Small Biopsy Specimens Reliably Indicate p16 Expression Status of Oropharyngeal Squamous Cell Carcinoma

Changqing Ma · James Lewis Jr.

Received: 15 October 2011 / Accepted: 13 December 2011 / Published online: 28 December 2011
© Springer Science+Business Media, LLC 2011

Abstract Human papillomavirus (HPV)—related oropharyngeal squamous cell carcinoma (SCC) is associated with favorable patient survival. Tumor HPV status at primary diagnosis is critical for proper management, and p16 immunohistochemistry (IHC) has emerged as a reliable, single, surrogate marker. It is not known, however, if small biopsy specimens are completely adequate for p16 evaluation. From a database of oropharyngeal SCC for which p16 IHC and histologic typing were already performed, all patients (32) who had available in-house primary tumor biopsy specimens and also subsequent surgical resections were analyzed. p16 IHC was performed along with histologic typing into: Type 1 keratinizing SCC, Type 2 nonkeratinizing SCC with maturation, and Type 3 nonkeratinizing SCC. Staining was graded on both biopsies and resections as follows: 0 = negative; 1+ = 1–25% of tumor cells positive; 2+ = 26–50%; 3+ = 51–75%; 4+ = 76–100%. Strictly considering p16 score, perfect biopsy-resection correlation was present in 28 of 32 cases (85%), including 6/9 (67%) Type 1, 6/7 (86%) Type 2, and 16/16 (100%) Type 3 cases. Considering p16 expression binarily as 51% tumor cell staining or more (3+ or 4+) being positive and lesser amounts (0, 1+, or 2+) as being negative, there was perfect biopsy-resection correlation for all 32 cases. With p16 expression in resection specimens considered the gold standard, p16 IHC in biopsies was both 100% sensitive and specific. Our results demonstrate that

p16 staining in diagnostic biopsies reliably reflects whole tumor staining results, and suggest that biopsies do not suffer from false negatives or positives.

Keywords Human papillomavirus · p16 immunohistochemistry · Squamous cell carcinoma · Oropharynx · Small biopsy

Introduction

Squamous cell carcinoma (SCC) of the head and neck was once considered a uniform group of tumors. The major risk factors are alcohol consumption and tobacco use, which also have synergistic effects [1]. However, over the last few decades, epidemiology, molecular biology, and outcome studies have shown that human papillomavirus (HPV)—related SCCs of the oropharynx are a distinct group of tumors clinically, morphologically, and biologically [2].

HPV-related oropharyngeal SCC arises from the base of the tongue and palatine tonsils [3–5] where lymphoid tissue is present and associated with crypt epithelium. HPV infection, particularly high-risk HPV type 16, is an important causal agent in these cancers [2, 6–9]. The major risk factors, as opposed to heavy tobacco and alcohol use, are sex-related, including high lifetime numbers of vaginal-sex partners and/or oral-sex partners [7, 10]. Although smoking rates have been declining in recent years as well as the rates of typical head and neck SCC, HPV-related oropharyngeal SCC rates are going up. Some have even termed this an epidemic [11, 12]. Smoking rates in HPV-related oropharyngeal SCC patients are lower, but still the majority are former or current smokers, making tobacco exposure a significant co-factor [9]. HPV-related SCC predominantly affects male patients who are 5–10 years younger than patients with typical head

C. Ma · J. Lewis Jr.
Department of Pathology and Immunology, Washington
University in St. Louis, St. Louis, MO, USA

J. Lewis Jr. (✉)
Otolaryngology Head and Neck Surgery, Washington University
in St. Louis, St. Louis, MO, USA
e-mail: jlewis@path.wustl.edu

and neck SCC [13]. Importantly, although it tends to present at high stage due to high rates of nodal metastasis at presentation, HPV-related SCC is associated with much better survival [5, 14–18]. Simply put, tumor HPV status has emerged as a strong, independent prognostic marker in oropharyngeal SCC [18].

Histologically, most HPV-related SCCs of the oropharynx have a non-keratinizing appearance, being composed of ovoid to spindled tumor cells with hyperchromatic nuclei without conspicuous nucleoli and lacking distinct cell borders. The tumor cells grow in large nests and sheets with pushing borders and typically elicit little or no stromal response (desmoplasia) in the surrounding tissue. High mitotic rates, apoptosis, and comedo necrosis are frequently observed [19–21]. In addition, a subset of the HPV-related SCC demonstrates “intermediate” features with nonkeratinizing areas with partial squamous maturation (nonkeratinizing SCC with maturation or hybrid SCC) [19, 22]. Chernock et al. [19] previously segregated SCC of the oropharynx based solely upon these histologic features (three types: Type 1 keratinizing SCC, Type 2 nonkeratinizing SCC with maturation, and Type 3 nonkeratinizing SCC) and showed that Types 2 and 3 SCC are highly HPV-related and, by histology alone, have better patient survival than Type 1 SCC [19, 23].

At the molecular level, HPV-related SCC harbors fewer TP53 mutations, less overall chromosomal mutations, and less chromosomal losses compared to non-HPV-related SCC [2, 8, 24, 25]. Also, HPV-related SCC almost always aberrantly over-expresses the tumor suppressor protein p16 [2, 8, 9, 25], which is usually inactivated in non-HPV-related SCC through either genetic or epigenetic mechanisms [26]. p16 over-expression in HPV-related SCC is strongly associated with better response to therapy and favorable clinical outcomes [27]. When compared to HPV-specific testing in oropharyngeal SCC, p16 expression has consistently shown equal or better patient outcome stratification [18]. Therefore, p16 expression is emerging as a reliable surrogate marker for HPV status of oropharyngeal SCC [2, 19].

In light of the unique biology, pathogenesis, and clinical behavior of HPV-related oropharyngeal SCC, a major focus of current research is to develop specific treatment regimens for these patients. Patients with HPV-related oropharyngeal SCC have very good outcomes whether primary surgical or primary non-surgical treatments are applied, and it is an active debate about which is optimal. Evaluation of HPV status in oropharyngeal SCC, particularly at primary diagnosis, is becoming increasingly important in clinical practice.

While both biopsy and resection specimens have been utilized for p16 IHC in numerous studies and shown to be prognostic, small biopsies are subject to potential sampling

issues. In some cases, it is possible that small biopsies might either yield false negative results for p16 expression, or even, potentially, false positives. While studies utilizing only small biopsy specimens and showing p16 IHC as prognostic are the norm in the literature, they cannot address the possibility that some small number of individual cases may have been misclassified for p16 expression. For example, in large studies of p16 IHC in oropharyngeal SCCs, if we made the assumption that a few percent of cases were misclassified for their p16 expression status, the entire study cohort likely would still show p16 IHC to have a statistically significant correlation with patient survival because p16 IHC is such a strong risk stratifier and prognosis predictor in these tumors. Evaluation of whole tumor sections from resections allows one to evaluate much more tumor tissue so, in theory, should better reflect the p16 expression of the tumors. The purpose of this study was to evaluate the effectiveness of p16 IHC staining of small diagnostic biopsies by comparing results with whole tumor resection specimens.

Materials and Methods

This study was approved by the Human Research Protection Office of Washington University. A 270 patient database of oropharyngeal SCC at Barnes-Jewish Hospital was searched for patients who had both an in-house primary tumor biopsy for diagnosis and then a corresponding surgical resection specimen. This subset was relatively small compared to the overall number of surgically-treated patients in the database. This was because most patients had either biopsy on the outside (then diagnosed at Washington University only in consultation) so that no biopsy tissue block was available for us to study, or patients had the diagnosis of SCC based on fine needle aspiration or open surgical biopsy of cervical nodal metastases.

Since biopsy specimens can vary from miniscule to sizeable, the sizes of these specimens were characterized as follows: The number of tissue pieces on each biopsy section was counted. Each piece in the specimen was measured in two dimensions, and the surface area was calculated. The total surface area of all pieces on a tissue section was then calculated by adding the surface area of all pieces together. The percentage of total surface area occupied by tumor on each tissue section was estimated visually for each biopsy as well.

Both histologic typing and p16 IHC had been previously performed on resection specimens as described [23]. Specifically, the histologic typing was as follows: Type 1 keratinizing SCC, Type 2 nonkeratinizing SCC with maturation, and Type 3 nonkeratinizing SCC. Keratinizing

SCC (Type 1) consists entirely of maturing squamous epithelium with no areas with nonkeratinizing or “basal” morphology (Fig. 1a, b). The cells have polygonal shapes with abundant, eosinophilic (keratinizing) cytoplasm, distinct cell borders, and intercellular bridges. The nests are usually angulated and irregular, and there is frequently marked stromal desmoplasia. Actual keratin formation is common but is not required as long as the cells have the prominent eosinophilic cytoplasm along with the other

features. This cytoplasm is filled with keratin intermediate filaments, so, despite sometimes lacking frank keratin formation, the cells still are “keratinizing.” Nonkeratinizing SCC with maturation (“hybrid SCC” or Type 2) is an intermediate group and consists of definitive areas with nonkeratinizing morphology showing sheets, nests, or trabeculae of oval and frequently spindled, hyperchromatic cells with indistinct cell borders and lacking prominent nucleoli (Fig. 2a, b). They have only modest amounts of

Fig. 1 Histologic features of Type 1 (keratinizing SCC). **a** Low-power image ($\times 100$) showing angulated and irregular nests of tumor cells in a desmoplastic background; **b** High-power image ($\times 200$) showing polygonal shaped tumors cells with abundant, eosinophilic cytoplasm, distinct cell borders, and intercellular bridges. (SCC = squamous cell carcinoma)

Fig. 2 Histologic features of Type 2 (nonkeratinizing SCC with maturation) and Type 3 (nonkeratinizing SCC). **a** Low-power image ($\times 100$) showing Type 2 tumor composed of sheets and nests or trabeculae of tumor cells without prominent stromal reaction; **b** High-power image ($\times 200$) showing oval-to-spindle shaped tumor cells with hyperchromatic nuclei, inconspicuous nucleoli, and indistinct cell borders. Focal squamous maturation is seen in greater than 10% of

tumor surface area; **c** Low-power image ($\times 100$) showing Type 3 tumor composed of nests of tumor cells with pushing borders and comedo necrosis; **d** High-power image ($\times 200$) showing oval-to-spindled tumor cells with hyperchromatic nuclei and indistinct nucleoli. There is no significant squamous maturation. Apoptosis and brisk mitotic activity are present. (SCC = squamous cell carcinoma)

eosinophilic cytoplasm. Comedo-type necrosis and brisk mitotic activity are usually present. There is typically no (or minimal) stromal reaction to the invading tumor. In addition, the tumor also has areas of maturing squamous differentiation comprising greater than 10% of the tumor surface area. Nonkeratinizing squamous cell carcinoma (NK SCC or Type 3) consists almost entirely of nonkeratinizing SCC as described above (Fig. 2c, d). Portions of the tumor can show squamous maturation, but these mature areas must constitute less than 10% of the total surface area. Other rare histologic types such as basaloid, spindle cell, undifferentiated, and adenosquamous carcinoma were present in the larger database and were diagnosed based on their published features. However, none of the 32 specimens in this study were of any of these unique histologic types.

p16 IHC was then performed on the diagnostic biopsy specimens using the identical methods as for the resection specimens in prior studies [19, 23]. Briefly, IHC was performed for p16 on representative 4 μ m sections cut from formalin-fixed, paraffin-embedded tissue blocks using a monoclonal antibody to p16 (MTM Laboratories; monoclonal; 1:1 dilution) on a Ventana Benchmark LT automated immunostainer (Ventana Medical Systems, Inc., Tucson AZ, USA) according to standard protocols. Detection involved Ventana's ultraView Universal DAB Detection Kit which utilizes a cocktail of enzyme labeled secondary antibodies

that locate the bound primary antibody. The complex is then visualized with hydrogen peroxide substrate and a 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. No biotin is involved. Antigen retrieval, standard on the machine, utilized the Ventana CC1, EDTA-Tris, pH 8.0 solution. A known p16 expressing head and neck SCC case was used as the positive control and sections of normal tonsil used for negative controls with each run.

Staining was graded in the same manner on both biopsies and resections. In particular, staining was both nuclear and cytoplasmic (Fig. 3a–d) and was independently evaluated by both study pathologists without knowledge of resection specimen results or clinical information. Cases were graded in a quartile manner for its extent in tumor cells as follows: 0 = negative (no staining in tumor cells); 1+ = 1–25% of tumor cells positive; 2+ = 26–50%; 3+ = 51–75%; 4+ = 76–100%. Discrepant results were resolved by consensus review.

In addition to considering staining by quartile (extent), we also divided the results binarily into extensively positive (3+ or 4+, i.e. >50% of tumor cells positive) and negative or focally positive (0, 1+, or 2+, i.e. negative or <50% of tumor cells positive) based on general head and neck pathology community practices and also on emerging data that suggests that most oropharyngeal SCC with transcriptionally-active HPV will have extensive p16 expression [28].

Fig. 3 Representative examples of the p16 IHC scores in oropharyngeal SCC. **a** 1+: 1–25% of tumor cells positive for p16; **b** 2+: 26–50% of cells positive; **c** 3+: 51–75% of cells positive; **d** 4+: 76–100% of cells positive (IHC = immunohistochemistry; SCC = squamous cell carcinoma)

Table 1 Clinical characteristics of patients with oropharyngeal carcinoma by histologic type

	Type 1 (9)	Type 2 (7)	Type 3 (16)	Total (32)	<i>P</i> value
Age (mean)	50.2	55.7	55.8	54.2	NP
Sex (%)					
Male	8 (88.9)	6 (85.7)	15 (93.8)	29 (90.6)	0.81
Female	1 (11.1)	1 (14.3)	1 (6.2)	3 (9.4)	
Smoker (%)					
Yes	9 (100)	3 (42.9)	10 (62.5)	22 (68.8)	0.06
No	0 (0.0)	3 (42.9)	6 (37.5)	9 (28.1)	
Unknown	0 (0.0)	1 (14.2)	0 (0.0)	1 (3.1)	
Stage (%)					
I	2 (22.2)	0 (0.0)	0 (0.0)	2 (6.3)	0.26
II	0 (0.0)	0 (0.0)	1 (6.2)	1 (3.1)	
III	3 (33.3)	1 (14.3)	4 (25.0)	8 (25.0)	
IVA	3 (33.3)	5 (71.4)	11 (68.8)	19 (59.3)	
IVB	1 (11.1)	1 (14.3)	0 (0.0)	2 (6.3)	
Lymph node metastases (%)					
Yes	4 (44.4)	6 (85.7)	14 (87.5)	24 (75.0)	0.04
No	5 (55.6)	1 (14.3)	2 (12.5)	8 (25.0)	

NP = not performed

Statistical tests were used for categorical data and thus analyzed with contingency tables and Chi-Square analysis utilizing an on-line calculator: <http://www.physic.csbsju.edu/stats/>.

Results

A total of 32 biopsy cases together with accompanying primary resections were captured and studied. The clinical and pathologic characteristics of the patients are provided in Table 1. They included 9 (28%) keratinizing SCC (Type 1), 7 (22%) nonkeratinizing SCC with maturation (Type 2), and 16 (50%) nonkeratinizing SCC (Type 3). Most of the patients were male (91%) and smokers (69%), although fewer smokers were present in patients with either Type 3 (63%) or Type 2 SCC (43%) compared to Type 1 (100%; *P* value = 0.06). Over 90% of patients presented at high stage (stage III and above) and 75% of patients had nodal metastases at presentation. Patients with Type 2 and 3 tumors were more likely to present with nodal metastasis

Table 2 Size and tumor content of the biopsy specimens

	Average	Minimum	Maximum
Number of pieces per specimen	6	2	12
Area for individual pieces of tissue (cm ²)	0.06	0.01	0.52
Total area of entire tissue (cm ²)	0.34	0.07	1.07
Percentage of overall tumor across entire tissue (%)	52	10	100
Total tumor area for entire tissue (cm ²)	0.17	0.014	0.65

(86 and 88%, respectively) than patients with Type 1 tumors (44%). This difference was statistically significant (*P* value = 0.04).

Size analysis and tumor content of the biopsy specimens are summarized in Table 2. Biopsy specimens contained 6 pieces of tissue, on average. The average two-dimensional area per tissue piece was 0.06 cm², and the total area of tissue across all pieces in the specimens ranged from 0.07 to 1.07 cm² (average 0.34 cm²). On average 52% of total tissue area consisted of tumor so the total area of tumor was, on average, 0.17 cm².

p16 IHC results for the diagnostic biopsy specimens are provided in Tables 3 and 4. Most of the Type 1 biopsies (78%) were negative (p16 score = 0, i.e. no tumor cells showed any staining for p16). Of the two Type 1 biopsies (22%) with some p16 positive IHC staining, one was 1+ (1–25% of tumor cells stained positive for p16) and the other was 2+ (26–50% of tumor cells stained positive for p16). In contrast, most of the Type 2 biopsies (71%), and all of the Type 3 (100%) biopsies were 4+ (greater than 75% of tumor cells positive). For the two Type 2 biopsies that were not p16 diffusely positive, one (14%) was 3+ (51–75% tumor cells positive) and the other (14%) was negative. In only two of the 32 (6%) cases did the biopsies and resection specimens differ in staining (no staining versus positive staining), including (1) a biopsy with 1+ staining and corresponding negative resection, and conversely (2) a negative biopsy with resection showing 1+ staining.

p16 results for the biopsies and corresponding resections were correlated in two ways (Table 4). When strictly considering the p16 IHC scores, biopsy-resection correlation was present in 28 of the 32 (85%) cases (Table 4). This

Table 3 p16 staining results by histologic type

p16 IHC	0 (%)	1+ (%)	2+ (%)	3+ (%)	4+ (%)
All biopsies (32)	8 (25.0%)	1 (3.1%)	1 (3.1%)	1 (3.1%)	21 (65.6%)
Type 1 (9)	7 (77.8%)	1 (11.1%)	1 (11.1%)	0 (0%)	0 (0%)
Type 2 (7)	1 (14.3%)	0 (0%)	0 (0%)	1 (14.3%)	5 (71.4%)
Type 3 (16)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	16 (100%)

Table 4 Correlation of p16 IHC scores between biopsies and resections

Histologic type	p16 IHC scores		p16 IHC interpretation (Positive = 3+ or 4+; Negative = 0, 1+ or 2+)	
	Resection	Biopsy	Resection	Biopsy
Type 1 (9)	1+	2+	Negative	Negative
	0	1+	Negative	Negative
	1+	0	Negative	Negative
	0	0	Negative	Negative
	0	0	Negative	Negative
	0	0	Negative	Negative
	0	0	Negative	Negative
	0	0	Negative	Negative
	0	0	Negative	Negative
Type 2 (7)	4+	3+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	0	0	Negative	Negative
Type 3 (16)	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive
	4+	4+	Positive	Positive

Discrepant results

included 6 of 9 (67%) Type 1 cases, 6 of 7 (86%) Type 2, and all 16 (100%) Type 3 cases. For the Type 1 cases, one differed as p16 0 (biopsy) versus 1+ (resection), and one case differed as p16 1+ (biopsy) versus 0 (resection). The third discrepant Type 1 case differed as p16 1+ (biopsy) versus 2+ (resection). The one discrepant Type 2 case differed as p16 3+ (biopsy) versus 4+ (resection). When considering p16 expression binarily as 51% staining or more (3+ or 4+) being positive and lesser amounts (0, 1+, or 2+) being negative, there was perfect biopsy-resection correlation for all 32 cases. With p16 expression (reflected

Table 5 Correlation table for p16 IHC in biopsies and resections

Test outcome p16 staining pattern in biopsies ^a	Gold standard p16 staining pattern in resections	
	Positive (22)	Negative (10)
Positive (22)	22	0
Negative (10)	0	10

With binary p16 results as negative (0, 1+, or 2+) or positive (3+ or 4+); ^a $P < 0.0001$

by p16 IHC score) in resection specimens considered the “gold standard,” p16 IHC on small biopsy specimens was both 100% sensitive and specific (Table 5).

Discussion

HPV-related oropharyngeal SCC is a distinct entity that is associated with good prognosis [14, 17] and caused by infection with high-risk HPV types, predominantly type 16 [4, 5, 29, 30]. HPV status has been shown as an independent prognostic factor for both overall and progression-free survival [17, 18]. Currently, SCC of the oropharynx receives essentially uniform treatment regardless of HPV status. However, targeted therapies and/or modified management tailored to HPV-related SCC are being studied with the goal of reducing the substantial morbidity associated with cancer treatment while maintaining the same excellent patient outcome. In light of this, correct identification and triage of patients with HPV-related SCC at initial workup have become critical in clinical practice.

p16 is a tumor suppressor that functions as a cell cycle checkpoint regulator [8]. p16 gene transcription is regulated by retinoblastoma (Rb) protein, the key regulator of cell cycle G1 checkpoint [8]. HPV genome encodes viral oncoprotein E7, which degrades Rb protein, thus leading to the nuclear and cytoplasmic expression of p16. Overexpression of p16 in HPV-related SCC correlates very well with HPV positivity [6]. The utility of p16 in evaluating HPV status of oropharyngeal SCC is well documented and p16 IHC is becoming an accepted single test for evaluating HPV infection status.

Diagnostic biopsies are frequently performed when patients first present with lesions of the oropharynx. Since the size of the diagnostic biopsies is usually small, it has been somewhat unclear whether or not the p16 IHC results of small diagnostic biopsies would be reliably representative of the expression for the entire primary tumor.

Our results show that p16 IHC staining in diagnostic biopsies perfectly reflects whole tumor staining results. In both biopsy and resection specimens, p16 staining is interpreted by simple visual inspection of the percentage of

positively stained tumor cells. When just quartile staining results are utilized, there is good correlation between p16 score of small diagnostic biopsies and resections (28/32 or 87%). The few discrepant cases were seen predominantly among the keratinizing SCC (Type 1) tumors that had patchy partial staining. The discrepancies do not appear to be related to the small sizes of biopsies. While the overall average tumor containing area on the specimens was 0.14 cm², the three 0 versus 1+ discrepancy cases had 0.22, 0.26, and 0.29 cm² of tumor (more than average amounts) while the one 3+ versus 4+ discrepancy case had 0.11 cm² (close to average amount).

The question of “what is the proper cutoff to establish positive p16 IHC?” is one that is garnering more attention as p16 emerges as a simple and reliable surrogate marker for HPV status [2, 19] and improved outcome in oropharyngeal SCC [23]. Fortunately, approximately 90–95% of oropharyngeal SCC are either completely negative or extensively positive for p16 [23, 31]. Since tumors with transcriptionally active HPV should have large amounts of p16 expression, many in the head and neck community only consider extensive p16 IHC as correlating with presence of biologically relevant HPV and improved prognosis. Their studies have already considered p16 IHC to be positive when extensively expressed (such as >70% of tumor cells) [32, 33]. However, there is very little data in the literature to actually support this position. A recent study by Schlecht et al. [28], which compared RT-PCR for high risk HPV E6 and E7 with p16 IHC, obtained results that do support this notion. Preliminary data from another of our studies also shows that most oropharyngeal SCC that have patchy, non-diffuse p16 expression of less than 50% of the tumor cells (which would be classified as 1+ or 2+ in this study) lack high risk HPV E6 and E7 mRNA by in situ hybridization (data accepted in abstract form but not published).

In summary, our study demonstrates the reliability of p16 IHC on small diagnostic oropharyngeal SCC biopsy specimens in predicting p16 expression status on whole sections of resected tumor. When a binary system with a 50% staining cutoff was used for p16 expression, there were no discrepant cases. As such, this provides further evidence that p16 testing of small biopsy specimens can reliably predict overall tumor status and can be used for treatment decisions in routine clinical practice.

Acknowledgments The authors would like to thank the Anatomic and Molecular Pathology (AMP) Core Lab (specifically, Lab Manager Neha Dahiya, M.D., M.B.A., Senior Research Technician Jianping Li, B.S., Research Lab Supervisor Autumn Watson, B.A., and Research Technician I Vernetta Layton) for their excellent work in preparing the histologic sections and immunohistochemical stains. This study was funded by discretionary research funds of the Department of Pathology and Immunology, Washington University in St. Louis.

References

1. Maier H, Dietz A, Gewelke U, Heller W, Weidauer H. Tobacco and alcohol and the risk of head and neck cancer. *Clin Invest*. 1992;70(3–4):320–7.
2. Adelstein DJ, Ridge JA, Gillison ML, Chaturvedi AK, D'Souza G, Gravitt PE et al. Head and neck squamous cell cancer and the human papillomavirus: summary of a national cancer institute state of the science meeting, November 9–10, 2008, Washington, D.C. *Head & Neck*. 2009;31(11):1393–422.
3. Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus (HPV) in head and neck cancer. *Cancer*. 1997;79(3):595–604.
4. Hammarstedt L, Lindquist D, Dahlstrand H, Romanitan M, Onelöv L, Joneberg J, et al. Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. *Int J Cancer*. 2006;119(11):2620–3.
5. Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E. Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. *Int J Cancer*. 2000;89(3):300–4.
6. Kim S-H, Koo B-S, Kang S, Park K, Kim H, Lee KR, et al. HPV integration begins in the tonsillar crypt and leads to the alteration of p16, EGFR and c-myc during tumor formation. *Int J Cancer*. 2007;120(7):1418–25.
7. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med*. 2007;356(19):1944–56.
8. Leemans CR, Braakhuis BJM, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011; 11(1):9–22.
9. Haffkamp HC, Manni JJ, Haesevoets A, Voogd AC, Schepers M, Bot FJ, et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. *Int J Cancer*. 2008;122(12):2656–64.
10. Applebaum KM, Furniss CS, Zeka A, Posner MR, Smith JF, Bryan J, et al. Lack of Association of Alcohol and Tobacco with HPV16-Associated Head and Neck Cancer. *J Natl Cancer Inst*. 2007;99(23):1801–10.
11. Näsman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer*. 2009;125(2):362–6.
12. Sturgis EM, Cinciripini PM. Trends in head and neck cancer incidence in relation to smoking prevalence. *Cancer*. 2007; 110(7):1429–35.
13. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and-unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol*. 2008;26(4):612–9.
14. Dahlgren L, Dahlstrand H, Lindquist D, Högmö A, Björnestål L, Lindholm J, et al. Human papillomavirus is more common in base of tongue than in mobile tongue cancer and is a favorable prognostic factor in base of tongue cancer patients. *Int J Cancer*. 2004;112(6):1015–9.
15. Hoffmann M, Görögh T, Gottschlich S, Lohrey C, Rittgen W, Ambrosch P, et al. Human papillomaviruses in head and neck cancer: 8 year-survival-analysis of 73 patients. *Cancer Lett*. 2005;218(2):199–206.
16. Ragin CCR, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. *Int J Cancer*. 2007;121(8):1813–20.
17. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, et al. Molecular classification identifies a subset of

- human papillomavirus-associated oropharyngeal cancers with favorable prognosis. *J Clin Oncol*. 2006;24(5):736–47.
18. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363(1):24–35.
 19. 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 Neck Pathol*. 2009;3(3):186–94.
 20. El-Mofty S, Zhang M, Davila R. Histologic identification of human papillomavirus (HPV)-related squamous cell carcinoma in cervical lymph nodes: a reliable predictor of the site of an occult head and neck primary carcinoma. *Head Neck Pathol*. 2008;2(3):163–8.
 21. El-Mofty SK, Patil S. Human papillomavirus (HPV)-related oropharyngeal nonkeratinizing squamous cell carcinoma: characterization of a distinct phenotype. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*. 2006;101(3):339–45.
 22. El-Mofty SK, Lu DW. Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: a distinct clinicopathologic and molecular disease entity. *Am J Surg Pathol*. 2003;27(11):1463–70.
 23. Lewis JS Jr, Thorstad WL, Chernock RD, Haughey BH, Yip JH, Zhang Q, et al. p16 Positive oropharyngeal squamous cell carcinoma: an entity with a favorable prognosis regardless of tumor HPV status. *Am J Surg Pathol*. 2010;34(8):1088–96.
 24. Braakhuis BJM, Snijders PJF, Keune W-JH, Meijer CJLM, Ruijter-Schippers HJ, Leemans CR, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. *J Natl Cancer Inst*. 2004;96(13):998–1006.
 25. Strati K, Pitot HC, Lambert PF. Identification of biomarkers that distinguish human papillomavirus (HPV)-positive versus HPV-negative head and neck cancers in a mouse model. *Proc Natl Acad Sci*. 2006;103(38):14152–7.
 26. Shintani S, Nakahara Y, Mihara M, Ueyama Y, Matsumura T. Inactivation of the p14ARF, p15INK4B and p16INK4A genes is a frequent event in human oral squamous cell carcinomas. *Oral Oncol*. 2001;37(6):498–504.
 27. Fischer CA, Zlobec I, Green E, Probst S, Storck C, Lugli A, et al. Is the improved prognosis of p16 positive oropharyngeal squamous cell carcinoma dependent of the treatment modality? *Int J Cancer*. 2010;126(5):1256–62.
 28. Schlecht NF, Brandwein-Gensler M, Nuovo GJ, Li M, Dunne A, Kawachi N, et al. A comparison of clinically utilized human papillomavirus detection methods in head and neck cancer. *Mod Pathol*. 2011;24(10):1295–305.
 29. Andl T, Kahn T, Pfuhl A, Nicola T, Erber R, Conradt C, et al. Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. *Cancer Res*. 1998;58(1):5–12.
 30. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst*. 2000;92(9):709–20.
 31. Reimers N, Kasper HU, Weissenborn SJ, Stützer H, Preuss SF, Hoffmann TK, et al. Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. *Int J Cancer*. 2007;120(8):1731–8.
 32. Singhi AD, Westra WH. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. *Cancer*. 2010;116(9):2166–73.
 33. Thavaraj S, Stokes A, Guerra E, Bible J, Halligan E, Long A, et al. Evaluation of human papillomavirus testing for squamous cell carcinoma of the tonsil in clinical practice. *J Clin Pathol*. 2011;64(4):308–12.