></td>
<td>2.36 (1.26-4.42)</td>
<td></td>
</tr>
<tr>
<td>p16 Status</td>
<td></td>
<td>&lt;.001</td>
<td></td>
<td>.046†</td>
</tr>
<tr>
<td>Negative</td>
<td>1 [Reference]</td>
<td></td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0.33 (0.21-0.54)</td>
<td></td>
<td>0.56 (0.32-0.99)</td>
<td></td>
</tr>
<tr>
<td>Basaloid</td>
<td></td>
<td>&lt;.001</td>
<td></td>
<td>.06</td>
</tr>
<tr>
<td>Negative</td>
<td>1 [Reference]</td>
<td></td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2.57 (1.53-4.34)</td>
<td></td>
<td>0.55 (0.30-1.02)</td>
<td></td>
</tr>
<tr>
<td>Keratinization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1 [Reference]</td>
<td>.005</td>
<td>1 [Reference]</td>
<td>.04†</td>
</tr>
<tr>
<td>Positive</td>
<td>0.52 (0.33-0.82)</td>
<td></td>
<td>1.66 (1.04-2.65)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: HR, hazard ratio.
* Denotes statistical significance with P < .05.
Figure 2. Disease-Specific Survival According to Keratinization With Subgroup Analysis of Basaloid Differentiation, p16 Status, and Smoking

A. Eighty-four patients with basaloid differentiated tumors (log rank, \( P = .74 \)) and 124 patients with nonbasaloid differentiated tumors (log rank, \( P = .002 \)).

B. One hundred eleven patients with p16-positive tumors (log rank, \( P = .88 \)) and 97 patients with p16-negative tumors (log rank, \( P = .01 \)).

C. Fifty-nine nonsmokers (log rank, \( P = .24 \)) and 146 smokers (log rank, \( P = .03 \)).
Patients with nonkeratinizing oral cavity SCC have previously been shown to have improved survival compared with those with keratinizing oral cavity cancers. Dissanayaka et al13 recently described keratinization as a prognostic indicator in a series of 193 patients with oral cavity SCC in univariate survival analysis. Similarly, Fountzilas et al12 found nonkeratinization to be strongly associated with improved survival on univariate but not multivariate analysis.

Interestingly, the survival advantage seen in those with nonkeratinizing tumors was not seen in patients who had basaloid differentiated tumors. In this subgroup of our series, both keratinizing and nonkeratinizing tumors had similar survival with 5-year DSS rates of 76.5% and 71.7%, respectively, a nonstatistically significant difference (P = .74). In those with nonbasaloid differentiated tumors, however, patients with nonkeratinizing tumors had a significant survival advantage with a 29.2% greater DSS compared with those with keratinizing, nonbasaloid tumors. Our previous research19 demonstrated a survival advantage based on basaloid differentiation on H&E staining that was independent of p16 status and keratinization. The molecular basis for basaloid differentiation and its survival advantage in OPSCC is not currently known. How this relates to keratinization is also uncertain. However, nonkeratinization was also shown to be an independent predictor of survival with Cox-regression analysis.

Similar to basaloid differentiation, the presence or absence of keratinization yielded nonstatistically significant differences in DSS in those patients who had p16-positive OPSCC. However, in those with p16-negative tumors, there was a 20.9% improvement in 5-year DSS in patients with nonkeratinizing tumors compared with those with keratinization. Based on these data, the prognostic value of the presence or absence of keratinization is important in p16-negative patients but does not change survival significantly in those with p16-positive tumors who already have a positive prognosis.

Survival in patients with OPSCC can be stratified by combining keratinization and basaloid differentiation observed on H&E staining. Patients with basaloid differentiated tumors have the best survival regardless of keratinization. Patients with nonbasaloid tumors have reduced survival, but those with nonkeratinizing tumors have an improved prognosis compared with those with keratinization. Important prognostic information can be obtained from the H&E stain alone prior to immunohistochemical staining for p16 and any other molecular markers or testing for HPV. Owing to its simplicity and the routine use of H&E staining on all oncolgic specimens submitted for pathologic analysis, this stratification system has universal applicability.16

There was a high number of smokers overall within this patient population, which was seen in both the groups with keratinizing and with nonkeratinizing tumors. Keratinization was predictive of increased mortality in subgroup analysis of smokers. However, owing to a smaller number of patients, the difference in 5-year DSS in nonsmokers based on keratinization was not statistically significant (P = .24).

Keratinization may also be combined with the classification of patients used by Ang et al18 to separate patients into low-, intermediate-, and high-risk categories. From our results, the lowest-risk patients were those who were nonsmokers with nonkeratinizing tumors, regardless of p16 status. The intermediate-risk group consisted of patients with p16-positive tumors who were either smokers or had keratinizing tumors. The high-risk group consisted of patients with p16-negative tumors who were either smokers or had keratinizing tumors. Our results are in agreement with those reported by Ang et al18 with the presence or absence of keratinization altering the risk categorization of 2 groups of patients. Nonsmokers with p16-positive tumors were classified as intermediate risk instead of low risk if they had keratinizing tumors. Also, nonsmokers with p16 tumors were classified as low risk instead of intermediate risk if they had nonkeratinizing tumors.

Previous studies19,20 have also shown that keratinizing OPSCC is associated with worsened survival outcomes, but the molecular basis for this is unclear. It has been presumed that keratinizing OPSCC may simply represent HPV/p16-negative tumors; however, our study and recent independent data suggest that this is not the case, despite some degree of correlation.20 A recent proteomic analysis suggests HPV-negative OPSCC have upregulated expression of proteins that may result in more differentiated, keratinizing tumors.21 Further work is needed to understand the molecular basis of keratinization, the interplay of this differentiation with HPV infection, and how this alters treatment response.

This study had several limitations. It was conducted retrospectively, and additional variables, such as comorbid status, were not included in the analysis. All patients were treated at a single institution. Our treatment outcomes and use of treatment modalities may differ from those at other head and neck centers. Some patients could not be included in the study owing to inadequate tissue availability for analysis. Histologic
analysis of the tumors for keratinization was performed on core samples from the TMA as opposed to the tumor itself. This may lead to sampling error owing to variability in histologic appearance within the tumor. However, the impact of this limitation was reduced through the use of duplicate and triplicate core samples taken from different areas of the tumor.

Conclusions

Similar to oral cavity SCC, nonkeratinization is a histologic feature observable on H&E staining that is indicative of improved prognosis in OPSCC. This prognostic advantage with nonkeratinization is seen in patients with nonbasaloid differentiated and p16-negative tumors. However, in those with tumors that are basaloid differentiated or p16 positive and have improved prognosis based on these features, keratinization does not have a significant impact on survival. Substantial prognostic information is available from initial H&E stains in OPSCC based on the presence or absence of 2 key features, keratinization and basaloId differentiation. Risk stratification of patients with OPSCC can be made by combining p16 status, keratinization, and smoking status.

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