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Association of *p73* G4C14-to-A4T14 Polymorphism with HPV16 Status in Squamous Cell Carcinoma of the Head and Neck in non-Hispanic Whites

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Abstract

Background—*p73* interacts with the HPV16 oncoproteins E6 and E7, and *p73* variation may modify the interaction between *p73* protein and HPV16 oncogenic proteins and contribute to cellular malignant transformation.

Methods—In this case-case comparison study, we analyzed HPV16 status in tumor specimens and genotyped the *p73* G4C14-to-A4T14 polymorphism using genomic DNA from blood of 202 non-Hispanic white patients with squamous cell carcinoma of the Head and Neck (SCCHN). We calculated odds ratio (OR) and 95% confidence intervals (CIs) in univariate and multivariable logistic regression models to examine the association between the *p73* polymorphism and HPV16 status in SCCHN.

Results—Compared with the *p73* GC/GC genotype, the AT/AT and combined GC/AT + AT/AT variant genotypes were significantly associated with tumor HPV16 positivity among SCCHN patients (adjusted OR = 5.32; 95% CI, 1.32-21.4 and adjusted OR = 1.91; 95% CI, 1.03-3.53, respectively). There was a significant dose-effect relationship between the AT allele and tumor HPV16-positive status in SCCHN patients (trend test: *P* = .014). Moreover, the stratified analyses showed that the association between tumor HPV16-positive status and the combined *p73* GC/AT + AT/AT genotypes was more pronounced among several subgroups of patients who were older, male, ever drinkers, and those with oropharyngeal cancer.

Conclusions—The *p73* polymorphism was associated with HPV16 status in SCCHN and may serve as a marker for tumor HPV16 positivity in patients with SCCHN, particularly those with oropharyngeal cancer.

Keywords

p73 polymorphism; genetic susceptibility; HPV; squamous cell carcinoma of the head and neck; molecular epidemiology

Introduction

Squamous cell carcinomas of the Head and Neck (SCCHN) are characterized by local tumor aggressiveness requiring morbidity-inducing local-regional therapies. Even with such therapy, these tumors are associated with moderately high recurrence rates, common medical comorbidities, and a high frequency of second primary tumors¹. These cancers are strongly associated with tobacco use and alcohol consumption. However, despite declining smoking rates in the United States, the incidence of SCCHN in young adults is increasing, and this increasing trend has been related to the increasing prevalence of human papillomavirus (HPV) infection^{2, 3}. Of the 120 known types of HPV, the high-risk HPV16 virus accounts for 90%–95% of HPV-positive tumors^{4, 5}.

Molecular studies have shown that oncogenic E6 and E7 proteins of HPV16 have a high binding affinity for p53 and Rb to promote the ubiquitination and complete degradation of these tumor suppressor proteins. These two oncogenic proteins also can interact with other key cell cycle regulators in both the p53 and Rb pathways, such as p73, a member of the *p53* family that shares three major domain organizations with and functions similarly to p53. Specifically, p73 activates the promoters of several p53-responsive genes participating in cell-cycle control, DNA repair, and apoptosis and inhibits cell growth in a p53-like manner by inducing apoptosis or G₁ cell-cycle arrest⁶. Also, p73 can promote apoptosis via the E2F-p73 pathway⁷ and upregulate cell proliferation through the activation of a silent allele⁸. The inactivation of p73 by oncogenic HPV16 E6 appears to be analogous to its inactivation of p53 without the modulation of the DNA-binding activities⁹. However, unlike p53, p73 is resistant to degradation by HPV16 E6, can suppress cell growth, and induce apoptosis in HPV16 E6-expressing cells¹⁰. In addition, the HPV16 E7 oncoprotein has been demonstrated to deregulate p73 and result in p73 overexpression via the HPV16 E7-mediated functional inactivation of pRb¹¹.

The evidence from molecular and epidemiologic studies indicates that HPV-positive SCCHN is a specific disease entity with distinct molecular, pathologic, and clinical characteristics and different risk factor profiles^{12, 13}. Compared with HPV-negative SCCHN, the molecular signature of HPV-positive SCCHN includes the presence of HPV DNA, wild-type *p53*, decreased expression of CCND1 and Rb, and upregulation of p16^{14, 15}. Interestingly, however, several investigators have observed better survival in patients with HPV-positive tumors than in patients with HPV-negative tumors^{16, 17}.

Survival data from studies of SCCHN patients have suggested that this observed better survival outcome in patients with HPV-positive tumors may result from their more favorable response to radiation therapy; indeed, patients with HPV-positive tumors were noted to be at less than half the risk of dying from their cancer or experiencing local failure than were patients with HPV-negative tumors¹⁸⁻²⁰. Thus, development of new biomarkers for identifying this unique subgroup of patients may ensure appropriate therapy for a better survival and improved quality of life.

Our previous molecular epidemiologic studies have found a significant association between polymorphisms of the cell cycle regulators such as *p73*, *MDM2*, *p27*, and *p21* and the risk of SCCHN²¹⁻²³. In particular, two linked, non-coding exon 2 polymorphisms of *p73* at positions 4 (G→A) and 14 (C→T) may influence p73 function by altering gene expression, perhaps by altering the efficiency of translation initiation²⁴. It is therefore possible that *p73* variants may alter the affinity of the E6 and E7 protein for p73 and thereby alter the degradation of p73 promoted by E6 or E7 oncogenic proteins. However, to the best of our knowledge, this hypothesis has not been examined. In the study reported here, we tested our hypothesis that a genetic polymorphism of *p73* at positions 4 (G→A) and 14 (C→T) in exon 2 is associated with

tumor HPV16 status in SCCHN. This involved our analyses of cancer specimens and blood samples in 202 SCCHN patients recruited for a molecular epidemiologic study of SCCHN.

Materials and Methods

Study Subjects

We studied 202 new patients with SCCHN who were consecutively recruited from an ongoing molecular epidemiologic study of SCCHN at The University of Texas M. D. Anderson Cancer Center. The subjects in this study, without restrictions on age, sex, and cancer stage or histology, had newly diagnosed, histopathologically confirmed, and untreated SCCHN. To avoid confounding due to ethnic characteristics, we included only non-Hispanic white patients in our study. All the patients signed institutional review board–approved informed consent and completed a questionnaire on demographic and risk factor information. In addition, all patients donated 30 ml of blood for *p73* genotyping. Paraffin-embedded tumor tissue samples were obtained for HPV16 detection.

HPV16 Detection

The paraffin-embedded tissues were assessed for the presence of HPV16 DNA. We extracted the DNA from the samples with a tissue DNA extraction kit (Qiagen Inc., Valencia, CA). Tumor tissues from the study subjects were tested for the presence of HPV16 DNA using PCR-based type-specific assays with modification and quality control for the E6 and E7 regions (9). Assays of the samples were run in triplicate, with positive (Siha cell line) and negative (TPC-1 cell line) controls and with β -actin as DNA quality control. Each subject was classified as HPV16-positive or HPV16-negative based on the PCR results.

Southern blotting analysis was performed to confirm HPV16 E6 and E7 specificity in a portion of the paraffin-embedded tissue samples, using a Roche Diagnostics labeling and hybridization system ¹⁶ (Roche Applied Science, Indianapolis, IN). Briefly, 5 μ l of PCR products were run on a 1.5% agarose gel. The DNA band was transferred to a nylon membrane overnight, after which the membrane was subjected to UV crosslink light treatment and dried at room temperature. This was followed by a prehybridization treatment consisting of exposure to 20 ml of DIG Easy solution at 40 °C for 30 min. For hybridization, a dUTP-DIG-labeled specific probe for the HPV16 E6 region 5'aactgtcaaaagccactgtgtcctgaagaaa and E7 region 5'acaagcagaaccggacagagcccat was utilized at 40 °C for 4 hours. After being washed twice with wash buffer for 15 min, each membrane was blocked, 1:10000 diluted anti-DIG antibodies were added, and samples were incubated at 37 °C for 1 hour. After two additional washings with wash buffer for 30 min each, autoradiography was performed with CDP-Star substrate. We also confirmed HPV16 E6 and E7 specificity in a portion of samples by digesting the PCR products with restriction enzymes Ban II and Msp I to verify the specific fragments for E6 and E7. The results of the two methods were 100% concordant.

p73 Genotyping

We extracted genomic DNA from a leukocyte cell pellet, which was obtained from the buffy coat by the centrifugation of 1 ml of whole blood, using the QIAGEN DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

We analyzed samples for the *p73* G4C14-to-A4T14 genotype using PCR with confronting two-pair primers, which makes it possible to determine genotypes by electrophoresis without restriction digestion ²⁵. The A4T14 allele was amplified with primers F1 (50CCACGGATGGGTCTGATCC30) and R1 (50GGCCTCCAAGGGCAGCTT30), which produced a 270-bp fragment; the G4C14 allele was amplified with primers F2 (50CCTTCCTTCCTGCAGAGCG30) and R2 (50TTAGCCCAGCGAAGGTGG30), which

amplified a 193-bp fragment. F1 and R2 also produced a common 428-bp fragment in each PCR^{25, 26}. The PCR was performed in 10- μ l volumes containing approximately 20 ng of genomic DNA, 0.1 mM each dNTP, 1 $\times$ PCR buffer (50 mM KCl, 10 mM Tris HCl and 0.1% Triton X-100), 1.5 mM MgCl₂, 0.5 U of Taq polymerase (Sigma-Aldrich Biotechnology, Saint Louis, MO), and 2 pmol of each of the four primers. The amplification conditions included 10 min of initial denaturation at 95 °C, 35 cycles of 1 min at 95 °C, 45 s at 62 °C and 1 min at 72 °C, and a final 5-min extension at 72 °C. All PCR products were visualized on a 2% agarose gel containing 0.25 mg/ml ethidium bromide. More than 10% of the samples were retested randomly, and the results were 100% concordant.

Statistical Analysis

Statistical Analysis System software (Version 9.1; SAS Institute, Cary, NC) was used for all of the statistical analyses. All tests were two-sided, and a *P* value of 0.05 was considered the cutoff for statistical significance. Participants who had smoked fewer than 100 cigarettes during their lifetimes were categorized as never smokers, and the rest were categorized as ever smokers. Participants who had drunk alcoholic beverages at least once a week for ≥ 1 year were categorized as ever drinkers, and the rest were defined as never drinkers. Subjects with HPV16-positive SCCHN were categorized as HPV16⁺ cases, while patients with HPV16-negative SCCHN were categorized as HPV16⁻ cases. Differences in the distributions of selected demographic characteristics, tobacco smoking and alcohol drinking status, and *p73* allele and genotype frequencies between HPV16⁺ and HPV16⁻ cases were evaluated using the χ^2 test. We estimated the association between *p73* genotypes and tumor HPV16 positivity among SCCHN by computing the ORs and 95% CIs using both univariate and multivariable logistic regression analyses. We further stratified the genotype data into subgroups by age, sex, smoking status, drinking status, and cancer site in multivariable logistic regression models. For logistic regression analyses, the *p73* genotype was also recoded as a dummy variable.

Results

For this study, complete *p73* genotype data, demographic characteristics, and smoking and drinking status were available for 202 non-Hispanic white patients with SCCHN (Table 1). No significant differences in drinking status were found between the HPV16-positive and HPV16-negative SCCHN patients. However, the distributions of age, sex and smoking status were significantly different between HPV16-positive and HPV16-negative patients (*P* < .01, *P* = .002 and *P* = .017, respectively), with young, males and nonsmokers being significantly overrepresented in the HPV16-positive group. The median age was 51 years for the tumor HPV16-positive SCCHN patients (mean = 51.4 years, range = 18-81 years) and 58 years for the tumor HPV16-negative SCCHN patients (mean = 58.1 years, range = 25-84 years).

The genotype distributions and allele frequencies of *p73* in the HPV16-positive and HPV16-negative patients are shown in Table 2. The AT variant allele was significantly more common among the HPV16-positive patients (36.5%) than among the HPV16-negative patients (23.5%; *P* = .005), implying that the AT allele may be associated with tumor HPV16 status among patients with SCCHN. Similarly, the difference in the *p73* GC/GC, GC/AT and AT/AT genotype distributions between the HPV16-positive and HPV16-negative groups was statistically significant (*P* = .007). In addition, the combined GC/AT heterozygote and the AT/AT homozygote subtypes were more common among the HPV16-positive patients (61.2%) than among the HPV16-negative patients (44.5%), and the difference in the combined genotypes (GC/AT + AT/AT) was statistically significant between the two groups (*P* = .019). In our multivariable logistic regression models, the association analyses were adjusted for age, sex, tobacco smoking, and alcohol drinking status. Compared with the GC/GC homozygote, the AT/AT homozygote was significantly associated with tumor HPV16 positivity among

SCCHN (adjusted OR = 5.32; 95% CI, 1.32-21.4). Alternatively, the AT/AT homozygosity is negatively associated (OR = 0.20, 95% CI, 0.05-0.82), whereas the GC/GC homozygosity is positively associated, with HPV16-negative SCCHN (OR = 5.04, 95% CI, 1.22-20.9). These two alleles may play different roles in the etiology of HPV16-positive and HPV16-negative SCCHN. Because the AT/AT variant homozygote was relatively uncommon, it was combined with the GC/AT variant heterozygote. A significant association between tumor HPV16 positivity and the combined variant genotype (GC/AT + AT/AT) compared with the GC/GC genotype was observed (adjusted OR = 1.91; 95% CI, 1.03-3.53). Furthermore, a dose-effect relationship between the number of the AT allele and tumor HPV16 positivity in SCCHN patients was statistically significant ($P = .014$).

To further evaluate the association of HPV16 positivity among SCCHN patients with the *p73* polymorphism, we performed the stratified analyses by age, sex, smoking status, drinking status, and tumor site with adjustment for the aforementioned variables. Therefore, we coded and analyzed the *p73* genotypes using the dominant model (i.e. wildtype = 0, heterozygote = 1 and homozygous variant = 1) by combined the GC/AT variant genotype with the AT/AT variant genotype for these stratification analyses (Table 3). Among HPV16 positive patients with oropharyngeal cancer, the combined-variant genotype (GC/AT+AT/AT) carriers exhibited a significant association with tumor HPV16 positivity (adjusted OR = 3.89, 95% CI, 1.40-10.80) than did the GC/GC genotype carriers while we did not find such significant association among the patients with non-oropharyngeal cancer. In addition, the combined-variant genotypes (GC/AT+AT/AT) were significantly associated with tumor HPV16 positivity among male patients (adjusted OR = 2.16, 95% CI, 1.08-4.33) and patients with alcohol drinking history (adjusted OR = 2.72, 95% CI, 1.31-5.67).

Discussion

In this case-case comparison study, we found a significant association between the *p73* G4C14-to-A4T14 polymorphism and tumor HPV16 status in patients with SCCHN, and the association showed a significant dose-effect relationship, indicating that this *p73* polymorphism could be a potential marker for tumor HPV16 status in patients with SCCHN. This is, as far as we are able to determine, the first molecular epidemiologic study to investigate the association between the *p73* polymorphism and tumor HPV16 status in SCCHN.

As an oncogenic agent, HPV is involved in the development of more than 10% of human cancers²⁷. These types of cancers include oral cavity cancer²⁸, laryngeal cancer²⁹, oropharyngeal cancer^{28, 30}, other head and neck cancers³¹, cervical cancer³², and others³³⁻³⁷. Moreover, HPV-positive cancers are distinct from HPV-negative cancers^{14, 15} in terms of the clinical course, prognostic signature, and survival duration^{16, 38, 39}. Although we cannot explain how this *p73* polymorphism influences the HPV16 status of SCCHN, our findings are biologically plausible for several reasons. First, it is possible that GC to AT change in *p73* G4C14-to A4T14 may result in formation of a stem-loop structure and so may affect the translation efficiency of *p73*²⁴ or that this *p73* polymorphism could be in linkage disequilibrium with other functional variants of *p73*, thereby altering the function of *p73*, or with alleles at other nearby susceptibility loci. While *p73* expression has been demonstrated to associate with the presence of HPV DNA in cancers⁴⁰. Additionally, *p73* interacts with both the HPV E6 and E7 oncoproteins, which contributes to the malignant transformation associated with HPV^{9, 10}. And, *p73* is involved in the regulation of the cell cycle, DNA repair, and apoptosis, particularly in a compensatory fashion when *p53* function is lost^{7, 41}. It is possible that this *p73* polymorphism may cause individual difference in resistance to apoptosis which might enable many HPV-infected cells to escape or counterattack against the immune system. Furthermore, the pathogenesis of SCCHN has been shown to differ depending on whether the tumor contains HPV genomic DNA⁴². It is also likely that this

p73 polymorphism plays a different role in susceptibility to HPV16 oncoproteins or metabolites of tobacco and alcohol, and thereby varies the prevalence of *p73* G4C14-to-A4T14 genotypes in HPV-positive versus negative cancers.

Although there are no reports on the relationship between this *p73* polymorphism and tumor HPV16 status in SCCHN, two studies with oropharyngeal and cervical cancer did suggest, however, that the *p53* codon 72 polymorphism was correlated with the HPV16 tumor and seropositive status, respectively^{43, 44}. Mechanically, genetic variants of this *p73* polymorphism alone and interplay between *p73* and E6 and E7 could lead to different levels of *p73* gene expression in HPV16-positive and HPV16-negative oesophageal squamous cell carcinomas⁴⁰.

Another finding in our study was that tumor HPV16 positive status associated with *p73* variant genotypes was significantly higher for patients with oropharyngeal cancer, but not for those with non-oropharyngeal cancer. The difference in tumor HPV16 status between patients with SCCHN at different sites may result from different etiologies for non-oropharyngeal and oropharyngeal cancers. This finding is in agreement with those in previous studies, in which most non-oropharyngeal cancers were found to be caused by smoking and drinking while the majority of oropharyngeal cancers were caused by HPV^{45, 46}. It is thus likely that the *p73* polymorphism plays a different role in the tumorigenesis of oropharyngeal and non-oropharyngeal cancers. This could be further explained by differences in the histologic characteristics of non-oropharyngeal and oropharynx tissues. For example, tonsillar cancer, the most common oropharyngeal cancer, has a reticulated squamous epithelium of the crypts that is infiltrated with a lymphoid element, but that is not found in oral cavity tissue⁴⁷. We speculate that the immune response generated against an HPV infection differs between the oropharynx and oral cavity, with the *p73* polymorphism possibly modulate the ability of the host to clear cells infected with HPV, which may affect the tumor HPV status. However, these hypotheses also need to be tested in future studies.

Interestingly, we found that the association between HPV16 positivity and *p73* variant genotypes in SCCHN were of significance in patients with a history of alcohol drinking (adjusted OR = 2.72, 95% CI, 1.31-5.67) but not in non-drinkers. Previous studies have shown that ethanol affects cell cycle regulation^{48, 49}, induces apoptosis⁵⁰, and inhibits DNA repair⁵¹. It is thus plausible that an ethanol-mediated cellular response affects *p73* activation and expression and that the *p73* polymorphism plays a role in this mechanism, resulting in the difference in presence of HPV16. Additionally, it is likely that ethanol consumption, which suppresses humoral and cellular immune responses to HPV16 infection and affects sexual behaviors, also partly influences HPV16 status in SCCHN. However, there is no biological mechanism to directly support this finding. We also found that the association between *p73* variant genotypes and HPV16 positivity is more evident among male patients. We speculate that differences in sexual behaviors and hormone characteristics between diverse groups may partially explain this finding. These findings may also be partially attributed to the selection bias of study patients and the interaction between HPV and smoking and/or alcohol drinking, but this hypothesis needs to be tested in future large studies.

Although we are the first to test the association between the *p73* polymorphism and HPV16 status in SCCHN tumor specimens, our study has some limitations. Because HPV16-positive cancer is distinct from HPV16-negative cancer, the clinical signatures, including age, sex and smoking status, were different and difficult to match, though we did recruit these patients from the same M. D. Anderson Cancer Center patient population. However, because our study included only non-Hispanic white patients and with adjustment for age, sex, tobacco smoking, and alcohol drinking, the potential effect of confounding factors on this association should be

minimal. The numbers of observations in some strata of the stratification analysis were relatively small and the positive results could have occurred by chance.

This case-case study also has other inherent limitations. We realized that this study was too small to test the interaction. The detection of HPV16 alone on tumor samples, cross contamination during the sampling in the laboratory, and misclassification of anatomic site may cause misclassification of tumor HPV status^{39, 52, 53}, which could bias the estimates of association^{54, 55, 56}. To minimize the misclassification of the tumor HPV status in tissues, our future studies should use the two highly specific methods^{16, 57} (Southern blot analysis and in situ hybridization) to further examine the presence of HPV16 in tumor samples.

We also can not exclude the possibility that the estimates of association we observed between *p73* polymorphism and tumor HPV16 status could be biased by the selection bias among hospital-based case subjects and other confounding factors such as sexual behavior, oral HPV16 infection, and serologic status of high-risk HPV types. Without the measures of exposure to HPV16, the control group of tumor HPV16-negative patients may not adequately represent the true prevalence of exposure to HPV16 because there is a discordance between the HPV16 serologic status and tumor HPV16 status. Therefore, our results also need to be interpreted as the study's limitations due to without adjustment with the information, and future prospective and well-designed studies with larger sample sizes and detailed information on sexual behavior, oral HPV16 infection, and serologic status for high-risk HPV types are needed to confirm our findings.

In conclusion, our study demonstrated that the *p73* G4C14-to-A4T14 polymorphism was significantly associated with the HPV16 status in non-Hispanic white patients with SCCHN, indicating that it may serve as a marker for tumor HPV16 status in patients with SCCHN, particularly in patients with oropharyngeal carcinoma, or with alcohol drinking history, or male SCCHN patients. Further validation of our findings in larger studies and exploration of the underlying molecular mechanisms are warranted. We are currently investigating potential roles of functional polymorphisms of other genes, which are crucial for cell cycle regulation and apoptosis, in HPV-associated cancers.

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TABLE 1
Distribution of Selected Variables in SCCHN Cases by HPV16 Status

Variable	HPV16 ⁺ Patients (N = 85)		HPV16 ⁻ Patients (N = 117)		P value*
	No.	%	No.	%	
Age					
≤58 years	67	78.5	60	51.3	< .01
>58 years	18	21.2	57	48.7	
Sex					
Male	71	83.5	75	64.1	.002
Female	14	16.5	42	35.9	
Tobacco smoking					
Ever	48	56.5	85	72.7	.017
Never	37	43.5	32	27.3	
Alcohol drinking					
Ever	62	72.9	89	76.1	.614
Never	23	27.1	28	23.9	

* Two-sided χ^2 test.

Risk of SCCHN Associated with the p73 Genotypes and Allele Frequencies in HPV16+ and HPV16- Patients

TABLE 2

p73 Genotype	HPV16+ Patients (N = 85)		HPV16- Patients (N = 117)		Crude OR (95% CI)	Adjusted OR (95% CI) ^a
	No.	%	No.	%		
G4C14-to-A4T14						
GC/GC ^b (c)	33	38.8	65	55.5	1.00	1.00
GC/AT ^b	42	49.4	49	41.9	1.69 (.94-3.04)	1.69 (.90-3.16)
AT/AT ^b	10	11.8	3	2.6	6.57 (1.69-25.5)	5.32 (1.32-21.4)
Trend test					P = .003	P = .014
GC/AT+AT/AT	52	61.2	52	44.5	1.97 (1.12-4.48)	1.91 (1.03-3.53)
AT allele	.365		.235			

^a Adjusted for age, sex, tobacco smoking and alcohol drinking status in a logistic regression model.

^b $\chi^2 = 9.937$, $P = .007$ for genotype distributions; $\chi^2 = 8.047$, $P = .005$ for allele frequency.

^c Reference group.

Stratification Analysis of p73 Genotypes, OR, and 95% CIs by Selected Variables

	HPV16 ⁺ Patients (N = 85)				HPV16 ⁻ Patients (N = 117)				Adjusted OR (95%CI) ^a	
	GC/GC		GC/AT + AT/AT		GC/GC		GC/AT + AT/AT		GC/GC ^c	GC/AT + AT/AT
	No.	%	No.	%	No.	%	No.	%		
	No.	%	No.	%	No.	%	No.	%		
All	33	38.8	52	61.2	65	55.6	52	44.4	1.00	1.91 (1.03-3.53)
Age										
≤58 years	25	37.3	42	62.7	33	55.0	27	45.0	1.00	1.85 (.87-3.93)
>58 years	8	44.4	10	55.6	32	56.1	25	43.9	1.00	2.66 (.79-8.92)
Sex										
Male	28	39.4	43	60.6	46	61.3	29	38.7	1.00	2.16 (1.08-4.33)
Female	5	35.7	9	64.3	19	45.2	23	54.8	1.00	1.42 (.36-5.63)
Smoking status										
Ever	21	43.7	27	56.3	50	58.8	35	41.2	1.00	2.01 (.94-4.30)
Never	12	32.4	25	67.6	15	46.9	17	53.1	1.00	2.39 (.74-7.74)
Drinking status										
Ever	25	40.3	37	59.7	54	60.7	35	39.3	1.00	2.72 (1.31-5.67)
Never	8	34.8	15	65.2	11	39.3	17	60.7	1.00	1.04 (.27-4.02)
Tumor site ^b										
Oropharynx	29	40.9	42	59.1	19	73.1	7	26.9	1.00	3.89 (1.40-10.8)
Non-oropharynx	4	28.6	10	71.4	46	50.6	45	49.6	1.00	2.06 (.54-7.91)

^a Adjusted for age, sex, tobacco smoking and alcohol drinking status in a logistic regression model.

^b 14.6% patients with cancer of the oral cavity were HPV16-positive; 73.2% of patients with cancer of the oropharynx were HPV16-positive; 6.3% of patients with cancer of the larynx and hypopharynx were HPV16-positive; $\chi^2 = 75.52$, $P < .0001$ for tumor site distribution in HPV16-positive versus HPV16-negative cancer.

^c Reference group.