### Title

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### Journal

Otolaryngology, 155(1)

### ISSN

0194-5998



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### Publication Date

2016-07-01

### DOI

10.1177/0194599816636612

Peer reviewed

HHS Public Access

Author manuscript

*Otolaryngol Head Neck Surg*. Author manuscript; available in PMC 2016 July 02.

Published in final edited form as:

*Otolaryngol Head Neck Surg*. 2016 July ; 155(1): 106–112. doi:10.1177/0194599816636612.

# Tumor Biomarkers in Spindle Cell Variant Squamous Cell Carcinoma of the Head and Neck

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## Abstract

**Objective—**To determine biomarkers of recurrence and survival in patients with spindle cell variant squamous cell carcinoma (SpSCC) of the head and neck.

**Study Design—**Retrospective case control study.

**Setting—**Tertiary academic center.

**Subjects and Methods—**Thirty-two SpSCC patients (mean age, 68.8) between 1987 and 2009 were identified and reviewed. A tissue microarray (TMA) was constructed from tumor specimens. Tumor biomarkers under study included p16, EGFR, p53, EZH2, Cyclin D1, CD104, HGFa, p21, and cMET. An additional TMA was constructed from patients with non-SpSCC oral cavity squamous cell carcinoma for comparative purposes. The main outcomes were overall survival (OS), disease specific survival (DSS) and recurrence free survival (RFS).

**Results—**In the SpSCC cohort, tumors positive for cMet had worse OS ($p<0.001$). Patients positive for cMet ($p=0.007$), Cyclin D1 ($p=0.019$), and p16 ($p=0.004$) had worse DSS. RFS was also worse in patients with tumors positive for cMet ($p=0.037$), Cyclin D1 ($p=0.012$), and p16 ($p<0.001$). Compared to the oral cavity cohort there was a significantly larger proportion of patients in the SpSCC group with tumors staining positive for cMet and a lower proportion of tumors positive for cyclin D1.

**Conclusion—**cMet, Cyclin D1, p16 are predictive tumor biomarkers for risk of recurrence and worse disease specific survival in patients with SpSCC.

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Financial disclosure: None

Conflict of interest: None

Presentation at meetings: AAO-HNS Annual Meeting, Oral Presentation, September 28, 2015, Dallas, TX

### Keywords

Spindle cell; Sarcomatoid; Head and neck cancer; Biomarkers; cMet; Cyclin D1; p16; EGFR

## INTRODUCTION

The spindle cell variant of squamous cell carcinoma (SpSCC) is a type of squamous cell carcinoma (SCC) with histologic features of both epithelial and mesenchymal origin. SpSCC can occur in all sites of the head and neck, though most occur in the oral cavity, larynx and oropharynx.[1] Previous studies have reported SpSCC to have a high propensity for locoregional recurrence, and no clinical or pathologic factors predicted recurrence or survival.[2] There is little known, however, regarding tumor biomarkers as predictors of recurrence. The identification of predictive biomarkers may allow for the pretreatment identification of patients who are at risk for treatment failure, or identify a molecular pathway unique to SpSCC that would allow for a targeted agent. With the propensity for locoregional recurrence, SpSCC is an ideal disease in which to study the pathways of this resistance. Therefore, our goal was to determine tumor biomarkers of recurrence and survival in patients with SpSCC of the head and neck, and to compare these patients to a group of oral cavity patients with non-spindle squamous cell carcinoma to understand how the prevalence of these biomarkers may differ.

## METHODS

### Study Population

A retrospective review of patients who underwent definitive treatment for pathologically confirmed SpSCC between 1987 and 2009 was performed. Patients were identified through the university's pathology department database searching under the terms "squamous cell carcinoma" with either "spindle" or "sarcomatoid" as modifiers. Cases not involving the upper aerodigestive tract (i.e. skin) were excluded. Patients were also excluded if they were never seen or did not follow up at the University of Michigan for their cancer surveillance. Patient information was collected through the electronic medical record. There were 62 patients initially identified; 9 of these patients were never seen at the University of Michigan (pathology only review) and 5 did not have follow-up after their initial consultation. Of the 48 remaining patients, 32 patients had adequate tissue to create a tissue micro array (TMA). As most patients' tumors arose in the oral cavity, a comparison TMA was constructed from cores of non-spindle cell oral cavity primary tumors from 76 patients. All patients were staged based on the 7$^{th}$ edition of the AJCC staging system.[3] This study was approved by the Institutional Review Board at the University of Michigan (HUM00033266).

### Tissue Microarray

A TMA was constructed in triplicate from cores of pretreatment biopsies of the primary tumor for the 32 patients with SpSCC. Tumor biomarkers analyzed were p16, EGFR, p53, EZH2, Cyclin D1, CD104, HGFa, p21, and cMET. Biomarkers were chosen based on common head and neck prognostic markers, as well as potential targetable pathways. The formalin-fixed paraffin-embedded sections from the TMAs were heated, and underwent

peroxidase blocking. Monoclonal antibodies to EGFR (31G7; Invitrogen, Camarillo, CA), TP53 (SP5; Cell Marque, Rocklin, CA), $p16^4$ (CINtec p16ink4a antibody; MTM Laboratories, Westborough, MA), and CMET (3D4; Invitrogen, Camarillo, CA) were applied. A FLEX + EnVision System (Dako, Carpinteria, CA) was used for staining. The TMAs were then counterstained with Harris Hematoxylin. Protein expression was scored by a board-certified pathologist in a blinded fashion. Scores from the 3 samples from each patient were averaged. As most of the patients in the spindle cell cohort came from oral cavity primary tumors, a comparison TMA was constructed from cores of non-spindle cell oral cavity primary tumors from 75 patients in the same fashion. Both TMAs were stained in the same laboratory under the identical conditions.

### Statistical Analysis

Variables under study included age, gender, tumor subsite, T classification, N classification, M classification, smoking status (never, former [quit >6 months ago], current) and tumor biomarkers. The outcomes of interest were recurrence free survival (RFS), disease specific survival (DSS) and overall survival (OS). Survival intervals were defined as the time to event from the beginning of treatment. All tumor markers were analyzed in groups based on an intensity of 0 (negative) versus 1–3 (positive). Survival estimates were computed using the Kaplan-Meier method. All statistical analyses were performed using IBM SPSS (version 20) software (IBM Corporation, Armonk, New York).

## RESULTS

Thirty-two patients who underwent definitive treatment of pathologically confirmed SpSCC between 1987 and 2009, with adequate tissue available for the creation of a TMA, were identified. Demographic and baseline characteristics are shown in Table 1. The mean age was 68.8 years (range 38–85). There were 19 oral cavity, 8 larynx, 4 oropharynx, and 1 maxillary sinus tumors. In situ hybridization was performed to assess for high risk HPV and all tumors were HPV negative. The Kaplan-Meier analysis of overall survival at 5 years in this cohort was 33.7%. The 5-year disease specific survival and recurrence free survival were 52.7% and 44.8% respectively. Of those who experienced a recurrence, the mean interval to recurrence was 21.8 months with a range of 0 (persistent disease) to 112 months.

In a univariate analysis, SpSCC tumors positive for cMet had worse overall survival (13.3% versus 52.9%, $p<0.001$) (Figure 1A). No other tumor biomarkers reached statistical significance (Table 2). Similarly, patients with tumors positive for cMet had worse disease specific survival (35.5% versus 68.1%, $p=0.007$) (Figure 2A). In addition, patients whose tumors stained positive for Cyclin D1 and p16 had worse disease specific survival (36.4% versus 59.3%, $p=0.019$ and 22.2% versus 66.4%, $p=0.004$ respectively) (Figure 2B and 2C). Recurrence free survival was also worse in patients with tumors positive for cMet (37.6% versus 55.2%, $p=0.037$), Cyclin D1 (14.0% versus 59.3%, $p=0.012$), and p16 (0.0% versus 68.0%, $p<0.001$) (Figure 3). The hazard ratios with 95% confidence intervals are show in Table 2. No other biomarkers were predictive of OS, DSS or RFS.

In a univariate analysis of overall stage, there were differences in overall survival (59.3% versus 11.8%, $p=0.001$), disease specific survival (77.4% versus 28.1%, $p=0.003$) and

recurrence free survival (70.7% versus 14.4%, p=0.007) when comparing stage 1and 2 versus stage 3 and 4 patients. We did not test differences between individual stages because of the limited power secondary to the small sample size.

We performed an analysis in a subset of our patient population, which included the 19 oral cavity patients from our SpSCC cohort. Analysis of this subgroup of SpSCC patients revealed no differences in recurrence rates or survival based on any tumor biomarkers tested, though the sample group was underpowered to detect a difference. No other subsites were tested given the small number of patients in each group.

In our comparison group of oral cavity patients there were 75 patients with a mean age of 54.2 years (range 21–86). Thirty-eight patients were male and 37 patients were female. At the time of treatment 28 patients were current smokers, 20 were former smokers, and 26 were lifetime non-smokers. Six patients were stage I, 14 patients were stage II, 9 patients were stage III, and 45 patients were stage IV. Complete staging data was missing in 1 patient (Table 1). Overall survival at 5 years was 48.5%, which was statistically better that the SpSCC cohort (p=0.028). The 5-year disease specific survival was 62.1% and recurrence free survival was 55.9%. These were not statistically different than the SpSCC cohort (p=0.092 and 0.264 respectively).

When we compared our spindle cell cohort to our comparison group of oral cavity patients, there was a statistically significant difference in age, with the spindle cell variant affecting older patients (68.8 years versus 54.2 years; p<0.001). There was no significant difference between the two groups in terms of smoking status, T classification, N classification, M classification or overall stage (Table 1). When we compared the tumor markers between the two groups there was a significantly larger proportion of patients in the spindle cell group with tumors staining positive for cMet (46.9% versus 2.7%; p<0.001) and a lower proportion of tumors positive for cyclin D1 (46.4% versus 77.3%; p=0.002). There was no difference in the percent of tumors positive for EGFR (p=0.27) or p16 (p=0.76). As with the spindle cell variant, cMet positivity was predictive of overall survival (0.0% versus 51.5%, p = 0.009), disease specific survival (0.0% versus 66.9%, p < 0.001) and recurrence free survival (0.0% versus 59.4%, p = 0.006), although the proportion of patients staining for cMet was significantly lower with only 2 tumors positive for cMet in this group. In contrast, neither cyclin D1, p16 nor EGFR positivity were predictive of overall survival (p = 0.70, p = 0.79, and p = 0.15 respectively), disease specific survival (p = 0.52, p = 0.60, and p 0.60 respectively), or recurrence free survival (p = 0.17, p = 0.52, and p = 0.92 respectively) (Table 2).

## DISCUSSION

There has been great vigor in the search for prognostic biomarkers in head and neck cancer. Biomarkers offer the possibility of identifying patients at risk for treatment failure, and thus may guide therapy based on pre-treatment risk stratification. Certain biomarkers may also identify patients who may be more or less susceptible to specific targeted treatments or chemotherapeutics and thus may inform treatment.

In this study potential tumor biomarkers were identified which may predict treatment failure in SpSCC of the head and neck. cMet was shown to be a predictor of recurrence, disease specific survival and overall survival. cMET, also known as hepatocyte growth factor receptor, is a receptor tyrosine kinase located on chromosome 7q31.[5] Hepatocyte growth factor (HGF) is the receptor's only known ligand.[6] Interestingly, HGF expression was not predictive of outcomes in our study. Amplification and mutations of cMET occur in several malignancies, including cancers of the lung and kidney in addition to head and neck SCC.[7,8] In the head and neck, cMet mutations occur in up to 25% of cancers.[9–11] In lung cancer, cMet mutations are present in up to 20% of patients with resistance to EGFR inhibitors, such as cetuximab, as these mutations lead to activation of pathways that induce resistance to EGFR inhibitors.[12,13] Similar studies are currently underway evaluating the role of cMet in EGFR inhibitor resistance in SCC of the head and neck.[14] There is also interest in developing agents targeting cMet, some of which are currently in clinical trials.[7,9,15] In our study, cMet staining was predictive of recurrence. While cMet was also predictive in the control group of oral cavity tumors in a univariate analysis, it is interesting that cMet positivity was significantly more prevalent in spindle cell variant tumors. We cannot make direct comparisons between the two groups, as there were only two patients who were cMet positive in our non-SpSCC group and thus we lack statistical power. Perhaps this contributes to the overall worse prognosis associated with spindle cell carcinoma versus typical squamous cell carcinomas. The increase in cMet positivity seen in the spindle cell group also makes this pathway an interesting candidate for targeted therapy.

Cyclin D1 positivity was also shown to be predictive of recurrence and worse disease specific survival in SpSCC. There was no statistically significant difference in terms of overall survival, though this study was likely underpowered to identify a difference. Cyclin D1 is encoded by the gene CCND1 on chromosome 11q13.[5] The CCND1 gene is amplified in as many as 40% of head and neck cancers with cyclin D1 over-expression in as many as 75% tumors.[5,16–18] Cyclin D1 over-expression has been associated with poor prognosis including worse overall survival and disease specific survival.[16,17,19,20] There has been speculation that the prognostic significance may be due to co-amplification of other genes located at the 11q13 locus.[5,21,22] Regardless of the pathway, this study supports the utility of cyclin D1 as a useful biomarker in SpSCC. Cyclin D1, however, was not shown to be predictive in the oral cavity group, though Cyclin D1 positivity was much more ubiquitous in this group.

Interestingly, p16 was associated with worse recurrence free survival and disease specific survival. There was no apparent association with overall survival, however. The protein p16 is one of the products of the CDKN2A gene on chromosome 9p21. It is a tumor suppressor that is over-expressed in response to human papilloma virus (HPV) expression of the E7.[23,24] Because of this, p16 is often used as a surrogate measure of HPV.[25] HPV related SCC of the head and neck has been shown in numerous studies to be associated with a much better prognosis.[4,26–30] Although most non-HPV-related carcinomas do not express p16, some HPV-negative express p16 via other mechanisms besides viral induced expression.[5] We performed in situ hybridization to assess for high risk HPV in all of the patients in this study. All 32 patients were HPV negative and thus p16 positivity in SpSCC of the head and neck is not related to HPV. In our oral cavity group p16 was not predictive of poor

outcomes, despite similar proportions of tumors positive for p16 when compared to SpSCC. This makes p16 an interesting biomarker in SpSCC.

EGFR was not found to be a predictive biomarker in our study, as this study was likely underpowered to detect a difference. EGFR is overexpressed in more than 90% of head and neck SCC. EGFR overexpression has been consistently associated with recurrence and decreased survival.[19,31–33] In addition to head and neck cancer, EGFR is mutated in several other tumors including lung and colon cancer and is targeted with anti-EGFR agents such as cetuximab.[33–36]

## CONCLUSION

In this study, cMet, Cyclin D1, and p16 were predictive tumor biomarkers for increased risk of locoregional recurrence and disease specific survival in patients with SpSCC. Future studies investigating the potential for targeted therapy or escalation of treatment based on the tumor biomarker profile are necessary to confirm the clinical applicability.

## Acknowledgments

Grant support: None

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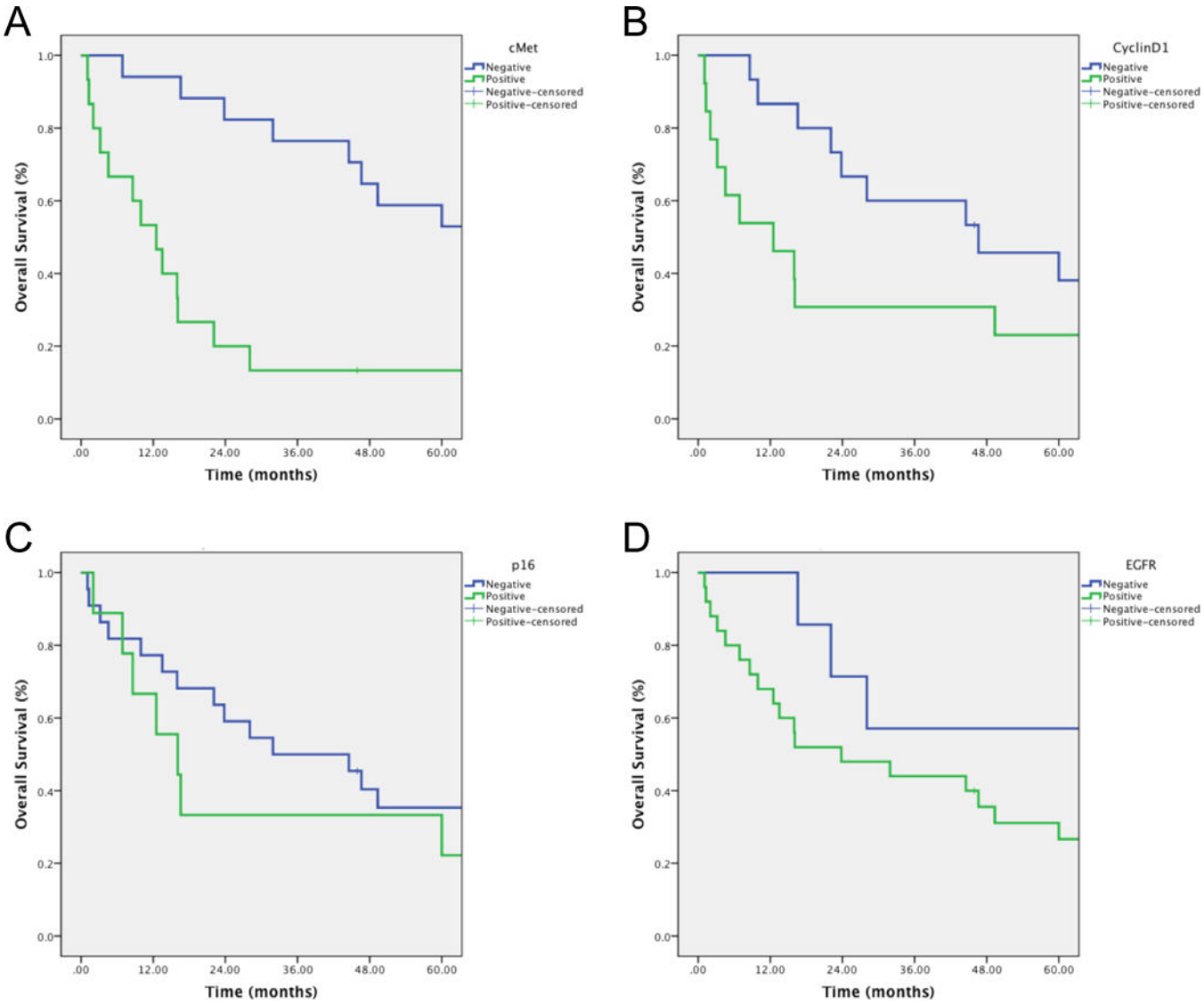


**Figure 1.**

Overall survival Kaplan-Meier curves based on tumor biomarkers cMet, p<0.001 (A), Cyclin D1, p=0.054 (B), p16, p=0.20 (C), and EGFR, p=0.079 (D).

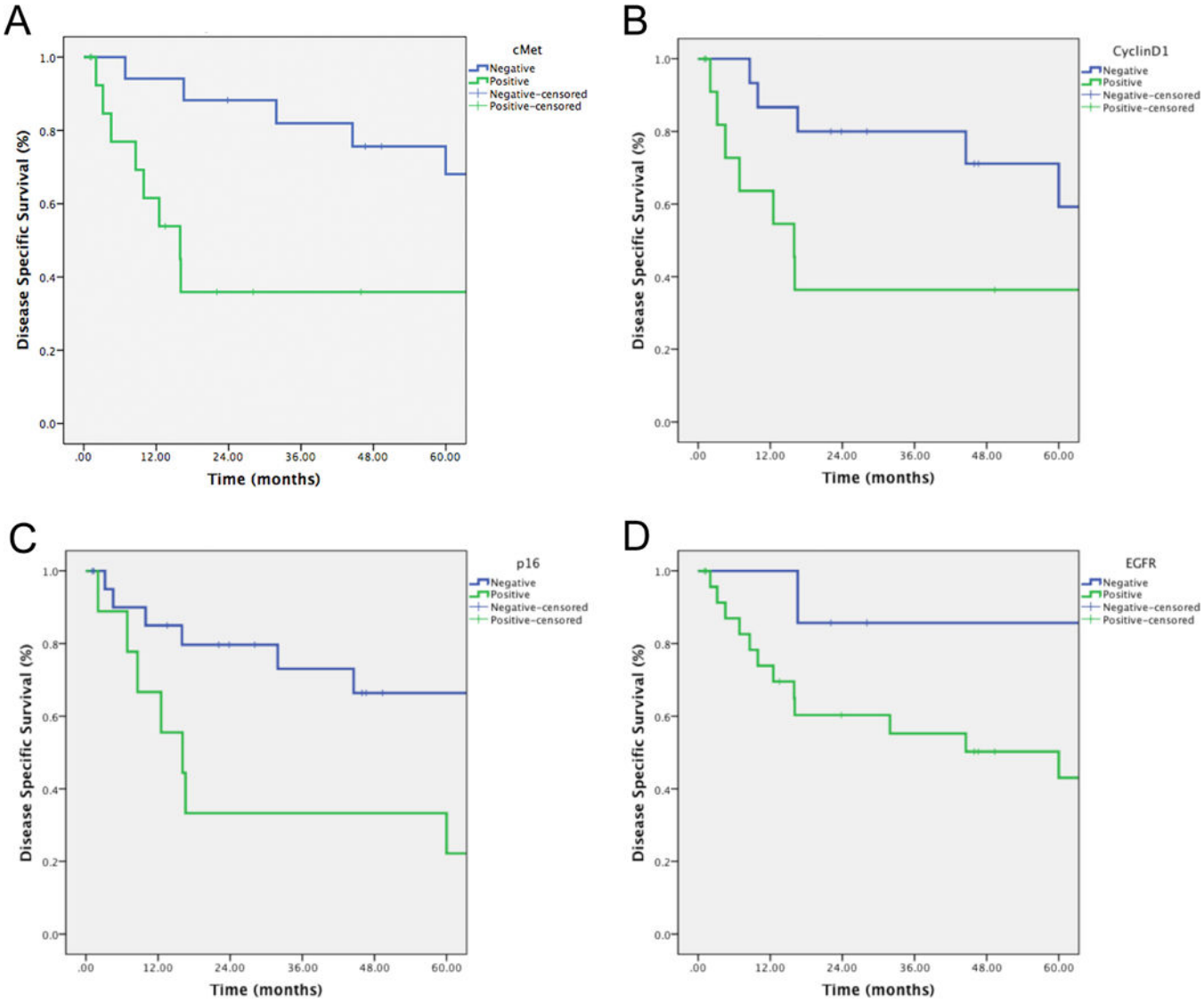


**Figure 2.**

Disease specific survival Kaplan-Meier curves based on tumor biomarkers cMet, p=0.007 (A), Cyclin D1, p=0.019 (B), p16, p=0.004 (C), and EGFR, p=0.072 (D).

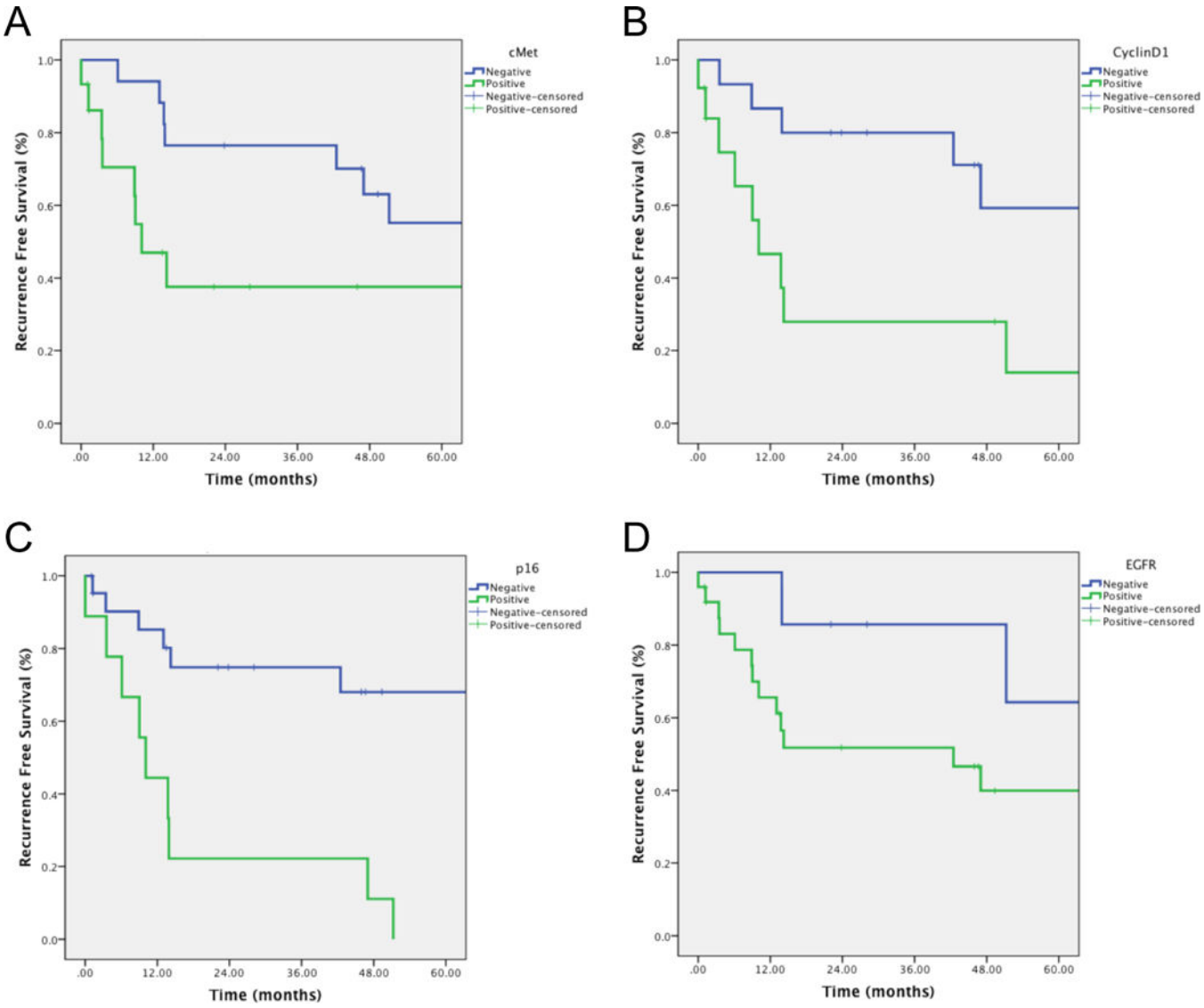


**Figure 3.**

Recurrence free survival Kaplan-Meier curves based on tumor biomarkers cMet, p=0.037 (A), Cyclin D1, p=0.012 (B), p16, p<0.001 (C), and EGFR, p=0.095 (D).

## Table 1

Patient and tumor characteristics of the SpSCC and non-SpSCC oral cavity comparison group.

| Variable | Spindle Cell Cohort (n=32) | Oral Cavity Cohort (n=75) | p – value |
|---|---|---|---|
| Age, years | 68.8 (range 38–85) | 54.2 (range 21–86) | p = < 0.001 |
| Gender | | | p = 0.086 |
| Male | 68.8% (22/32) | 50.7% (38/75) | |
| Female | 31.2% (10/32) | 49.3% (37/75) | |
| Tobacco Use | | | p = 0.714 |
| Current | 40.6% (13/32) | 37.8% (28/74) | |
| Former | 28.1% (9/32) | 27.0% (20/74) | |
| Never | 31.3% (10/32) | 35.1% (26/74) | |
| Missing data | | 1.3% (1/75) | |
| Overall Stage | | | p = 0.166 |
| I | 26.7% (8/30) | 8.1% (6/74) | |
| II | 16.7% (5/30) | 18.9% (14/74) | |
| III | 20.0% (6/30) | 12.2% (9/74) | |
| IV | 36.7% (11/30) | 60.8% (45/74) | |
| Missing data | 6.3% (2/32) | 1.3% (1/75) | |
| cMet Positive | 46.9% (15/32) | 2.7% (2/75) | p < 0.001 |
| Cyclin D1 Positive | 44.6% (14/32) | 77.3% (58/75) | p = 0.002 |
| p16 Positive | 29.0% (9/31) | 26.1% (18/69) | p = 0.76 |
| EGFR Positive | 78.1% (25/32) | 86.7% (65/75) | p = 0.27 |

**Table 2**

Hazard ratios (HR) with 95% confidence intervals (CI) and log-rank p-values evaluating the tumor biomarkers cMet, Cyclin D1, p16, EGFR, and OSS DSS and RFS in both the SpSCC and non-SpSCC cohort.

| | | Spindle Cell | | Non-Spindle Cell | |
|---|---|---|---|---|---|
| **Marker** | **Outcome** | **HR (95% CI)** | **p-value** | **HR (95% CI)** | **p-value** |
| cMet | OSS | 5.24 (2.05–13.39) | <0.001 | 5.80 (1.31–25.66) | 0.009 |
| | DSS | 4.49 (1.39–14.57) | 0.007 | 12.27 (2.52–59.69) | <0.001 |
| | RFS | 2.96 (1.03–8.53) | 0.037 | 6.18 (1.38–27.54) | 0.006 |
| Cyclin D1 | OSS | 2.26 (0.97–5.27) | 0.054 | 1.17 (0.51–2.67) | 0.71 |
| | DSS | 3.47 (1.15–10.45) | 0.019 | 1.42 (0.48–4.14) | 0.52 |
| | RFS | 3.77 (1.25–11.37) | 0.012 | 2.26 (0.68–7.49) | 0.17 |
| p16 | OSS | 1.72 (0.75–3.95) | 0.20 | 0.88 (0.38–2.02) | 0.76 |
| | DSS | 4.13 (1.46–11.68) | 0.004 | 0.74 (0.25–2.17) | 0.58 |
| | RFS | 5.49 (1.92–15.7) | <0.001 | 1.33 (0.56–3.17) | 0.52 |
| EGFR | OSS | 2.56 (0.86–7.59) | 0.079 | 0.53 (0.22–1.28) | 0.15 |
| | DSS | 3.67 (0.81–16.57) | 0.072 | 0.72 (0.21–2.44) | 0.6 |
| | RFS | 3.33 (0.75–14.79) | 0.095 | 1.06 (0.32–3.55) | 0.92 |