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p16 Status, Pathologic and Clinical Characteristics, Biomolecular Signature, and Long Term Outcomes in Unknown Primary Carcinomas of the Head and Neck

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Abstract

Background—To report associations between p16 status, clinicopathologic characteristics, and outcomes for unknown primary head and neck squamous cell carcinoma (SCCUPS).

Methods—Specimens of SCCUPS were re-analyzed. HPV status was determined by p16 stain. A tissue microarray (TMA) was constructed to evaluate biomarkers potentially prognostic in HNSCC.

Results—A majority of the population (n = 26, 74%) was p16+. Prognostic factors benefitting survival were p16+ status (p < 0.0001), absence of macroscopic extracapsular extension [ECE] (p = 0.004), younger age (p = 0.01), and higher grade (p = 0.007). The prognostic implication of worse OS with macroscopic ECE (p = 0.009) remained significant when limited to p16+ patients (p=0.002). Exploratory TMA between unknown primary and controls suggested a biomolecular difference between SCCUPS and known-primary cancer.

Conclusions—The majority of SCCUPS patients were p16+, indicative of HPV association. p16 staining and ECE appear to be the most prognostic features in SCCUPS.

Background

Unknown primary head and neck squamous cell carcinoma (SCCUPS) describes a clinical scenario in which gross neck adenopathy demonstrates squamous cell carcinoma but no primary tumor can be identified despite pursuit of thorough history, physical examination,

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and both non-invasive and invasive investigations, including biopsies¹. It currently accounts for 5% of new diagnoses of head and neck squamous cell carcinoma (HNSCC) (range 1 – 15%)^{1,2}, although the incidence may be lower following the adoption of functional imaging to help direct biopsies³. Two theories have been advanced to explain SCCUPS: 1) the primary tumor spontaneously regresses as a result of an immune reaction⁴ or 2) a subclinical primary cancer remains viable, but dormant, and therefore never becomes clinically significant⁵.

While the pathogenesis of SCCUPS is uncertain, numerous reports show two features of the disease. First, most primary tumors not detected on physical examination reside in the oropharynx⁶. Even when directed oropharynx biopsies are unrevealing, the most likely location of the primary tumor is still the oropharynx (sparing the hypopharynx and larynx with radiotherapy fields does not adversely affect the low rate of primary mucosal emergence)⁷. Second, unknown primary cancer seems to have a favorable prognosis when compared to other cancers of the upper aerodigestive tract, when treated with a variety of different regimens. As a consequence, there is increasing enthusiasm for de-intensification of treatment for unknown primary squamous cancers⁸.

In multiple recent multi-institution prospective analyses^{9–12} human papillomavirus (HPV) association was found to be the strongest independent determinant of progression free survival (PFS) and overall survival (OS) among patients with oropharyngeal cancer. Analysis of banked tumor specimens has demonstrated that HPV associated oropharyngeal cancer has been present for decades¹³, although its incidence is increasing¹⁴.

HPV associated oropharyngeal cancer typically presents with a smaller primary tumor and greater nodal bulk than are encountered in patients with tumors caused by substance abuse^{4,9,11}. HPV associated oropharyngeal carcinoma seems to have a favorable prognosis when compared to HPV negative oropharyngeal carcinoma. As a consequence, de-intensification of HPV associated oropharyngeal cancer treatment has been addressed in clinical trials (NCT01084083 [ECOG 1308] and NCT01302834 [RTOG 1016]). In view of the favorable prognosis and large nodal burden (by comparison to the primary) the possibility that SCCUPS represents a variant of HPV associated oropharyngeal carcinoma has been entertained.

At Fox Chase Cancer Center, SCCUPS has typically been managed with neck dissection, followed by adjuvant radiotherapy for N2-N3 disease. As a consequence, the institution has banked specimens of SCCUPS treated with initial resection. This monograph reports the association between SCCUPS and presence of p16 stain by IHC, the method determined to be the best (and favored) surrogate for ascertaining HPV status^{9,15}. It includes detailed pathologic and clinical characteristics, long-term outcomes, and identification of a biomolecular signature for SCCUPS via tissue microarray (TMA).

Methods

We queried the tumor registry and an institutional tumor bank for patients with a history of SCCUPS treated with neck dissection. The relevant medical records and specimens were

reviewed in accordance with a Fox Chase Cancer Center Institutional Review Board-approved protocol and the Health Insurance Portability and Accountability Act (HIPAA). The medical chart was abstracted for data regarding patient presentation and tumor burden at diagnosis. Hematoxylin and eosin-stained slides were again reviewed and re-analyzed for typical morphologic features including but not limited to: the size of largest involved lymph node, number of involved lymph nodes, presence and extent of extracapsular extension (ECE), presence or absence of keratinization, and grade. The extent of extracapsular extension (distance in millimeters [mm] of tumor from lymph node capsule) was measured in every case of extracapsular extension by a co-author (DF). Both 1 mm and 2 mm of extension were separately analyzed as potential demarcations between micro- and macroscopic ECE. Immunohistochemistry was performed for p16 on representative 4 μ m sections cut from formalin-fixed, paraffin-embedded tissue blocks using a purified mouse anti-human monoclonal antibody to p16^{INK4A} (G175-405, 1:400 dilution, BD Pharmingen, USA) on a Ventana Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ). A positive test was defined as intermediate/strong nuclear and cytoplasmic p16 staining in 70% of cells and results were compared to both positive and negative controls. Patient demographics, tumor characteristics, and treatment-related outcomes were obtained from chart review.

A tissue microarray (TMA) was constructed from the formalin fixed, paraffin-embedded tissue blocks. Included in this TMA are 34 samples, plus two samples of normal mucosa, three samples of known p16+ oropharynx carcinoma, and three samples of known HPV-head and neck squamous cell carcinoma. For this analysis we included well-validated biomarkers in head and neck squamous cell carcinoma (p16, p53, EGFR, ERCC1) and additional markers considered related to validated pathways (Rb [p16], survivin [p53], RRM1, Her-2, PTEN, pAKT, ACK, pACK, Met, PARP [EGFR]). The TMA was stained by a modified indirect immunofluorescence method. Automated image capture was performed by the HistoRx PM-2000 (HistoRx, New Haven, CT) using the AQUAsition software. High-resolution monochromatic digital images of the cytokeratin staining visualized with AF555, DAPI and target staining with Cy5 were captured and saved for every histospot on the arrays. Tumor mask was created from the cytokeratin image of each histospot, representing areas of epithelial tumor. Histospots were excluded if the tumor mask represented less than 5% of the total histospot area. DAPI immunoreactivity defined the nuclear compartment. The cytoplasmic compartment was defined by the tumor mask with specific exclusion of the nuclear compartment. Before automatic analysis images were visually inspected and cropped for unfavorable findings such as being out of focus or presence of debris or damaged tissue. Target expression was quantified by calculating Cy5 fluorescent signal intensity within each image pixel. An AQUA score was generated by dividing the sum of target signals within the tumor mask. AQUA scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different exposure times to be directly comparable.

Clinical characteristics were compared by p16+ protein status using Fisher's Exact Tests and Wilcoxon rank sum tests as appropriate. Differences in ECE status by p16+ protein status were determined using Fisher's Exact Test. Hazard ratio estimates for ECE definitions were estimated using Cox proportional hazards regression for time to death from all causes. The

Kaplan-Meier product-limit method estimated overall survival and cause-specific survival functions by p16 status and ECE status. Surviving patients were censored at their last available follow-up date. Differences in the curves were assessed using the log rank test. Cox proportional hazards regression was employed for inferences about the relationship of survival time to p16 status, adjusting for age at diagnosis. Statistical analyses were performed using SAS statistical software, version 9.2 (Cary, NC) and survival plots generated using the open-source R software (www.r-project.org). Due to the exploratory nature of this analysis, a Type I error of 5% was used and there was no correction for multiple testing. The collection, storage, and retrieval of data were all done in compliance with the hospital's Institutional Review Board and the Health Insurance Privacy and Portability Act.

Results

We identified a total of 39 cases of SCCUPS treated with neck dissection between 1990 – 2010, 35 of which had sufficient material available for pathologic analysis. The median follow-up was 60 months (range 3.5 – 226). Cases were most commonly (71%) from the second half of the study period (2000–2010). All patients were staged with pre-operative imaging and endoscopy with systematic biopsy performed in 97%. The majority of patients (83%) were men and most (57%) had a > 10 pack year smoking history. Most patients (63%) presented with cN2 disease and 23% presented with cN3 disease. Level II (74%) was the most commonly involved station.

Initial neck dissection was the most common treatment approach (n = 31, 89%). The type of neck operation performed was determined by the extent of disease at the time of surgery, but dissection of levels I–V was customary. All but one (97%) of patients treated with initial lymphadenectomy received adjuvant radiotherapy. Almost all of the samples (89%) manifested keratinization and a majority of the specimens (63%) were noted to have a component of necrosis. The unadjusted hazard ratio estimate for extracapsular extension (ECE) with overall survival was influenced by the definition of ‘macroscopic ECE’ (Table 1). Pathologic nodal staging was 20% N1, 14% N2a, 51% N2b, 9% yN2b, 3% N3, 3% yN3. The typical indication for adjuvant radiation of an N1 neck was diagnosis by virtue of an excisional biopsy undertaken prior to arrival at the institution. In all cases RT was delivered to the mucosal subsites and both sides of the neck. Median dose to the high-risk volume was 60 (range 58 – 70) in an adjuvant setting and 65 (range 50 – 70) with preoperative radiation. A total of four patients (two of whom were treated pre-operatively) received concurrent systemic therapy (11%). The median 5-yr overall survival (OS) for the study cohort was 77% (95% CI 56%–88%) and cause specific survival (CSS) was 86% (95% CI 70%–98%) (Figure 1). Failures were rare. One p16+ patient developed a tonsil primary. There were no recurrences in the neck and three patients developed distant metastases.

Pathologic re-analysis of the tumor specimens demonstrated that a majority of samples (74%; n = 26) stained positive for the p16 protein (p16+). p16+ patients were more likely male (p = 0.03) and younger (median age 50 vs. 66 yrs.; p = 0.04) in comparison to p16– patients. In contrast, smoking history (p = 0.82), neck stage at presentation (p = 0.36), and use of adjuvant therapy was not associated with p16 status (100% of p16+ patients received

RT, 89% of p16– patients received RT). Necrosis was present in 100% of p16– samples compared to 50% of p16+ samples ($p = 0.01$). Any extracapsular extension was more common among HPV negative patients, although this did not reach statistical significance (78% v 46%, $p = 0.13$), and keratinization was common regardless of p16 status (88%). There was a statistically significant overall survival benefit for patients who were p16+ ($p < 0.0001$). This benefit persisted after controlling for age ($p = 0.01$). The 5-yr OS was 92% (95% CI 70%–98%) for p16+ patients as compared to 30% (95%CI 5%–63%) for p16– patients. The 5-yr CSS was 92% (95% CI 70%- 98%) for p16+ patients as compared to 60% (95%CI 13% – 88%); this did not reach statistical significance (Figure 2) in our small sample ($p = 0.09$). Higher-grade cancer was found to have a better prognosis ($p = 0.006$), likely secondary to rare failures in poorly differentiated tumors, which were frequently p16+ (82%).

Given the known prognostic significance of ECE in a postoperative setting^{16,17} and the hypothesis that degree of ECE may be significant, we graded all ECE as either micro- or macroscopic and analyzed the significance of ECE as a function of p16 staining. Detailed pathologic measurement of degree of ECE (Table 1) demonstrated that a cutoff of 2 mm was the best determinant of ‘clinically significant’ ECE. Survival was significantly better ($p = 0.004$) among patients without macroscopic ECE (Figure 3). Macroscopic ECE was observed in 19% of p16+ and 44% of p16– patients. Among p16+ patients, macroscopic ECE was still associated ($p = 0.002$) with a difference in OS (Figure 4).

Of the 35 patients comprising the study cohort, 26 were consented for TMA analysis: 21 were p16+ and 5 were p16–. The protein expression levels of various biomarkers were analyzed in this TMA using an advanced automated quantitative analysis (AQUA) technology. AQUA uses antibodies and fluorescent stains as molecular identification of compartments to quantify biomarker expression in specific tissues or sub-cellular components. This approach combines fluorescent immunoassay sensitivity and dynamic range with the location information typically provided by traditional immunohistochemistry (IHC). Quality staining of some biomarkers are shown in Figure 5. It has been demonstrated that AQUA scores are directly proportional to molecular unit area and protein concentration¹⁸. Statistically significant increases of nuclear p53, survivin, ERCC, and RRM1 levels were recorded in p16– samples as compared to p16+ samples (Table 1). By contrast, both cytoplasmic and nuclear levels of AuroraA, HER2, pAKT, PARP, pAuroraA, and PTEN were quite similar regardless of p16 IHC. The remaining evaluated biomarkers (EGFR, pEGFR, and MET) had small differences that did not reach statistical significance in our small group.

An exploratory analysis demonstrated a surprisingly different biomarker profile between samples of known p16+ oropharyngeal carcinoma and unknown primary p16+ carcinoma. Statistically significant differences in nuclear expression of PTEN, RRM1, pAKT, and HER2 were demonstrated in a relatively small set of tumors. A similar exploratory analysis between p16– unknown primary carcinoma and p16– known HNSCC demonstrated a more similar staining pattern, with only EGFR and pEGFR demonstrating a difference approaching significance (Table 2).

Discussion

There is no standard treatment paradigm for head and neck SCCUPS. As a consequence, management of this disease is institution-specific and generally based upon single center retrospective series. The common loci of controversy include determination of which modalities should be employed to afford the best chance for cure with lowest possible long-term toxicity. An important goal of this study was better to understand patients with SCCUPS in the hope that it would permit more sophisticated choice of therapy.

Traditional radiotherapy volumes for unknown primary carcinoma targeted the both sides of the neck, the nasopharynx, oropharynx, larynx, and hypopharynx. These fields, still recommended by the NCCN for adjuvant therapy¹⁹, result in satisfactory mucosal control (with a primary tumor emergence rate essentially the same as that expected for a second primary cancer), but likely cause unnecessary toxicity. Because the most common site of an occult primary is the oropharynx⁶, some centers have removed the larynx and hypopharynx from the field (when the nodal disease is in level II or III) without a rise in the rate of development of mucosal primary tumors⁷, suggesting that this is a safe approach to limiting toxicity. SCCUPS is uncommon, and prospective trials to address management questions have not been undertaken. In their absence the standard at our institution has been to perform a comprehensive cervical lymphadenectomy, followed by radiation to both sides of the neck, the oropharynx, and the nasopharynx (generally radiated as a consequence of covering the ipsilateral retrostyloid space and retropharyngeal nodes).

During the past 20 years three-fourths of SCCUPS at the Fox Chase Cancer Center has been HPV associated. The percentage of HPV associated tumors was much higher in the most recent decade (1990 – 2000: 50% p16+ v 2000 – 2010: 84% p16+). Despite the keratinizing character of the most such lesions (a feature uncommon among HPV associated oropharynx cancers), SCCUPS today is overwhelmingly referable to HPV²⁰.

What radiation dose is necessary to assure mucosal control in the setting of primary cancers quite sensitive to non-surgical therapy? Recent series demonstrate decreasing mucosal doses^{7,21,22} (median 55 Gy) with low mucosal emergence rates (< 10%), similar to that (3%) in this report. Even lower mucosal doses may warrant consideration for virus associated SCCUPS, perhaps even sparing the mucosa entirely and relying instead on dose “fall off” from treatment of the neck using an intensity-modulated approach²³.

It would seem that either chemoradiation or neck dissection followed by post-operative radiation will suffice to treat most patients with N2 and N3 disease from SCCUPS. Radiation alone may permit single modality therapy for selected N2 patients²⁴. Recent use of transoral surgical techniques^{25,26} suggests a majority of unknown primary cancer is in fact occult base of tongue cancer. Thus, it is reasonable to suspect that most unknown primary cases in this series (and others) are in fact small T1 base of tongue cancers. HPV associated base of tongue cancer with N2-N3 neck nodes is generally managed with primary chemoradiation. While it is anticipated that outcomes with both combined modality treatment techniques (primary chemoradiation v neck dissection + adjuvant radiation) will have favorable success rates in the HPV associated unknown primary population, it is

unclear which treatment paradigm is 'best'. Regardless of preferred treatment technique, the results of this experience and others suggest that a majority of patients stand to benefit little from the more costly and morbid tri-modality therapy.

With an up-front neck dissection management technique, decisions regarding adjuvant chemoradiation are predicated on the pathology findings. Though not systematically reported, it seems that most centers employ for SCCUPS the same indicators for chemoradiation used for traditional postoperative radiation therapy (this is the recommendation of the NCCN¹⁹). Our present series demonstrates conclusively that many cases of ECE are relatively small (47% of cases of ECE were < 2 mm) and suggests that so-called 'microscopic ECE' has a different risk profile that does not justify the use of adjuvant chemoradiation.

A series of treatment intensification protocols^{9–12} have attributed prognostic significance to p16 protein expression as an indicator of HPV associated cancers with better treatment response. The present analysis demonstrates a similarly significant overall survival benefit among p16+ patients with SCCUPS. In addition, this small unknown primary series suggests that the degree of extracapsular extension is similarly significant; the survival difference between p16+ patients with (n = 12) and without any extracapsular extension (n = 14) was not significant (80% v 100%, p = 0.2) while the same comparison among patients with (n = 5) and without (n = 21) macroscopic ECE was significant (95% v 40%, p = 0.0018). Hence, there is little justification for administration of concurrent chemoradiation on the basis of ECE alone. Based on our analysis, it would seem that only macroscopic ECE could potentially justify the added morbidity (Figure 6).

The 13 biomarkers that we studied comprise a novel panel potentially important for risk stratification in locally advanced head and neck cancer. With only rare treatment failure it proved impossible to divide patients into different prognostic groups based upon their markers. Hence, analysis was restricted to comparisons of the biomarker profiles of SCCUPS as a function of p16 status and in comparison to controls.

We demonstrated a statistically significant increase in both p53 and survivin nuclear expression among p16– unknown primary cancers, similar to that in known primary oropharynx cancer^{27,28}. It has been hypothesized that the dramatic difference in response to non-surgical therapy is in part a consequence of differential p53 expression, and it appears that a similar principle governs unknown primary cancer. Also similar to known primary cancers was the demonstration of significant nuclear ERCC1 over-expression in p16– SCCUPS²⁹. By contrast, the expression of both EGFR and downstream EFGR biomarkers (pEGFR and pAKT)³⁰ was not significantly different, in contrast to previous reports that high EGFR is either a poor prognostic factor, or associated with p16– disease, or both^{31,32}. Like EGFR, c-MET expression, associated with a poor prognosis in oral tongue cancer (an anatomic site characterized as HPV-negative)³³, was not significant in our small sample.

Using the 13 biomarker panel between p16+ patients and p16+ controls (in an attempt to determine whether or not the unknown primary tumors appeared to be the same disease as the known primary HPV associated tumors) with a limited number of controls (n = 3), a

difference was noted observed pAKT, PTEN, and RRM1. Coupled with the difference between EGFR staining in our unknown primary patients and prior EGFR staining in cancers of known primary, these findings raise the possibility that SCCUPS represents a different form of HPV associated carcinoma that is highly responsive to therapy.

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References

1. Issing WJ, Taleban B, Tauber S. Diagnosis and management of carcinoma of unknown primary in the head and neck. *European archives of oto-rhino-laryngology: official journal of the European Federation of Oto-Rhino-Laryngological Societies*. Sep; 2003 260(8):436–443.
2. Pavlidis N, Pentheroudakis G, Plataniotis G. Cervical lymph node metastases of squamous cell carcinoma from an unknown primary site: a favourable prognosis subset of patients with CUP. *Clinical & translational oncology: official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*. Jun; 2009 11(6):340–348. [PubMed: 19531448]
3. Johansen J, Buus S, Loft A, et al. Prospective study of 18FDG-PET in the detection and management of patients with lymph node metastases to the neck from an unknown primary tumor. Results from the DAHANCA-13 study. *Head & neck*. Apr; 2008 30(4):471–478. [PubMed: 18023031]
4. Challis GB, Stam HJ. The spontaneous regression of cancer. A review of cases from 1900 to 1987. *Acta oncologica*. 1990; 29(5):545–550. [PubMed: 2206563]
5. van de Wouw AJ, Jansen RL, Speel EJ, Hillen HF. The unknown biology of the unknown primary tumour: a literature review. *Annals of oncology: official journal of the European Society for Medical Oncology / ESMO*. Feb; 2003 14(2):191–196. [PubMed: 12562643]
6. Cianchetti M, Mancuso AA, Amdur RJ, et al. Diagnostic evaluation of squamous cell carcinoma metastatic to cervical lymph nodes from an unknown head and neck primary site. *The Laryngoscope*. Dec; 2009 119(12):2348–2354. [PubMed: 19718744]
7. Wallace A, Richards GM, Harari PM, et al. Head and neck squamous cell carcinoma from an unknown primary site. *American journal of otolaryngology*. Jul-Aug; 2011 32(4):286–290. [PubMed: 20719404]
8. Nieder C, Gregoire V, Ang KK. Cervical lymph node metastases from occult squamous cell carcinoma: cut down a tree to get an apple? *International journal of radiation oncology, biology, physics*. Jul 1; 2001 50(3):727–733.
9. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *The New England journal of medicine*. Jul 1; 2010 363(1):24–35. [PubMed: 20530316]
10. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *Journal of the National Cancer Institute*. Feb 20; 2008 100(4):261–269. [PubMed: 18270337]
11. Rischin D, Young RJ, Fisher R, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. Sep 20; 2010 28(27):4142–4148. [PubMed: 20697079]
12. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. Apr 20; 2009 27(12):1992–1998. [PubMed: 19289615]

13. Gillison ML, Zhang Q, Jordan R, et al. Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. Jun 10; 2012 30(17):2102–2111. [PubMed: 22565003]
14. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. Nov 10; 2011 29(32):4294–4301. [PubMed: 21969503]
15. Shi W, Kato H, Perez-Ordóñez B, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. Dec 20; 2009 27(36):6213–6221. [PubMed: 19884544]
16. Huang DT, Johnson CR, Schmidt-Ullrich R, Grimes M. Postoperative radiotherapy in head and neck carcinoma with extracapsular lymph node extension and/or positive resection margins: a comparative study. *International journal of radiation oncology, biology, physics*. 1992; 23(4):737–742.
17. Ang KK, Trotti A, Brown BW, et al. Randomized trial addressing risk features and time factors of surgery plus radiotherapy in advanced head-and-neck cancer. *International journal of radiation oncology, biology, physics*. Nov 1; 2001 51(3):571–578.
18. McCabe A, Dolled-Filhart M, Camp RL, Rimm DL. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. *Journal of the National Cancer Institute*. Dec 21; 2005 97(24):1808–1815. [PubMed: 16368942]
19. Pfister DG, Ang KK, Brizel DM, et al. Head and neck cancers. *Journal of the National Comprehensive Cancer Network: JNCCN*. Jun 1; 2011 9(6):596–650. [PubMed: 21636536]
20. Chernock RD, El-Mofty SK, Thorstad WL, Parvin CA, Lewis JS Jr. HPV-related nonkeratinizing squamous cell carcinoma of the oropharynx: utility of microscopic features in predicting patient outcome. *Head and neck pathology*. Sep; 2009 3(3):186–194. [PubMed: 20596971]
21. Sher DJ, Balboni TA, Haddad RI, et al. Efficacy and toxicity of chemoradiotherapy using intensity-modulated radiotherapy for unknown primary of head and neck. *International journal of radiation oncology, biology, physics*. Aug 1; 2011 80(5):1405–1411.
22. Klem ML, Mechalakos JG, Wolden SL, et al. Intensity-modulated radiotherapy for head and neck cancer of unknown primary: toxicity and preliminary efficacy. *International journal of radiation oncology, biology, physics*. Mar 15; 2008 70(4):1100–1107.
23. Fried DL-DM, Hackman T, et al. Dosimetric effect of sparing the primary site for oropharyngeal squamous cell carcinoma after transoral laser microsurgery. *International Journal of Radiation, Oncology, Biology, and Physics*. 2012; 84(3, Supplement):S472.
24. Frank SJ, Rosenthal DI, Petsuksiri J, et al. Intensity-modulated radiotherapy for cervical node squamous cell carcinoma metastases from unknown head-and-neck primary site: M. D. Anderson Cancer Center outcomes and patterns of failure. *International journal of radiation oncology, biology, physics*. Nov 15; 2010 78(4):1005–1010.
25. Karni RJ, Rich JT, Sinha P, Haughey BH. Transoral laser microsurgery: a new approach for unknown primaries of the head and neck. *The Laryngoscope*. Jun; 2011 121(6):1194–1201. [PubMed: 21495044]
26. Mehta V, Johnson P, Tassler A, et al. A new paradigm for the diagnosis and management of unknown primary tumors of the head and neck: a role for transoral robotic surgery. *The Laryngoscope*. Jan; 2013 123(1):146–151. [PubMed: 23154813]
27. Smith EM, Wang D, Rubenstein LM, Morris WA, Turek LP, Haugen TH. Association between p53 and human papillomavirus in head and neck cancer survival. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. Feb; 2008 17(2):421–427.
28. Haraf DJ, Nodzenski E, Brachman D, et al. Human papilloma virus and p53 in head and neck cancer: clinical correlates and survival. *Clinical cancer research: an official journal of the American Association for Cancer Research*. Apr; 1996 2(4):755–762. [PubMed: 9816227]

29. Mehra RCK, Zhu F, et al. Analysis of ERCC1 (excision repair cross complementing group 1) in squamous cell carcinoma of the head and neck (SCCHN) by quantitative immunohistochemistry (IHC) using FL297. ASCO Meeting Abstracts. 2010; 28(15_suppl):5539.
30. Nijkamp MM, Hoogsteen IJ, Span PN, et al. Spatial relationship of phosphorylated epidermal growth factor receptor and activated AKT in head and neck squamous cell carcinoma. Radiotherapy and oncology: journal of the European Society for Therapeutic Radiology and Oncology. Oct; 2011 101(1):165–170. [PubMed: 21775008]
31. Husain H, Psyrri A, Markovic A, et al. Nuclear epidermal growth factor receptor and p16 expression in head and neck squamous cell carcinoma. The Laryngoscope. Dec; 2012 122(12): 2762–2768. [PubMed: 23086695]
32. Worden FP, Kumar B, Lee JS, et al. Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. Jul 1; 2008 26(19):3138–3146. [PubMed: 18474879]
33. Kim CH, Koh YW, Han JH, et al. c-Met expression as an indicator of survival outcome in patients with oral tongue carcinoma. Head & neck. Dec; 2010 32(12):1655–1664. [PubMed: 20848408]

Figure 1.
Overall and Cause-Specific Survival for the study cohort

Figure 2.
Overall and Cause-Specific Survival as a function of p16 status

Figure 3.
Overall and Cause-Specific Survival as a function of macroscopic extracapsular extension in lymph nodes

Figure 4. Overall and Cause Specific Survival among p16+ patients (n = 26) as a function of macroscopic extracapsular extension in lymph nodes

Figure 5.
Representative AQUA staining of some biomarkers in SCCUPS. Target (biomarker) stainings are p53, survivin, ERCC, and RRM1. Cytokeratin: Tumor mask. Dapi: nuclear staining

Figure 6.
Typical treatment regimen for patients with unknown primary carcinoma of the head and neck

Table 1

Hazard ratio estimates for all-cause mortality as a function of degree of extracapsular extension (ECE).

ECE Definition	N	deaths	Hazard Ratio Estimate (95% CI)	p value
Model 1:				
None	16	2	1.00 (ref)	0.068
Any ECE	19	9	4.19 (0.90–19.48)	
Model 2:				
None/Micro (<1mm)	22	4	1.00 (ref)	0.031
Gross (≥ 1mm)	13	7	3.95 (1.14–13.72)	
Model 3:				
None/Micro (<2mm)	26	5	1.00 (ref)	0.009
Gross (≥ 2mm)	9	6	4.99 (1.49–16.75)	

Table 2

Tissue microanalysis comparison of biomarkers as a function of p16 status

Cytoplasmic			Nucleic		
Marker	N	Median (range)	p	Median (range)	p
p53					
p16-	6	3390 (2094 – 3754)		6808 (5760 – 9869)	
p16+	23	2119 (1397 – 4537)	p16- vs p16+	3836 (2626 – 5130)	p16- vs p16+ <0.0001
Control p16+	3	2278 (1611 – 3076)	p16+ vs control p16+	3838 (3659 – 4629)	p16+ vs control p16+ 0.70
Control p16-	3	1937 (844 – 5943)	p16- vs control p16-	5445 (1757 – 7228)	p16- vs control p16- 0.20
Survivin					
p16-	6	4012 (391.6 – 3760)		5057 (4294 – 5336)	
p16+	23	3644 (397.5 – 2757)	p16- vs p16+	4537 (3351 – 5122)	p16- vs p16+ 0.02
Control p16+	3	3352 (3345 – 3907)	p16+ vs control p16+	4449 (4278 – 4816)	p16+ vs control p16+ 1.00
Control p16-	3	3234 (3018 – 4312)	p16- vs control p16-	4449 (4278 – 4816)	p16- vs control p16- 0.17
ERCC 1					
p16-	6	2732 (1369 – 3774)		5019 (3822 – 6284)	
p16+	23	2158 (941.5 – 3773)	p16- vs p16+	3749 (1421 – 5694)	p16- vs p16+ 0.02
Control p16+	3	2958 (2478 – 3456)	p16+ vs control p16+	1598 (976.3 – 2160)	p16+ vs control p16+ 0.06
Control p16-	3	1885 (1600 – 2295)	p16- vs control p16-	4566 (3957 – 4916)	p16- vs control p16- 0.38
PTEN					
p16-	6	5744 (4992 – 7253)		6221 (1545 – 8218)	
p16+	23	6264 (1889 – 8632)	p16- vs p16+	6221 (1545 – 8218)	p16- vs p16+ 0.98
Control p16+	3	7789 (7459 – 8577)	p16+ vs control p16+	7175 (7106 – 7872)	p16+ vs control p16+ 0.05
Control p16-	3	4824 (4485 – 7248)	p16- vs control p16-	5217 (4829 – 6527)	p16- vs control p16- 0.71
RRM1					
p16-	6	3430 (2912 – 4404)		3669 (3492 – 4425)	
			p16- vs p16+		p16- vs p16+ 0.01

Marker	N	Cytoplasmic		Nucleic	
		Median (range)	p	Median (range)	p
p16+	23	3198 (2454 – 3841)		3326 (2256 – 3751)	
Control p16+	3	3594 (3482 – 3785)	p16+ vs control p16+	3783 (3629 – 3800)	p16+ vs control p16+ 0.003
Control p16–	3	3420 (3028 – 3640)	p16– vs control p16–	3682 (3210 – 3815)	p16– vs control p16– 0.71
pAKT					
p16–	6	2056 (1312 – 2941)		2626 (1756 – 3302)	
p16+	23	1927 (938.2 – 3387)	p16– vs p16+	2456 (1067 – 3372)	p16– vs p16+ 0.77
Control p16 +	3	2761 (2004 – 3062)	p16+ vs control p16+	3459 (2496 – 3985)	p16+ vs control p16+ 0.05
Control p16–	3	2945 (1702 – 3259)	p16– vs control p16–	3088 (2100 – 3230)	p16– vs control p16– 0.55
Aurora A					
p16–	6	3790 (3574 – 4104)		4055 (3990 – 4376)	
p16+	23	3849 (2535 – 4643)	p16– vs p16+	4080 (2892 – 5003)	p16– vs p16+ 0.90
Control p16+	3	4190 (3835 – 4546)	p16+ vs control p16+	4193 (3918 – 4468)	p16+ vs control p16+ 0.71
Control p16–	3	3603 (3384 – 4022)	p16– vs control p16–	4516 (4009 – 4528)	p16– vs control p16– 0.25
EGFR					
p16–	6	968.3 (844 – 2041)		749.4 (718.6 – 1492)	
p16+	23	1414 (848.1 – 3672)	p16– vs p16+	1147 (690.14 – 2663)	p16– vs p16+ 0.14
Control p16+	3	1218 (1025 – 1331)	p16+ vs control p16+	995.9 (820.8 – 1100)	p16+ vs control p16+ 0.31
Control p16–	3	2182 (1160 – 3090)	p16– vs control p16–	1598 (976 – 2160)	p16– vs control p16– 0.10
Her2					
p16–	6	5305 (3793 – 5603)		3248 (2678 – 4070)	
p16+	23	4997 (3965 – 5837)	p16– vs p16+	3309 (2459 – 4410)	p16– vs p16+ 0.98
Control p16+	3	5311 (5130 – 5651)	p16+ vs control p16+	3508 (3427 – 3610)	p16+ vs control p16+ 0.08
Control p16–	3	5143 (4013 – 5580)	p16– vs control p16–	3290 (2882 – 3595)	p16– vs control p16– 0.71
MET					
p16–	6	4174 (2580 – 6062)		2647 (1721 – 3618)	
			p16– vs p16+		p16– vs p16+ 0.09

Marker	N	Cytoplasmic		Nucleic		p
		Median (range)		Median (range)		
p16+	23	5199 (2440 – 8453)		3069 (1971 – 5302)		
Control p16+	3	6897 (4688 – 6997)	p16+ vs control p16+	4292 (3627 – 4299)	p16+ vs control p16+	0.21
Control p16–	3	4905 (4797 – 7852)	p16– vs control p16–	3351 (3024 – 4825)	p16– vs control p16–	0.14
PARP						
p16–	6	1395 (1232 – 2539)		4580 (3241 – 7605)		
p16+	23	1923 (756.7 – 5324)	p16– vs p16+	4879 (2757 – 7104)	p16– vs p16+	0.85
Control p16+	3	2416 (1577 – 3084)	p16+ vs control p16+	5295 (4519 – 7432)	p16+ vs control p16+	0.35
Control p16–	3	1759 (1108 – 1870)	p16– vs control p16–	5014 (4877 – 6820)	p16– vs control p16–	0.55
pAurora A						
p16–	6	3590 (3077 – 5182)		4677 (4011 – 5969)		
p16+	23	3691 (2677 – 5081)	p16– vs p16+	4443 (3601 – 6071)	p16– vs p16+	0.33
Control p16+	3	3796 (3499 – 3929)	p16+ vs control p16+	4581 (3676 – 5427)	p16+ vs control p16+	0.78
Control p16–	3	3603 (3384 – 4022)	p16– vs control p16–	4516 (4009 – 4528)	p16– vs control p16–	0.25
pEGFR						
p16–	6	1340 (502.4 – 5424)		1531 (525.7 – 3474)		
p16+	23	2815 (570 – 6068)	p16– vs p16+	1127 (505.3 – 3501)	p16– vs p16+	0.45
Control p16+	3	1366 (1018 – 5517)	p16+ vs control p16+	1118 (831.8 – 4215)	p16+ vs control p16+	0.88
Control p16–	3	4295 (4280 – 6634)	p16– vs control p16–	2711 (2450 – 4549)	p16– vs control p16–	0.10