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Biomarkers of HPV in Head and Neck Squamous Cell Carcinoma

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Abstract

Human papillomavirus (HPV) is an accepted cause of head and neck squamous cell carcinoma (HNSCC), and patients with HPV-associated HNSCC have a favorable prognosis. Currently, there is no general guidance on the most appropriate biomarkers for clinical assessment of HPV in these malignancies. We compared PCR-based and serologic HPV assays, as well as p16 immunohistochemistry, individually and in combination in a single population-based study to assess their associations with overall survival among patients with HNSCC, and thus their potential value as biomarkers. HPV16 serology was determined for 488 patients; immunohistochemical detection of p16 expression in tumors was conducted in a subset of 233 cases, and PCR-based methods to assess the presence of HPV16 DNA in a subset of 179 cases of tumors. Considering each biomarker individually in the subset of patients studied for all endpoints, seropositivity for the E6 and E7 proteins was significantly associated with enhanced all-cause survival in oropharyngeal disease [$HR_{E6/E7+} = 0.1$, 95% confidence interval (CI) = 0.02–0.3]. Neither the presence of HPV16 DNA nor p16 immunostaining was associated with significant enhanced overall survival in oropharyngeal disease ($HR_{DNA} = 0.9$, 95% CI = 0.3–2.9; $HR_{p16} = 0.3$, 95% CI = 0.1–1.1). However, the combination of HPV-positive DNA and E6 or E7 serology was associated with enhanced overall survival in oropharyngeal disease ($HR_{DNA+/E6/E7+} = 0.1$, 95% CI = 0.02–1.0), whereas E6/E7 seronegative patients with evidence of HPV in tumor DNA did not show any evidence of favorable survival ($HR_{DNA+/E6-/E7-} = 3.4$, 95% CI = 0.6–18.1). Furthermore, patients with p16 staining and E6 or E7 seropositivity had favorable survival from oropharyngeal disease ($HR_{p16+/E6/E7+} = 0.1$, 95% CI = 0.02–0.4), whereas patients who were p16 positive and E6/E7 seronegative had significantly increased hazard of all causes of death ($HR_{p16+/E6-/E7-} = 3.1$, 95% CI = 1.2–7.7). A stronger association of HPV presence with prognosis (assessed by all-cause survival) is observed when "HPV-associated" HNSCC is defined using tumor status (HPV DNA status or P16) and HPV E6/E7 serology in combination rather using tumor HPV status alone. *Cancer Res; 72(19); 5004–13. ©2012 AACR.*

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Introduction

HPV16 infection is now a known cause of head and neck squamous cell carcinoma (HNSCC; refs. 1–6), and HPV-positive patients have a favorable prognosis (7–10). However, the ability of clinically available biomarkers to predict prognosis remains uncertain, attributable to both the varied ways of measuring current or past HPV infection and to the paucity of population-based studies of the biomarkers of HPV exposure and infection.

Numerous HPV biomarkers exist, including detection of HPV DNA in tumors and serologic markers indicative of cumulative viral exposure (antibodies to HPV16 L1, the virus' capsid protein) or expressed oncoproteins (antibodies to HPV16 E6 and E7 proteins; refs. 6, 11–14). In addition, p16 overexpression in the tumor has been used as an indirect biomarker of HPV, as expression of the E7 oncoprotein suppresses pRb and increases the level of p16 protein via a negative feedback mechanism (15–17).

HPV is sexually transmitted in the general population, and HPV exposure and infection can occur without development of head and neck disease. Furthermore, carcinogen exposure

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(tobacco and alcohol in this case) is known to induce somatic alterations of the *Rb* gene as part of the genesis of some HNSCCs (16) leading to overexpression of the p16 protein that would be indistinguishable from that arises when HPV16 is present. Finally, the virus can enter cells and not drive transformation, generating an immune response unrelated to the initiation of the cancer (although it is possible that the virus can later contribute to maintenance of a tumor after initiation of the cancer; ref. 18). Although there have been numerous markers (including HPV16 DNA detection in tumors, serologic response to the L1, E6, and E7 proteins, and p16 immunohistochemical staining) used to detect HPV infection, there is a paucity of data comparing them in any one population where there is also data on disease outcome. Thus, choosing which marker or combination of HPV markers is preferred for predicting overall survival among patients with HNSCC remains a compelling clinical problem. We have assessed different measurements of HPV exposure and infection, evaluating different markers [serologic response (L1, E6, E7), presence of HPV DNA in tumor detected using PCR, and p16 immunostaining] to assess their relationships to overall survival in a population-based study of incident HNSCC in the greater Boston area.

Materials and Methods

Study subjects

Study subjects were patients with HNSCC from a population-based study conducted in the greater Boston, Massachusetts area between December 1999 and December 2003 (19). Eligible cases were residents in the study area and 18 years or older with a pathologically confirmed incident cancer identified from clinics at 9 medical facilities in the study area. The state cancer registry was also queried to insure that all eligible cases were identified. Patients with carcinoma *in situ*, lip, salivary gland, or nasopharyngeal cancer, or recurrent HNSCC were excluded. Tumors were classified as oral cavity, oropharynx, or larynx based on recommendations by the American Joint Committee on Cancer (AJCC). According to the AJCC, tumors at the base of the tongue were classified as oropharyngeal and those located at the anterior of the tongue were classified as those of the oral cavity. The pathology reports for subjects with carcinoma of the tongue were reviewed and further classified as located at the anterior or the base of the tongue. This review was conducted blinded to history of alcohol consumption, tobacco use, and HPV16 status. Oral cavity tumors corresponded to ICD-9 codes 143, 144, 145, and, if located at the anterior of the tongue, 141; oropharyngeal tumors corresponded to ICD-9 codes 146, 148, 149, and, if at the base of the tongue, 141; and laryngeal tumors corresponded to ICD-9 code 161. Institutional Review Boards of all the medical facilities and Brown University approved the study, and written informed consent was obtained from all study subjects. A self-administered questionnaire was used to collect information about demographic characteristics and the standard risk factors for HNSCC. All the subject responses were reviewed by study personnel during in-person visits. The calculation of tobacco and alcohol use has been described previously (20). Survival time and vital status were collected for all subjects from the social security death index and from active search of public databases, including telephone confirmation of vital status, retirement records, and work history.

Biospecimen collection and detection of viral DNA

Tumor samples from all incident cases where the tissue remained for evaluation after surgery were microscopically examined, and the sections confirmed to be more than 70% tumor content by the study pathologist were selected for study. Blood was drawn as soon as feasible after diagnosis of incident disease. Serum from venous blood was separated within 12 to 24 hours of blood drawing and stored at $-80°C$. Two laboratories assessed HPV DNA in tumors. Formalin-fixed paraffin-embedded tissue was processed for DNA extraction by proteinase K digestion as described previously (21, 22). The first laboratory used PCR amplification of a specific short fragment of the L1 element and was conducted as previously described (HPV16 DNA SFPCR; ref. 21). The second laboratory used the Multiplex Human Papillomavirus Genotyping (MPG; refs. 23, 24) assay. This assay amplifies HPV DNA by a broad spectrum general primer PCR (BSGP5+/6+, the backward GP6+ primers are biotinylated), and includes β-globin primers for quality control. The biotinylated strand was detected with probes coupled to fluorescence-labeled polystyrene beads (Luminex technology). For each probe, median fluorescence intensity (MFI) values of the reactions with water control PCR products (4–6/each plate) were defined as background. Net MFI values for the samples were computed by subtraction of 1.2 times the median background value. Net MFI values equal to or above 5 MFI (above the mean background plus 3 times the SD) were defined as positive reactions. Rigorous and standard quality analysis/quality check procedures were routinely used in both labs to minimize contamination. Of the 300 samples, 180 (60%) were of sufficient DNA quality for luminex analysis (i.e., are positive either for HPV DNA and/or for β-globin DNA); 120 samples of 300 (40%) were negative for both HPV DNA and for β-globin even after being analyzed with both 10 and 2 μL of DNA PCR input.

Detection of antibodies by cLIA

The detection of HPV16 L1 antibodies via competitive Luminex immunoassay (cLIA) by Merck has been described previously (25). This assay was designed to detect a robust, clinically significant immune response to prior vaccination rather than to detect subtle variation in community acquired infection, as was the case for the assay designed by Pawlita and colleagues described below (26–29).

Detection of antibodies by multiplex serology

To detect antibodies against HPV type 16 L1, E6, and E7 proteins, a glutathione *S*-transferase capture enzyme-linked immunosorbent assay was used in combination with fluorescent bead technology (26–29). MFI values were dichotomized as antibody positive or negative. A standardized cut-off for HPV16 L1 antibodies was defined earlier (30) and applied here (422 MFI). Seropositivity cut-offs for HPV16 early proteins were determined using serum of 117 female, HPV DNA negative, self-reported virgins from a cross-sectional study among Korean students (30). The mean plus 5 SDs was calculated, and the

resulting cut-off was doubled to stringently separate seropositive and seronegative reactions (HPV16 E6, 484 MFI; HPV16 E7, 1096 MFI; refs. 6, 31).

Detection of p16 immunostaining

The immunohistochemical staining for p16 in tumors was conducted using a purified mouse anti-human p16^{INK4} monoclonal antibody kit (clone E6H4, prediluted 1:2; mtm laboratories) with heat-induced antigen retrieval in citrate buffer (pH = 6.0) using pressure cooker pretreatment with positive and negative cell lines (and normal mouse serum) as controls stained in parallel, as described in detail previously (32). The intensity of staining of positive cells was scored by one pathologist blind to patient serology and other data (J.F. Krane).

Statistical analysis

The characteristics were tabulated in frequency and percentage by different HPV markers. κ values describe the concordance of HPV markers. For each patient, the overall survival was measured in days from the date of diagnosis to censoring (the earlier of either the date lost to follow-up, date achieving 5 years of follow-up, or December 31, 2008) or death, whichever occurred first. Survival analysis used the Kaplan–Meier method, and the difference between strata was assessed using log-rank tests. Cox proportional hazards models were conducted to calculate the hazard ratios (HR) and corresponding 95% CIs of events (death) controlling for confounders. Receiver operating characteristic (ROC) curve was plotted and area under the ROC curve (AUC) was calculated using the

method provided by Heagerty and colleagues (33). The significance tests (the DeLong test) comparing 2 ROC curves and confidence intervals (CI) were generated using package "pROC" developed by Robin and colleagues (34). Statistical analyses used SAS9.1 and *R*, and all tests were 2-sided with a 0.05 significance level.

Results

Characteristics of subjects

A total of 844 patients with incident HNSCC were identified as eligible for the study. Of these, 57 patients refused to participate and 44 did not complete their questionnaire, leaving 722 enrolled case subjects and a case participation rate of 86%. Blood samples were obtained from 81% of the case subjects. The presence of HPV16 antibodies was successfully determined for 488 case subjects. Among 488 cases, 179 and 274 samples with available tumor DNA were successfully assayed for HPV viral DNA using the MPG and SFPCR techniques, and 233 samples were assayed for tumor p16 immunostaining. The subsets of cases with available data from each assay were similar to each other and the overall case group (Supplementary Table S1). The 488 HNSCC cases (196 oral cavity cancers, 199 oropharyngeal cancers, 92 laryngeal cancers, and 1 nonspecified) represent the vast majority of the disease occurring in Eastern Massachusetts between 1999 and 2003. The demographic characteristics by HPV16 biomarkers are presented in Table 1. Cases that were HPV16 L1, E6/E7 seropositive detected by MS method and p16 positive tended

Table 1. Characteristics of HNSCC patients by HPV markers, Eastern Massachusetts, 1999–2003

HPV markers	Age Mean ± SD	P	Sex Female	Male	P	Smoking Never	Ever	P	Alcohol < 8 drinks	≥ 8 drinks	P
HPV16 DNA (MPG)		0.74			0.05			0.85			0.64
Negative	59.5 ± 10.7		25 (49.0)	43 (33.6)		18 (39.1)	50 (37.6)		30 (40.0)	38 (36.5)	
Positive	60.1 ± 10.9		26 (51.0)	85 (66.4)		28 (60.9)	83 (62.4)		45 (60.0)	66 (63.5)	
HPV16 DNA (SFPCR)		0.60			0.68			0.21			0.13
Negative	60.4 ± 10.8		49 (70.0)	148 (72.6)		33 (64.7)	164 (73.5)		70 (66.7)	127 (75.2)	
Positive	59.7 ± 11.7		21 (30.0)	56 (27.5)		18 (35.3)	59 (26.5)		35 (33.3)	42 (24.9)	
HPV16 L1 (MS)		0.02			0.14			<0.01			<0.01
Negative	60.1 ± 11.5		108 (82.4)	272 (76.2)		55 (59.8)	325 (82.1)		133 (71.1)	247 (82.1)	
Positive	57.3 ± 10.2		23 (17.6)	85 (23.8)		37 (40.2)	71 (17.9)		54 (28.9)	54 (17.9)	
HPV16 L1 (cLIA)		0.28			0.10			0.02			0.07
Negative	59.9 ± 11.7		97 (74.1)	234 (66.1)		53 (58.2)	278 (70.6)		118 (63.4)	213 (71.2)	
Positive	58.7 ± 10.4		34 (26.0)	120 (33.9)		38 (41.8)	116 (29.4)		68 (36.6)	86 (28.8)	
HPV16 E6/E7		0.08			<0.01			<0.01			0.01
Negative	60.0 ± 11.8		111 (84.7)	247 (69.2)		51 (55.4)	307 (77.5)		124 (66.3)	234 (77.7)	
Positive	58.2 ± 9.6		20 (15.3)	110 (30.8)		41 (44.6)	89 (22.5)		63 (33.7)	67 (22.2)	
P16		0.01			0.01			<0.01			0.01
Negative	60.3 ± 12.3		59 (88.1)	120 (72.3)		28 (60.9)	151 (80.8)		63 (68.5)	116 (82.3)	
Positive	56.4 ± 9.2		8 (11.9)	46 (27.7)		18 (39.1)	36 (19.2)		29 (31.5)	25 (17.7)	

NOTE: the sample size for each assay was different (179 for DNA (MPG), 274 for DNA (SFPCR), 488 for HPV16 L1, E6 and E7 using multiplex serology detecting method, 485 for L1 (cLIA) and 233 for P16.

to be younger and more likely to be never smokers and light drinkers (less than 8 drinks/week) compared with those who were HPV seronegative, patterns that were not apparent in patients positive for HPV DNA.

Concordance between HPV16 biomarkers

In tumor tissues, the overall prevalence of HPV16 DNA by the MPG method was 62% (111/179), whereas the prevalence of HPV16 DNA (SFPCR) was 28%. The concordance between HPV16 DNA (SFPCR) and HPV16 DNA (MPG) was 53.2% with an κ equivalent to 0.2 (Supplementary Table S2). The overall concordance between HPV16 L1 serologic methods was 83.7% with a κ value equal to 0.6 (data not shown). The concordance between p16 immunohistochemistry and HPV16 DNA was low (κ = 0.1–0.3) but higher with serologic markers (κ = 0.5–0.7; Supplementary Table S2). The concordance of HPV16 serologic markers, p16 immunostaining, and their combinations with measures of HPV16 DNA is shown in Table 2. The overall concordance was comparable among all pairs of individual markers or marker combinations, but concordance adjusted by chance was fairly low. For example, HPV16 E6 seropositive cases were identified in 47% of HPV16 DNA (MPG) positive cases and in 7% of the DNA-negative cases. Slightly lower prevalences of seropositive HPV16 E7 were observed in HPV16 DNA (MPG)

positive (36%) and negative cases (6%). Although the combination of 2 or 3 markers increased the κ values, this increase was modest.

Overall survival of HNSCC by HPV16 markers

Kaplan–Meier survival analyses showed that cases seropositive for L1 and E6/E7 showed better overall survival compared with seronegative cases (Fig. 1A and B), whereas HPV16 DNA (MPG) was not significantly associated with overall survival (Fig. 1C). Comparing ROC curves for E7 (AUC = 0.64, 0.57–0.68) and HPV DNA (AUC = 0.57, 0.48–0.65) showed serology was a superior model of clinical outcome ($P = 0.024$, DeLong test; Supplementary Fig. S1). Positive p16 immunostaining alone also was not associated with improved survival in Kaplan–Meier analysis (Fig. 1D). When we combined HPV16 DNA (MPG) status with E6 or E7 serology, patients with HPV16 DNA+/E6/E7+ had the most favorable overall survival, whereas patients with DNA-/E6/E7- and DNA+/E6/E7- had the worse overall survival with no substantial difference between these latter 2 strata (Fig. 2A). When we combined p16 immunostaining and E6/E7 serology measures (Fig. 2B), patients with both positive p16 immunostaining and positive E6/E7 serology had the most favorable survival overall, followed by biomarkers indicating p16-/E6/E7+ and p16-/E6/E7-. Patients with p16+/E6/E7- had the worst

Table 2. Concordance of HPV16 DNA (MPG) and HPV16 markers, Eastern Massachusetts, 1999–2003

| HPV Markers	Status	HPV16 DNA (MPG) Negative n (%)	HPV16 DNA (MPG) Positive n (%)	Kappa
HPV16 DNA (SFPCR)				0.18
	Negative	37 (77.1)	50 (55.6)	
	Positive	11 (22.9)	40 (44.4)	
HPV16 L1 (MS)				0.25
	Negative	61 (88.4)	66 (59.5)	
	Positive	8 (11.6)	45 (40.5)	
HPV16 L1 (cLIA)				0.28
	Negative	58 (85.3)	59 (53.1)	
	Positive	10 (14.7)	52 (46.9)	
HPV16 E6				0.34
	Negative	64 (92.8)	59 (53.2)	
	Positive	5 (7.3)	52 (46.9)	
HPV16 E7				0.26
	Negative	65 (94.2)	71 (64.0)	
	Positive	4 (5.8)	40 (36.0)	
HPV16 E6/E7				0.36
	Negative	63 (91.3)	56 (50.5)	
	Either positive	6 (8.7)	55 (49.6)	
P16				0.27
	Negative	43 (86.0)	40 (56.3)	
	Positive	7 (14.0)	31 (43.7)	
HPV16 E6/E7/P16				0.34
	Negative	43 (86.0)	35 (49.3)	
	Either positive	7 (14.0)	36 (50.7)	
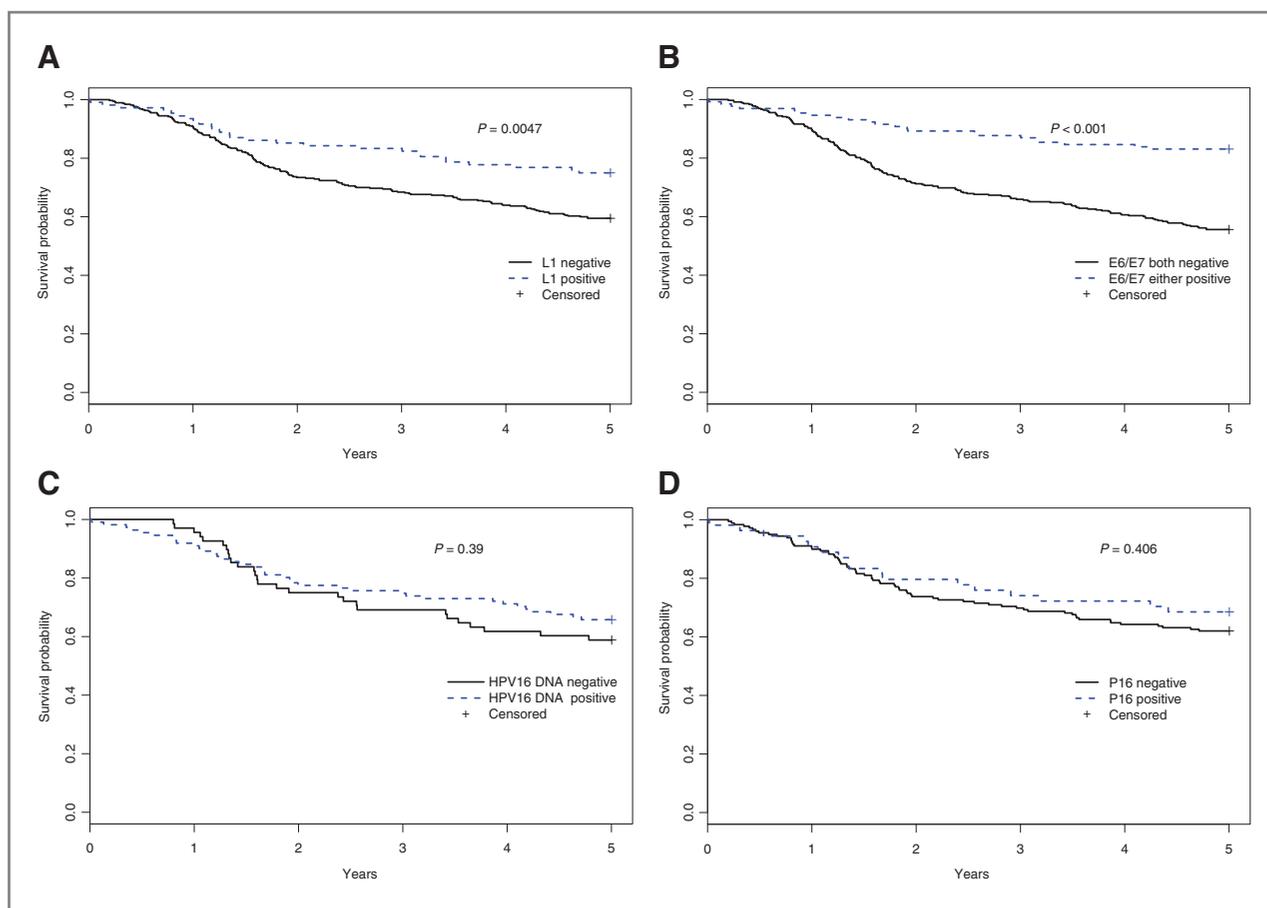
Figure 1. Kaplan–Meier estimates and log-rank tests for overall survival. A, HPV16 L1 (MS) serology status, events/subjects = 154/380 for seronegative, and events/subjects = 27/108 for seropositive, $P = 0.0047$. B, HPV16 E6/E7 serology status, events/subjects = 159/358 for both seronegative and events/subjects = 22/130 for either seropositive, $P < 0.001$. C, HPV16 DNA (MPG) status, events/subjects = 28/68 for negative, and events/subjects = 38/111 for positive, $P = 0.39$. D, P16-staining status, events/subjects = 68/179 for negative, and events/subjects = 17/54 for positive, $P = 0.406$.

survival and this was consistent across anatomic sites. Supplementary Table S3 presents the HRs for these comparisons by anatomic site.

Table 3 presents the adjusted HRs evaluating the associations between HPV16 DNA biomarkers, HPV16 serologic markers, p16 immunostaining, and 5-year overall survival restricted to the 94 participants with data for each biomarker. Detection of HPV DNA in the tumor using the multiplex genotyping assay was not significantly associated with overall mortality ($HR_{MPG} = 1.0$, 95% CI = 0.5–2.0), whereas using the short fragment PCR to interrogate the tumor DNA for the presence of the virus was associated with a 30% nonsignificant reduction of overall mortality ($HR_{SFPCR} = 0.7$, 95% CI = 0.3–1.5). This result did not differ when different thresholds were applied to the positive cutoff for the HPV DNA MPG assay (Supplementary Table S4). A decreased hazard of overall death was observed for all HPV16 serologic markers: a 40% and 20% nonsignificant reduction of overall mortality was detected for HPV16 L1 seropositivity using multiplex serology ($HR_{MS} = 0.6$, 95% CI = 0.2–1.5) and cLIA ($HR_{cLIA} = 0.8$, 95% CI = 0.4–1.6), respectively. There were significant 80% reductions in overall mortality in patients seropositive for E6 or E7 compared with

E6 or E7 seronegativity (both HRs = 0.2, 95% CI = 0.1–0.6 for E6 and 0.1–0.7 for E7). These associations were unchanged when either early proteins were present ($HR_{E6+/E7+} = 0.2$, 95% CI = 0.1–0.6). Combining the serologic markers and HPV16 DNA biomarkers detection revealed that patients with DNA+/E6/E7+ had better survival than patients who were/DNA- E6/E7- (HR = 0.3, 95% CI = 0.1–0.9). The results of the similar analysis for SFPCR were almost identical (data not shown). In these patients ($N = 94$ with complete data), no significant association with survival was observed for p16-positive immunostaining ($HR_{p16+} = 0.6$, 95% CI = 0.3–1.5; Table 3). However, cases that were p16 positive and seropositive for E6/E7 had significantly favorable survival ($HR_{p16+/E6/E7+} = 0.3$, 95% CI = 0.1–0.8). Interestingly, in all cases, the point estimates for the HRs were smaller when restricted to the oropharynx, although not all were significant as the sample size was diminished (Table 3).

Supplementary Table S5 shows these comparisons unrestricted to those with all biomarkers conducted; the associations are consistent with those presented in Table 3. Among those who had PCR HPV DNA data and serology data ($N = 488$), being seropositive for either early protein or positive for tumor

Figure 2. A, Kaplan–Meier estimates and log-rank tests for overall survival for the combination of HPV16 DNA (MPG) and E6/E7, events/subjects = 26/62 for DNA-/E6/E7-, events/subjects = 2/6 for DNA-/E6/E7+; events/subjects = 30/56 for DNA+/E6/E7-, and events/subjects = 8/55 for DNA+/E6/E7+, $P < 0.001$; E6/E7- means both E6 and E7 seronegativity; E6/E7+ means either E6 or E7 seropositivity. B, Kaplan–Meier estimates and log-rank tests for overall survival for the combination of p16 staining and E6/E7, events/subjects = 65/170 for p16-/E6/E7-, events/subjects = 3/9 for p16-/E6/E7+; events/subjects = 11/17 for p16+/E6/E7-, and events/subjects = 6/37 for p16+/E6.E7+, $P = 0.004$; E6/E7- means both E6 and E7 seronegativity; E6/E7+ means either E6 or E7 seropositivity.
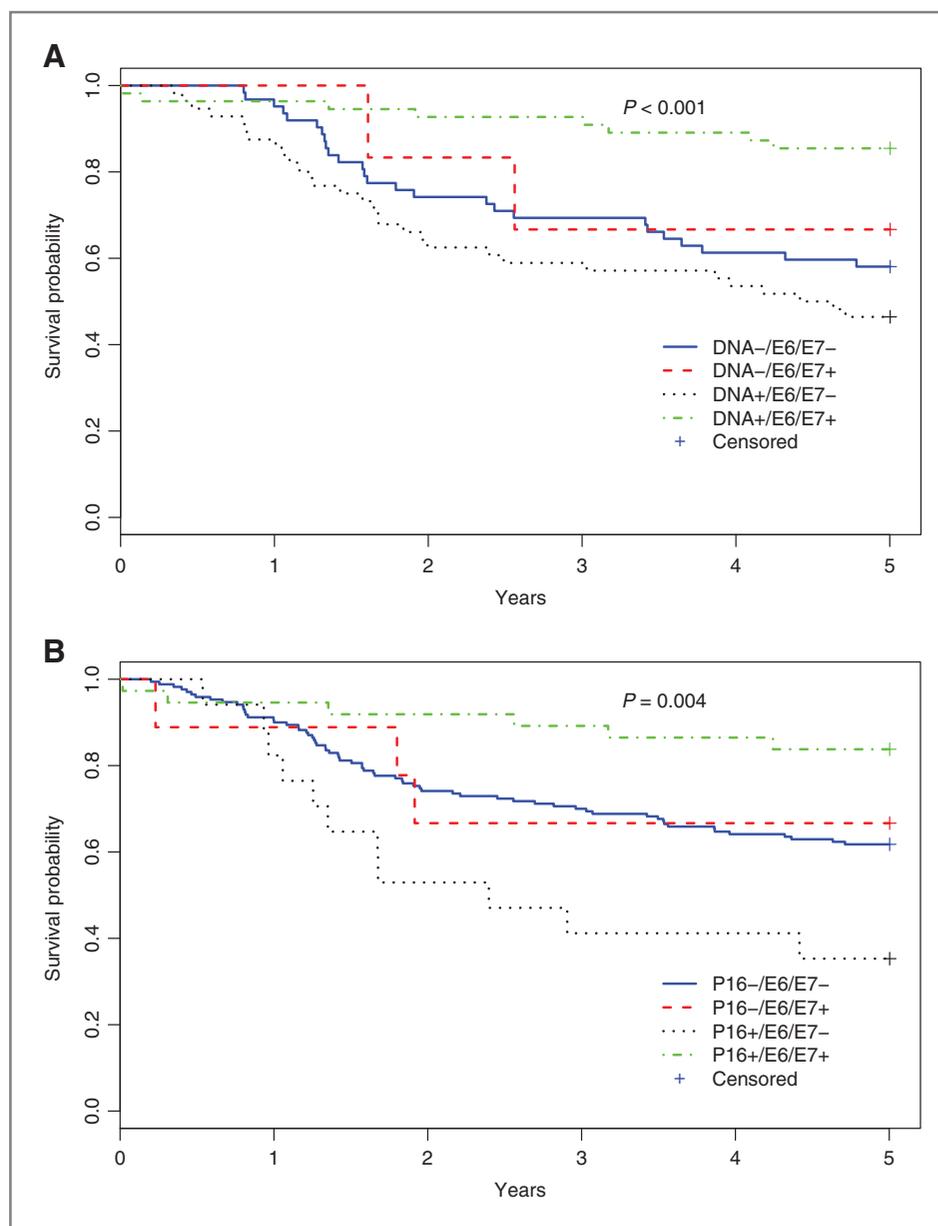
DNA consistently indicated a reduced mortality, as indicated in Supplementary Table S5 ($HR_{E6+/DNA+}$ = 0.3, 95% CI = 0.1–0.6; $HR_{E7/DNA+}$ = 0.2, 95% CI = 0.1–0.6). Supplementary Table S6 also indicates that in this same larger population, among E6 and E7 seronegative patients, individuals whose tumors were positive for HPV DNA–positive did not have different overall survival from patients whose tumors were HPV DNA–negative ($HR_{E6-/E7-/DNA+}$ = 1.2, 95%CI = 0.7–2.1). P16−/E6+ was not associated with an enhanced survival (HR = 0.5, 0.1–2.2). In contrast, an increased hazard of death was observed for those patients with p16+/E6- ($HR_{p16+/E6-}$ = 2.0, 95% CI = 1.0–3.9; data not shown). This, again, almost exactly paralleled the E7 data. When compared with p16-/E6-/E7-, patients with p16+/E6-/E7- had an increased hazard of death ($HR_{p16+/E6-/E7-}$ = 2.1, 95% CI = 1.1–4.2), whereas patients with p16+/E6+/E7+

had a reduced hazard of death ($HR_{p16+/E6+/E7+}$ = 0.3, 95% CI = 0.1–0.9; Supplementary Table S5).

Discussion

In this well-defined, large population with incident HNSCC, we assessed the associations between biomarkers of HPV16 and overall survival of patients with HNSCC. In agreement with previous findings (35, 36), our data show that all serologic biomarkers that we examined were associated with improved overall survival and this improvement is particularly enhanced for oropharyngeal cancer (9). In our study, the HPV16 E6 and E7 serology markers were superior to all other biomarkers that we examined, taken alone, in predicting the prognosis of HNSCC. Importantly, we

Table 3. Overall survival among HNSCC patients by HPV markers, overall and by oropharynx, Eastern Massachusetts, 1999–2003[a]

HPV16 markers	Overall (n = 94)		Oropharynx (n = 45)	
	Death/cases	HR (95% CI)	Death/cases	HR (95% CI)
HPV16 DNA (MPG)				
Negative	13/35	ref.	5/15	ref.
Positive	23/59	1.0 (0.5–2.0)	10/30	0.9 (0.3–2.9)
HPV16 DNA (SFPCR)				
Negative	28/65	ref.	12/30	ref.
Positive	8/29	0.7 (0.3–1.5)	3/15	0.4 (0.1–1.6)
HPV16 L1 (MS)				
Negative	29/67	ref.	9/23	ref.
Positive	7/27	0.6 (0.2–1.5)	6/22	0.5 (0.2–1.6)
HPV16 L1 (cLIA)				
Negative	24/59	ref.	9/20	ref.
Positive	12/35	0.8 (0.4–1.6)	6/25	0.3 (0.1–1.0)
HPV16 E6/E7				
Negative	31/64	ref.	11/19	ref.
Either Positive	5/30	0.2 (0.1–0.6)	4/26	0.1 (0.02–0.3)
P16 IHC				
Negative	25/60	ref.	8/18	ref.
Positive	11/34	0.6 (0.3–1.5)	7/27	0.3 (0.1–1.1)
P16/E6/E7[†]				
P16-/E6/E7-	24/56	ref.	8/15	ref.
P16-/E6/E7+	1/4	0.4 (0.05–2.9)	0/3	-
P16+/E6/E7-	7/8	3.1 (1.2–7.7)	3/4	2.0 (0.4–10.0)
P16+/E6/E7+	4/26	0.3 (0.1–0.8)	4/23	0.1 (0.02–0.4)
HPV16 DNA (MPG)/E6/E7[b]				
DNA-/E6/E7-	12/32	ref.	4/12	ref.
DNA-/E6/E7+	1/3	0.4 (0.05–3.2)	1/3	0.3 (0.02–3.4)
DNA+/E6/E7-	19/32	1.5 (0.7–3.3)	7/7	3.4 (0.6–18.1)
DNA+/E6/E7+	4/27	0.3 (0.1–0.9)	3/23	0.1 (0.02–1.0)

Abbreviation: ref., group is referent for computing the hazard ratio (HR).
[a]Analysis restricted to participants for whom data on each marker was available.
[b]E6/E7- indicates both E6 and E7 seronegativity; E6/E7+ means either E6 or E7 seropositivity.

observed that p16 overexpression by immunohistochemical staining or HPV16 DNA by PCR, as individual biomarkers, are not informative for predicting HNSCC prognosis in this population-based sample. Patients whose tumors were HPV16 DNA–positive but E6 and/or E7 seronegative did not have any reduction in overall mortality, whereas patients with E6 and/or E7 seropositive showed a substantial and significant reduction in overall mortality regardless of DNA status. P16 overexpression was only informative when serologic status for E6 and E7 was known. Importantly, E6 and/or E7 seropositive patients without p16 overexpression did not have enhanced survival. In contrast, the E6 and E7 seronegative patients with p16 overexpression were at a significantly increased hazard of death. This striking relationship is consistent with the known multiple mechanisms of inactivation of the retinoblastoma pathway, including the action of HPV16 or deletion, methylation, or mutation of *Rb* or other genes in this pathway.

It has been suggested that the favorable prognosis associated with HPV is specific to DNA-positive HNSCC patients with transcriptionally active HPV genomes (37). Our finding of the clear importance of the combination of HPV16 DNA and seropositivity to early proteins for predicting survival strongly supports that view and thus the need to choose particular biomarkers that will be clinically useful. Our results also quite strongly suggest that the mere presence of HPV16 DNA in tumors (by PCR) is not an informative biomarker for HPV16 in predicting overall survival. Consistent with prior studies (38–41), our data indicated patients with HPV16 serologic positivity tended to be younger, male, nonsmokers, and nondrinkers or light drinkers. However, when we include HPV DNA status with E6/E7 serologic status, we found that patients with tumor DNA–positive, but E6/E7 seronegative tended to be older (62.1 vs. 57.8), females (33.9% vs. 11.5%), smokers (78.0 vs. 71.2%), and heavy drinkers (64.4% vs. 53.9%) compared with DNA-positive and E6/E7 seropositive. Thus, our finding supports the

hypothesis that cases whose tumors are HPV DNA positive, but without evidence of transcriptional activity (as evidenced by a lack of serologic response to the HPV early genes), are tumors that are not driven by HPV16.

Given our higher prevalence of HPV16 DNA positivity compared with other studies (40, 42), we assessed the impact of assay threshold on survival by increasing the cutoff of MFI level (see Supplementary Table S4). There was no difference in the overall survival across different thresholds of DNA positivity (by the MPG method), although a slightly enhanced survival was observed in the cases of orophryngeal cancer when the cut-off was set to 700 MFI (30% of cases DNA positive). The prevalence of HPV16 DNA (MPG)–positive oropharyngeal cancer (72%) and the percentage of E6/E7 seropositivity among DNA-positive oropharyngeal cases (71%) in our study are similar to those of D'Souza and colleagues (43) in that HPV16 DNA was detected in 72% of tumor specimens and 64% were seropositive for HPV16 E6/E7. Smith and colleagues (35) reported anti-E6 and/or E7 antibodies detectable in 75% of tumor HPV-16–positive cases and 5% of HPV-16–negative cases. We found E6/E7 seropositivity in 50% of HPV-16 DNA–positive cases and 9% of negative cases. Smith and colleagues also compared the HPV serology assays with tumor tissue findings in association with survival, indicating that E6/E7 seropositive cases have similar prognostic advantage as that based on tumor DNA status. This discrepancy is probably related to the concordance between HPV16 DNA and E6/E7 serology, which was 0.74 in their study and 0.36 in our study. This differing concordance can result from populations with different prevalence of HPV infection [our sample was population-based and Smith and colleagues (35) used a hospital-based design] or potentially with distinct immunologic status, as both studies used the same methods to measure serology and HPV DNA in the tumor.

Conflicting results exist regarding the prognostic significance of immunostaining for p16. P16 overexpression has been associated with improved outcome in head and neck cancer (17, 38, 44), whereas other investigators have reported no association (44–47). Few studies have directly compared the prognostic significance in subgroups of HNSCC by p16 and HPV infection. Smith and colleagues (45) reported a worse overall survival (HR = 4.1, 1.7–9.9) and disease-specific survival (HR = 4.0, 1.5–10.7) for the group of p16-/p53+/HPV- compared with p16+/p53−/HPV+. However, we found that patients with p16+/E6-/E7- had an increased hazard of death, whereas Smith and colleagues (48) reported no association with survival. Our data are consistent with that of Weinberger and colleagues (46) who reported that tumors that were HPV DNA–negative with low p16 protein expression had a poor 5-year survival of 20%. Similar results were observed in tumors that were HPV DNA–positive with low p16 protein expression (46).

A major strength of this study is the fact that, in a single, population-based study, we examined the concordance between tumor DNA and serologic markers, compared the prognostic findings among the markers, and investigated their joint effects in predicting the overall survival of HNSCC cases. We were able to detect tumor DNA and L1 serology using different detection methods to determine whether the discrepancy of findings is due to the difference in detection methods. The results show remarkable consistency regardless of detection methods used. Because a serologic assay is not site-specific, it can be argued that infections outside the head and neck may influence the estimates (albeit, this is a bias to the null). Also, not all individuals exposed to HPV seroconvert or maintain detectable antibody levels over time (49). Serum antibodies elicited by distant, past infection may wane over time (and the antibody response for different proteins may wane in a different fashion) leading to potential exposure misclassification. To examine the expression levels of E6/E7 oncogenes, mRNA should be considered as a potential biomarker. Furthermore, tumor DNA and p16 immunostaining were only measured in a subset of the entire sample. However, this subset was comparable with the entire sample in demographics and clinical characteristics (Supplementary Table S1) and the HR estimates were similar when restricting to subjects with complete data (Table 3 and Supplementary Table S5). In addition, we were not able to adjust for tumor stage for the estimates presented in Table 3 given the paucity of available data for tumor stage. However, for each marker, hazard estimates were similar when comparing models adjusted for tumor stage with those that were not (Supplementary Table S6). Furthermore, we were not able to evaluate disease-specific survival because these data were not collected. It is possible that overall survival is subject to competing risk, especially tobacco use. Yet, we expect that this bias is small and nondifferential with respect to HPV status and potentially results in a reduction of the observed estimates, as tobacco use has been shown to be independent of HPV in its association with outcome (20, 39, 50). Last but not the least, the conclusions we draw were based on our data that were analyzed using specific detection methods. In particular, HPV DNA assays were PCR-based. Our results may not be generalized to the studies that used other detection methods (e.g., in situ hybridization, viral load) to assess HPV biomarkers.

In conclusion, our study strongly suggests that the combination of detection of HPV16 DNA in HNSCC tumors or p16 immunostaining with E6/E7 antibodies represents the most clinically valuable surrogate markers for the identification of patients with HNSCC who have a better prognosis.

Disclosure of Potential Conflict of Interest

Authors' Contributions

Conception and design: C.J. Marsit, H.H. Nelson, R.I. Haddad, J.R. Clark, M. Pawlita, J.F. Krane
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Marsit, M.D. McClean, B.C. Christensen, R.I. Haddad, J.R. Clark, R.O. Wein, G.A. Grillone, G. Halec, T. Waterboer, M. Pawlita, J.F. Krane, K.T. Kelsey
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Liang, C.J. Marsit, H.H. Nelson, B.C. Christensen, R.I. Haddad, E.A. Houseman, G. Halec, M. Pawlita, J.F. Krane

References

1. Pintos J, Black MJ, Sadeghi N, Ghadirian P, Zeitouni AG, Viscidi RP, et al. Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. Oral Oncol 2008;44:242–50.
2. Smith EM, Ritchie JM, Summersgill KF, Klussmann JP, Lee JH, Wang D, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. Int J Cancer 2004;108:766–72.
3. Zumbach K, Hoffmann M, Kahn T, Bosch F, Gottschlich S, Görögh T, et al. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head-and-neck squamous-cell carcinoma. Int J Cancer 2000;85:815–8.
4. Smith EM, Ritchie JM, Pawlita M, Rubenstein LM, Haugen TH, Turek LP, et al. Human papillomavirus seropositivity and risks of head and neck cancer. Int J Cancer 2007;120:825–32.
5. Santin AD, Hermonat PL, Ravaggi A, Chiriva-Internati M, Pecorelli S, Parham GP, et al. Radiation-enhanced expression of E6/E7 transforming oncogenes of human papillomavirus-16 in human cervical carcinoma. Cancer 1998;83:2346–52.
6. Herrero R, Castellsagué X, Pawlita M, Lissowska J, Kee F, Balaram P, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J Natl Cancer Inst 2003;95:1772–83.
7. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008;100:261–9.
8. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer 2001;92:805–13.
9. Ragin CC, 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;121:1813–20.
10. Sedaghat AR, Zhang Z, Begum S, Palermo R, Best S, Ulmer KM, et al. Prognostic significance of human papillomavirus in oropharyngeal squamous cell carcinomas. Laryngoscope 2009;119:1542–9.
11. Syrjänen S. HPV infections and tonsillar carcinoma. J Clin Pathol 2004;57:449–55.
12. Sun Y, Eluf-Neto J, Bosch FX, Muñoz N, Walboomers JM, Meijer CJ, et al. Serum antibodies to human papillomavirus 16 proteins in women from Brazil with invasive cervical carcinoma. Cancer Epidemiol Biomarkers Prev 1999;8:935–40.
13. Olsen AO, Dillner J, Gjøen K, Magnus P. Seropositivity against HPV 16 capsids: a better marker of past sexual behaviour than presence of HPV DNA. Genitourin Med 1997;73:131–5.
14. Ho GY, Studentsov YY, Bierman R, Burk RD. Natural history of human papillomavirus type 16 virus-like particle antibodies in young women. Cancer Epidemiol Biomarkers Prev 2004;13:110–6.
15. Wittekindt C, Gültekin E, Weissenborn SJ, Dienes HP, Pfister HJ, Klussmann JP. Expression of p16 protein is associated with human papillomavirus status in tonsillar carcinomas and has implications on survival. Adv Otorhinolaryngol 2005;62:72–80.
16. Singhi AD, Westra WH. Comparison of human papillomavirus *in situ* hybridization and p16 immunohistochemistry in the detec-

tion of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer 2005;116:2166–73.
17. 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;120:1731–8.
18. Feller L, Khammissa RA, Wood NH, Lemmer J. Epithelial maturation and molecular biology of oral HPV. Infect Agent Cancer 2009;4:16.
19. Liang C, McClean MD, Marsit C, Christensen B, Peters E, Nelson HH, et al. A population-based case-control study of marijuana use and head and neck squamous cell carcinoma. Cancer Prev Res (Phila) 2009;2:759–68.
20. 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;99:1801–10.
21. Furniss CS, McClean MD, Smith JF, Bryan J, Nelson HH, Peters ES, et al. Human papillomavirus 16 and head and neck squamous cell carcinoma. Int J Cancer 2007;120:2386–92.
22. de Koning MN, Quint WG, Pirog EC. Pirog, Prevalence of mucosal and cutaneous human papillomaviruses in different histologic subtypes of vulvar carcinoma. Mod Pathol 2008;21:334–44.
23. Schmitt M, Dondog B, Waterboer T, Pawlita M. Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. J Clin Microbiol 2008;46:1050–9.
24. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T, et al. Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol 2006;44:504–12.
25. Dias D, Van Doren J, Schlottmann S, Kelly S, Puchalski D, Ruiz W, et al. Optimization and validation of a multiplexed luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16, and 18. Clin Diagn Lab Immunol 2005;12:959–69.
26. Sehr P, Müller M, Höpfl R, Widschwendter A, Pawlita M. HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. J Virol Methods 2002;106:61–70.
27. Sehr P, Zumbach K, Pawlita M. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. J Immunol Methods 2001;253:153–62.
28. Waterboer T, Sehr P, Michael KM, Franceschi S, Nieland JD, Joos TO, et al. Multiplex human papillomavirus serology based on *in situ*-purified glutathione s-transferase fusion proteins. Clin Chem 2005;51:1845–53.
29. Waterboer T, Sehr P, Pawlita M. Pawlita, Suppression of non-specific binding in serological Luminex assays. J Immunol Methods 2006;309:200–4.
30. Clifford GM, Shin HR, Oh JK, Waterboer T, Ju YH, Vaccarella S, et al. Serologic response to oncogenic human papillomavirus types in male and female university students in Busan, South Korea. Cancer Epidemiol Biomarkers Prev 2007;16:1874–9.
31. Herrero R. Chapter 7: Human papillomavirus and cancer of the upper aerodigestive tract. J Natl Cancer Inst Monogr 2003;47–51.
32. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and

neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001;92: 276–84.

33. Heagerty PJ, Lumley T, Pepe MS. Time-dependent ROC curves for censored survival data and a diagnostic marker. Biometrics 2000; 56:337–44.

34. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.

35. Smith EM, Rubenstein LM, Ritchie JM, Lee JH, Haugen TH, Hamsikova E, et al. Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers? Cancer Epidemiol Biomarkers Prev 2008;17:2087–96.

36. Rubenstein LM, Smith EM, Pawlita M, Haugen TH, Hamšíková E, Turek LP. Human papillomavirus serologic follow-up response and relationship to survival in head and neck cancer: a case-comparison study. Infect Agent Cancer 2011;6:9.

37. Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 2007;121:2465–72.

38. 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;363:24–35.

39. Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 2008;100:407–20.

40. Ringström E, Peters E, Hasegawa M, Posner M, Liu M, Kelsey KT, et al. Human papillomavirus type 16 and squamous cell carcinoma of the head and neck. Clin Cancer Res 2002;8:3187–92.

41. Smith EM, Pawlita M, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer. Int J Cancer 2009;127:111–7.

42. Sethi S, Ali-Fehmi R, Franceschi S, Struijk L, van Doorn LJ, Quint W, et al. Characteristics and survival of head and neck cancer by HPV status: a cancer registry-based study. Int J Cancer 2012;131: 1179–86.

43. 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;356:1944–56.

44. Geisler SA, Olshan AF, Weissler MC, Cai J, Funkhouser WK, Smith J, et al. p16 and p53 Protein expression as prognostic indicators of survival and disease recurrence from head and neck cancer. Clin Cancer Res 2002;8:3445–53.

45. Smith EM, Rubenstein LM, Hoffman H, Haugen TH, Turek LP. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. Infect Agent Cancer 2010;5:4.

46. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Sasaki C, et al. Prognostic significance of p16 protein levels in oropharyngeal squamous cell cancer. Clin Cancer Res 2004;10: 5684–91.

47. Smith IM, Mithani SK, Mydlarz WK, Chang SS, Califano JA. Inactivation of the tumor suppressor genes causing the hereditary syndromes predisposing to head and neck cancer via promoter hypermethylation in sporadic head and neck cancers. ORL J Otorhinolaryngol Relat Spec 2010;72:44–50.

48. Smith EM, Rubenstein LM, Hoffman H, Haugen TH, Turek LP. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. Infect Agent Cancer 5:4.

49. Carter JJ, Koutsky LA, Wipf GC, Christensen ND, Lee SK, Kuypers J, et al. The natural history of human papillomavirus type 16 capsid antibodies among a cohort of university women. J Infect Dis 1996;174: 927–36.

50. Smith EM, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Tobacco and alcohol use increases the risk of both HPV-associated and HPV-independent head and neck cancers. Cancer Causes Control 2010;21:1369–78.

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