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## The Relationship Between Human Papillomavirus Status and Other Molecular Prognostic Markers in Head and Neck Squamous Cell Carcinomas

Christina S. Kong, M.D.<sup>\*</sup>, Balasubramanian Narasimhan, Ph.D.<sup>†</sup>, Hongbin Cao, M.S.<sup>‡</sup>, Shirley Kwok, B.S.<sup>\*</sup>, Julianna P. Erickson, B.S.<sup>§</sup>, Albert Koong, M.D., Ph.D.<sup>‡</sup>, Nader Pourmand, Ph.D.<sup>§,¶</sup>, and Quynh-Thu Le, M.D.<sup>‡</sup>

<sup>\*</sup>Department of Pathology, Stanford University, Santa Cruz, CA

<sup>†</sup>Department of Statistics, Stanford University, Santa Cruz, CA

<sup>‡</sup>Department of Radiation Oncology, Stanford University, Santa Cruz, CA

<sup>§</sup> Department of Biochemistry-Genome Center, Stanford University, Santa Cruz, CA

<sup>¶</sup>Department of Biomolecular Engineering, University of California, Santa Cruz, CA

### Abstract

**Purpose**—To evaluate the relationship between human papillomavirus (HPV) status and known prognostic makers for head and neck cancers including tumor hypoxia, epidermal growth factor receptor (EGFR) expression and intratumoral T-cell levels and to determine the prognostic impact of these markers by HPV status.

**Methods and Materials**—HPV status in 82 evaluable head and neck squamous cell carcinomas patients was determined by pyrosequencing and related to p16<sup>INK4a</sup> staining and treatment outcomes. It was correlated with tumor hypoxia (tumor pO<sub>2</sub> and carbonic anhydrase [CAIX] staining), EGFR status, and intratumoral lymphocyte expression (CD3 staining).

**Results**—Forty-four percent of evaluable tumors had strong HPV signal by pyrosequencing. There was a significant relationship between strong HPV signal and p16<sup>INK4a</sup> staining as well as oropharynx location. The strong HPV signal group fared significantly better than others, both in time to progression (TTP,  $p = 0.008$ ) and overall survival (OS,  $p = 0.004$ ) for all patients and for the oropharyngeal subset. Positive p16<sup>INK4a</sup> staining was associated with better TTP ( $p = 0.014$ ) and OS ( $p = 0.00002$ ). There was no relationship between HPV status and tumor pO<sub>2</sub> or CAIX staining. However, HPV status correlated inversely with EGFR reactivity ( $p = 0.0006$ ) and directly with CD3 (+) T-lymphocyte level ( $p = 0.03$ ). Whereas CAIX and EGFR overexpression were negative prognostic factors regardless of HPV status, CD3(+) T-cell levels was prognostic only in HPV(–) tumors.

**Conclusion**—HPV status was a prognostic factor for progression and survival. It correlated inversely with EGFR expression and directly with T-cell infiltration. The prognostic effect of CAIX and EGFR expression was not influenced by HPV status, whereas intratumoral T-cell levels was significant only for HPV(–) tumors.

Reprint requests: to Quynh-Thu Le, M.D., Department of Radiation Oncology, 875 Blake Wilbur Drive, MC 5847, Stanford, CA 94305-5847. Tel: (650) 498-5032; Fax: (650) 725-8231; qle@stanford.edu.

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## Keywords

HPV; Head and neck cancer; p16<sup>INK4a</sup>; EGFR; Hypoxia; CD3

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## Introduction

Head and neck squamous cell carcinomas (HNSCC) are heterogeneous and traditionally associated with tobacco and alcohol use (1). Recently, human papillomavirus (HPV) has been implicated in the development of certain HNSCC, specifically oropharyngeal carcinomas (OP) (2–4). HPV positive [HPV(+)] tumors differ from HPV negative [HPV(-)] tumors in many aspects including histologic appearance, differentiation, risk factors and prognosis (5).

At the molecular level, several differences are linked to HPV status. HPV integration leads to increased expression of the E6 and E7 oncogenes, which neutralize the p53 and retinoblastoma (Rb) tumor suppressor pathways, causing perturbation in key cell-cycle proteins (6,7). One protein is p16<sup>INK4A</sup>, which acts by binding to cell-cycle regulators. Loss of p16<sup>INK4A</sup> is associated with poor prognosis in HNSCC and the converse is true for its overexpression (8, 9). In tobacco-related HNSCC, p16<sup>INK4a</sup> is often absent because of homologous deletion or promoter hypermethylation. However, in HPV(+) HNSCC tumors, p16<sup>INK4a</sup> is over-expressed because of Rb loss and is subsequently used as a surrogate marker for HPV.

The improved apoptotic response to chemoradiation due to less p53 mutations and functional p16<sup>INK4a</sup> may explain the improved outcomes for HPV(+) tumors. However, these mechanisms alone cannot explain the better prognosis when these tumors were treated with surgery alone (10). Other hypotheses for improved outcomes in HPV(+) tumors include the lack of field cancerization and enhanced immune surveillance (5). Although several molecular markers have been studied for prognostication in HNSCC (11–15), few have been validated in large prospective studies. Certain validated factors include tumor hypoxia and epidermal growth factor receptor (EGFR) status (14,15). The relationship between HPV infection and these factors are unclear. Therefore, in this study, we assessed HPV presence in HNSCC using high throughput pyrosequencing and p16<sup>INK4a</sup> staining, evaluated the prognostic significance of HPV and p16<sup>INK4a</sup> in these tumors, explored the relationship between HPV and tumor hypoxia (by tumor pO<sub>2</sub> and tumor expression of carbonic anhydrase IX [CAIX], a hypoxia-induced protein), EGFR expression, and intratumoral T-cell levels (by staining for CD3, a pan-T-cell marker) and determined the prognostic impact of these three biomarkers in relation to HPV status.

## Methods and Materials

### Patients

Criteria for patient participation included (1) newly diagnosed HNSCC, (2) available tissue block, and (3) willingness to sign an informed consent. All tumors were staged using the 2002 American Joint Committee on Cancer staging system (16).

### DNA extraction

Four-micron-thick hematoxylin-and-eosin (H&E) sections were first examined by a pathologist (C.K.) for tumor. Eighty-nine of 99 cases (90%) had 50% or more tumor in the 4-micron-thick H&E sections examined. The remaining 10/99 cases (10%) had at least 25% tumor. Three to four 10-micron-thick scrolls were then cut from each block and used for DNA extraction. DNA was isolated from paraffin-embedded tumors using Ambion's RecoverAll Total Nucleic Acid Isolation kit (Applied Biosystem, Austin, TX) as instructed. The eluted

DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific, Pittsburgh, PA), and samples were stored at  $-20^{\circ}\text{C}$ .

### HPV PCR/Pyrosequencing

Polymerase chain reaction (PCR) for the L1 region of HPV was performed using the forward primer GP5+ (5'-TTTGTTACTGTTG TTGATACTAC-3') and the biotinylated reverse primer GP6+ (5'-GAAAAATAAACTGTAAATCATATTC-3') as described (17).  $\beta$ -globulin was used as an internal amplification control to ensure the DNA integrity. Seventeen of 99 samples were inevaluable for HPV analysis: 4 with negative and 13 with weak  $\beta$ -globulin amplification signals, yielding 82 samples for pyrosequencing analysis (17). The quantitative gel-based exACTGene 50-bp Mini DNA Ladder (Fisher Scientific, Pittsburgh, PA) was used to measure DNA concentration of HPV-fragment amplicons. Amplicon concentration  $<100\text{ ng}/10\ \mu\text{L}$  PCR was scored as "weak" and  $\geq 100\text{ ng}/10\ \mu\text{L}$  PCR as "strong." The amplicons were thereafter pyrosequenced as described subsequently.

Pyrosequencing was performed by immobilizing the biotinylated PCR products onto streptavidin-coated High Performance Sepharose beads (Amersham Biosciences/GE Healthcare Biosciences Corp., Piscataway, NJ). The beads were subjected to the following: washing with 70% ethanol, denaturing DNA with 0.2 M sodium hydroxide and washing with TE-buffer to remove nonimmobilized complementary strands. The beads were incubated in 12  $\mu\text{L}$  annealing buffer containing 0.4 pmol sequencing primer at  $95^{\circ}\text{C}$  for 2 min,  $50^{\circ}\text{C}$  for 5 min, and  $25^{\circ}\text{C}$  for 5 min (17).

For each sample, four reactions were prepared, one for each primer pool. GP5+ is a general primer designed to provide sequence signals for any HPV type. Three multiple sequencing primer pools (MSP), each containing four type-specific primers, were also used. MSP1 contains primers specific to HPV16, 31, 59, and 39; MSP2 for HPV18, 33, 52, and 56; and MSP3 for HPV35, 45, 51, and 58. These pools were designed to target the most common high-risk HPV types.

Following primer hybridization, samples were sequenced using the PSQ HS96A System (Biotage, Charlottesville, VA) as described (17). Ten cycles of A-C-G-T were dispensed for each assay. Nucleotide incorporation events were determined by detection of photons generated upon pyrophosphate release during polymerase extension. Sample type was determined by comparing sequence signals for each primer pool to known data.

### Tissue array generation and immunohistochemical staining

The tissue microarray (TMA) was constructed as previously described (18). Immunoperoxidase stains for p16<sup>INK4a</sup> (clone E6H4, Dako, Carpinteria, CA), EGFR (31G7 mouse monoclonal, Zymed, South San Francisco, CA), CD3 (rabbit monoclonal, Cell Marque, Rocklin, CA), and CAIX (M75 mouse monoclonal, Bayer, Pittsburgh, PA) were performed on 4- $\mu\text{M}$ -thick sections of the TMA. CD3 was also performed on corresponding 4- $\mu\text{M}$ -thick whole tumor sections.

The TMA and whole sections were interpreted by a pathologist (CSK), who was blinded to the clinical data, and scored for EGFR and CAIX staining as follows: 0 as negative, 1 as uninterpretable (when the core was either missing or heavily folded, making it impossible to interpret), 2 as weak and 3 as strong staining. For EGFR, scoring was based on membrane staining; the percentage of positive cell staining was also scored as follows: 1 for 0%–10% positive, 2 for 11%–50%, 3 for 51%–80%, and 4 for 81%–100%. For CAIX, scoring was performed separately for cytoplasmic and membrane staining but interpreted as strong if either was scored strongly positive. For p16<sup>INK4a</sup>, weak cytoplasmic staining in  $<5\%$  of the cells was

interpreted as negative, focal strong nuclear and/or cytoplasmic staining in 5%–80% of the cells as focally positive, and diffuse strong staining in >80% of the cells as diffusely positive. For the purpose of multivariate analysis, both focally and diffusely positive tumors were considered positive. Previous studies on cervical dysplasia have shown that diffusely strong p16<sup>INK4a</sup> reactivity correlated with high-risk HPV, whereas focally strong reactivity correlated with both high and low-risk HPV (19–21).

### Tumor pO<sub>2</sub> measurement

All measurements were performed using a computerized histogram (Sigma Eppendorf PO2 Histogram, Hamburg, Germany) as described (22).

### Statistical analysis

Statistical analyses were performed using the R version 2.5.1 (23). The Fisher exact test was used to correlate HPV PCR status to different parameters. Time to progression (TTP), disease-specific survival (DSS), and overall survival (OS) (24). Log-rank statistics were used to compare survival curves (25). Variables evaluated on univariate model were age, sex, smoking status, hemoglobin, N-classification, HPV status, p16<sup>INK4a</sup> staining, and treatment. Only those that achieved a *p* value <0.05 were included in a Cox proportional hazard model for multivariate analysis (25).

## Results

### HPV status and typing

Tissues from 99 patients including 61 with OP primary and 38 with HNSCC from other primary sites (13 oral cavity, 8 larynx, 11 hypopharynx, 3 nasopharynx, 2 paranasal sinuses, and 1 unknown primary) were tested for the presence of HPV using pyrosequencing. Eighty-two of 99 patients had evaluable HPV pyrograms and formed the final cohort of this study. Of these 82 patients, 26 had undetectable PCR signal, 20 had weak signal defined as PCR amplicon concentration <100 ng/10 μL, and 36 had strong signals defined as PCR amplicon concentration ≥100 ng/10 μL (Fig. 1A). The classification of weak and strong signal was further validated in a blinded fashion using quantitative real-time PCR and normalized against β-globulin in 12 randomly selected samples. As shown by the box-plot in Fig. 1B, all strong samples has a normalized ratio >1.5, whereas all but one of the weak samples had normalized ratio <1; the difference between the two groups was highly statistically significant (*p* < 0.0001), confirming the validity of the classification. Twelve HPV subtypes were simultaneously evaluated; infection by a single type was noted in 51 patients and mixed infection in 5 patients (1 with 16/18, 1 with 16/33, and 3 with multiple strains). Fifty of 51 patients with single infection harbored HPV16.

### Relationship between HPV status, p16<sup>INK4a</sup> staining, and clinical parameters

Table 1 shows the relationship between p16<sup>INK4a</sup> expression and HPV status by pyrosequencing. p16<sup>INK4a</sup> expression strongly correlated with HPV status; specifically most tumors with strong HPV signal showed diffuse-strong staining for p16<sup>INK4a</sup>, and those with negative or weak HPV signal were predominantly p16<sup>INK4a</sup> negative. Of the six tumors that displayed strong HPV pyrosequencing signal but negative p16<sup>INK4a</sup> staining, only one patient fit the HPV-related tumor profile as a nonsmoker with an oropharyngeal carcinoma. The other five patients were all heavy smokers with 30–60 pack-year of cigarette use, and their tumors were located in the oropharynx in 3 patients, hypopharynx in 1 patient, and oral cavity in 1 patient.

We also evaluated the relationship between HPV status by pyrosequencing and tumor site. Similar to prior reports, 92% of strong HPV tumors were located in the oropharynx. However, only 35% of the tumors with weak HPV signal were located in the oropharynx, a frequency that was identical to that of the HPV(-) group. Because of the similarities between the HPV (-) and weak groups in p16<sup>INK4a</sup> staining and primary tumor site, we combined these two groups together when analyzing the relationship between HPV status and various clinical factors (Table 2). In general, patients with strong HPV signal were younger, had more advanced nodal stages, and had higher hemoglobin levels. With respect to treatment strategy, patients were classified as receiving either definitive nonsurgical therapy (64 patients) with either radiation alone (3 patients) or chemoradiation (61 patients) or definitive surgical therapy (18 patients) without any adjuvant therapy (2 patients), with adjuvant radiation (9 patients), or with adjuvant chemoradiation (7 patients) for high-risk pathologic features such as extracapsular nodal extension or involved surgical margins. Because of our institutional preference of treating most oropharyngeal carcinomas with nonsurgical therapy, the majority of patients with strong HPV signal (33 of 36) received nonsurgical treatment, whereas only three patients were treated with primary surgery for tonsillar carcinoma.

### Association between HPV, p16<sup>INK4a</sup>, and treatment outcomes

Figure 2 shows the TTP (2A,  $p = 0.008$ ) and OS (2B,  $p = 0.004$ ) by HPV status by pyrosequencing. Patients with strong HPV signal fared significantly better than those with weak or negative signal, confirming our hypothesis that the latter two were biologically similar. An analogous pattern was noted for DSS by HPV status; the 5-year DSS was 89% for strong, 49% for weak and 42% for negative HPV signals ( $p = 0.0005$ ). Subsequently, the weak/negative HPV cohorts were grouped together. Pattern of failure study suggested that patients whose tumor yielded strong HPV signal had better local control, regional control, and freedom from distant metastasis than the rest (Table 3). We also analyzed treatment outcomes by HPV status within the 49 patients with OP tumors and evaluable HPV programs. As shown in Fig. 2C and 2D, patients with OP tumors harboring strong HPV signal had significantly better TTP (Fig. 2C) and overall survival (Fig. 2D) compared with those with OP tumors harboring weak or negative signal. Because of the small number of patients and events, multivariate analysis was not performed for this patient subgroup.

Figure 3A and 3B show TTP and OS by p16<sup>INK4a</sup> status. Patients with positive p16<sup>INK4a</sup> staining, regardless of staining pattern, did significantly better than those with negative staining. A similar pattern was also noted for DSS by p16<sup>INK4a</sup> status ( $p = 0.002$ , data not shown). Subsequently, all p16<sup>INK4a</sup> positive (focal or diffuse strong) tumors were combined together for further analysis.

Given the strong association between HPV and p16<sup>INK4a</sup>, we grouped the patients on the basis of these markers' status and scored the combinations as follows: 0 for neither being strongly positive [HPV(negative-weak)/p16<sup>INK4a</sup>(-)], 1 for either marker being strongly positive [HPV (strong)/p16<sup>INK4a</sup>(-) or HPV(negative-weak)/p16<sup>INK4a</sup>(+)], and 2 for both being strongly positive [HPV(strong)/p16<sup>INK4a</sup>(+)]. Figure 4 shows TTP (4A) and OS (4B) for the three groups. Patients with both markers strongly positive fared the best, and those with both markers being either weak or negative the worst. We then considered this new HPV/p16<sup>INK4a</sup> classification as a categorical variable on multivariate analysis, adjusting for potential clinical prognostic factors that achieved a  $p$  value  $<0.05$  on univariate analyses. Patients with both markers strongly positive fared significantly better than those with both negative on multivariate analysis (Table 4).



### Relationship between HPV status, tumor hypoxia, EGFR expression, and T cell level

To investigate the possible reason why HPV(+) tumors fared better than others, we determined the relationship between HPV pyrosequencing status and tumor hypoxia, EGFR expression, and T-cell tumoral infiltration, three factors that may affect outcomes. Table 5 summarizes the results. There was no significant association between HPV status and tumor hypoxia as detected by either pO<sub>2</sub> measurements or immunohistochemical (IHC) staining for CAIX. In contrast, there was a strong association between HPV status and EGFR staining intensity ( $p = 0.0006$ ), as well as % of EGFR(+) tumor cells ( $p = 0.0002$ ). Most tumors with strong HPV signal were EGFR negative and vice versa. Similarly, HPV status significantly correlated with the level of intratumoral T lymphocytes (by IHC for CD3) ( $p = 0.03$ ). Tumors with strong HPV signal exhibited extensive CD3 reactivity.

To determine whether the prognostic impact of CAIX, EGFR, and CD3 is influenced by HPV status, we evaluated the effect of these markers on OS in these patients, stratified by HPV status. Table 6 summarizes the results. Patients with tumors stained negative or weak for these markers were grouped together because they had similar 5-year survival rates. In general, within each HPV category, patients with strong CAIX staining had relatively lower survival compared with those with negative or weak staining; however, the number of patients with strong CAIX staining for each subgroup was too small to reach statistical significance. Similar observation was noted for EGFR, yet there were only five patients in the HPV strong group with intense EGFR staining. With regard to CD3 staining, its impact was mainly confined to the HPV negative/weak group with significantly better survival noted for patients with diffusely strong lymphocyte infiltration. There was no obvious survival difference by CD3 status noted for the HPV-strong patients.

### Discussion

Since the implication of HPV in HNSCC development was reported, several studies have found better prognosis for HPV(+) tumors (2,10,26–28). The first study (2) reported that HPV status was an independent prognostic factor for DSS. Other studies reported similar findings in patients treated with either primary surgical or nonsurgical therapies (10,26–28). A meta-analysis confirmed that HPV(+) oropharyngeal cancer patients had a 28% lower risk of death than their negative counterparts (29). HPV status was also associated with a better response to induction chemotherapy in two recent studies (30,31). The reasons for improved survival are unclear but have been attributed to several factors as discussed earlier. Our study suggests an additional potential mechanism, involving lower EGFR expression for HPV(+) tumors.

Some studies have evaluated the relationship between EGFR and HPV in HNSCC. Two studies found no EGFR mutation and one reported less EGFR gene amplification for HPV(+) compared with HPV(-) tumors (6% vs. 43%) (32–34). However, the relationship between HPV and EGFR protein expression is controversial. Almadori *et al.* (33,35), using a radioligand receptor assay, noted that HPV(+) laryngeal cancers had higher EGFR levels than HPV(-) tumors. Perrone *et al.* observed no difference in EGFR IHC expression by HPV status despite more EGFR gene amplification in HPV(-) tumors; however, most tumors in their study had high EGFR protein expression (33). In contrast, Reimers *et al.* (36) reported that p16<sup>INK4a</sup>(+) oropharyngeal carcinomas had lower EGFR expression than p16<sup>INK4a</sup>(-) tumors. Kumar *et al.* (37) reported in a brief communication that EGFR intensity was inversely related to HPV titer in 42 OP patients, but they did not provide details of this inverse correlation. In an expanded study of 66 OP patients, they confirmed this observation and found that the combination of EGFR and HPV was useful in stratifying DSS in these patients (38). These findings are highly consistent with our data, showing an inverse relationship between EGFR expression and HPV status both in intensity and percent positive cell staining. This finding suggests that improved prognosis for HPV(+) tumors may be partially due to a larger proportion of these tumors having

negative or low EGFR expression. The reason HPV(+) tumors have less EGFR expression is unknown but could be because of the lesser EGFR amplification in these tumors. However, within the HPV(+) group, EGFR staining intensity continued to be prognostic because survival of the five patients with strong EGFR staining was significantly worse than those with negative or weak staining. These data are consistent with prior reports (36,38) and suggested that aggressive therapy, including EGFR targeting, should still be considered in a small subset of tumors with high HPV and EGFR expression.

Patients with HNSCC have cell-mediated immune defects (39,40). Low levels of circulating natural killer cells are associated with poorer survival (41). Here we evaluated the intratumoral T-cell levels in relation to HPV status. We noted a larger proportion of HPV(+) tumors with diffusely strong CD3(+) T-cell infiltration and that CD3 status was prognostic only for HPV (-) but not HPV(+) tumors. This suggested that the impact of T-cell-mediated immunity for tumor control is more significant in HPV(-) than HPV(+) tumors. However, the limitations of using CD3 as a pan T-cell marker is the lack of information on the different T-cell populations and the functional significance of the T-cell infiltrate with regard to antitumor immunity. Direct comparison of the global and intratumoral immune function between HPV(+) and (-) tumors is necessary to confirm these findings.

We found no obvious relationship between HPV status and either tumor pO<sub>2</sub> measurements or CAIX staining, suggesting that HPV infection does not influence tumor hypoxia. In addition, the CAIX expression intensity had a similar prognostic effect on OS, regardless of HPV status. To our knowledge, this is the largest study evaluating the association between HPV and tumor oxygenation. One caveat in studying tumor hypoxia is the lack of uniform agreement in its assessment. We and others have found a lack of association between tumor pO<sub>2</sub> and IHC expression of known hypoxia-induced proteins such as CAIX (11,42). To minimize the potential biases associated with each approach, we used both to assess hypoxia. Regardless of the approach used, we did not observe any association between HPV status and tumor hypoxia in this small cohort of patients. This needs to be validated in a larger patient group to rule out Type II error.

DNA sequencing is the most accepted standard for viral typing. However, traditional sequencing techniques can be challenging for HPV genotyping when specimens harbor multiple genotypes. A type-specific, multiple-sequencing-primers method, combined with pyrosequencing, was developed to address this issue (17,43). This approach was as robust and accurate in detecting several HPV subtypes in mixed infections. We have previously employed this approach to characterize HPV genotypes in 20 cervical cancer cases (43). This is the first and the largest series in which pyrosequencing was used to genotype HPV in HNSCC from paraffin embedded tissues. It revealed that most HPV(+) tumors harbored HPV16 subtypes. Because this is a PCR-based approach, different signal intensities are detected for HPV(+) tumors. The distinction between weak vs. strong signal was defined a priori on the basis of PCR amplicon concentration and was further validated by quantitative RT PCR assay. Although it is possible that the weaker HPV signal is due to contamination, it is also possible that it represents a low-level, clinically irrelevant infection. Of interest, Worden *et al.* (30), using a combination of real-time PCR and mass spectroscopy for HPV detection, noted that HPV copy number correlated with induction chemotherapy response and DSS, with the best results observed in patients with the highest copy numbers (>65 copies/cell). With more sensitive HPV detection methods such as the PCR-based approach, a distinction needs to be made between clinical and analytic sensitivity (44). Therefore, HPV results by pyrosequencing are best interpreted in combination with a functional marker such as p16<sup>INK4a</sup>.

In summary, we found that the HPV/p16<sup>INK4a</sup> grouping, as used here, was an independent prognostic factor for relapse and survival in HNSCC. HPV(+) status had no direct correlation

with hypoxia but had an inverse correlation with EGFR expression, which remained prognostic for both HPV groups. Intratumoral T-cell infiltration was more likely to be absent or weaker in HPV(-) tumors and was prognostic only in the HPV(-) subset. Drawbacks from this study include its retrospective nature, the mix of primary sites, the small sample size, and the heterogeneity of treatment employed. These preliminary findings need to be validated in larger and more homogeneous HNSCC patient cohorts.

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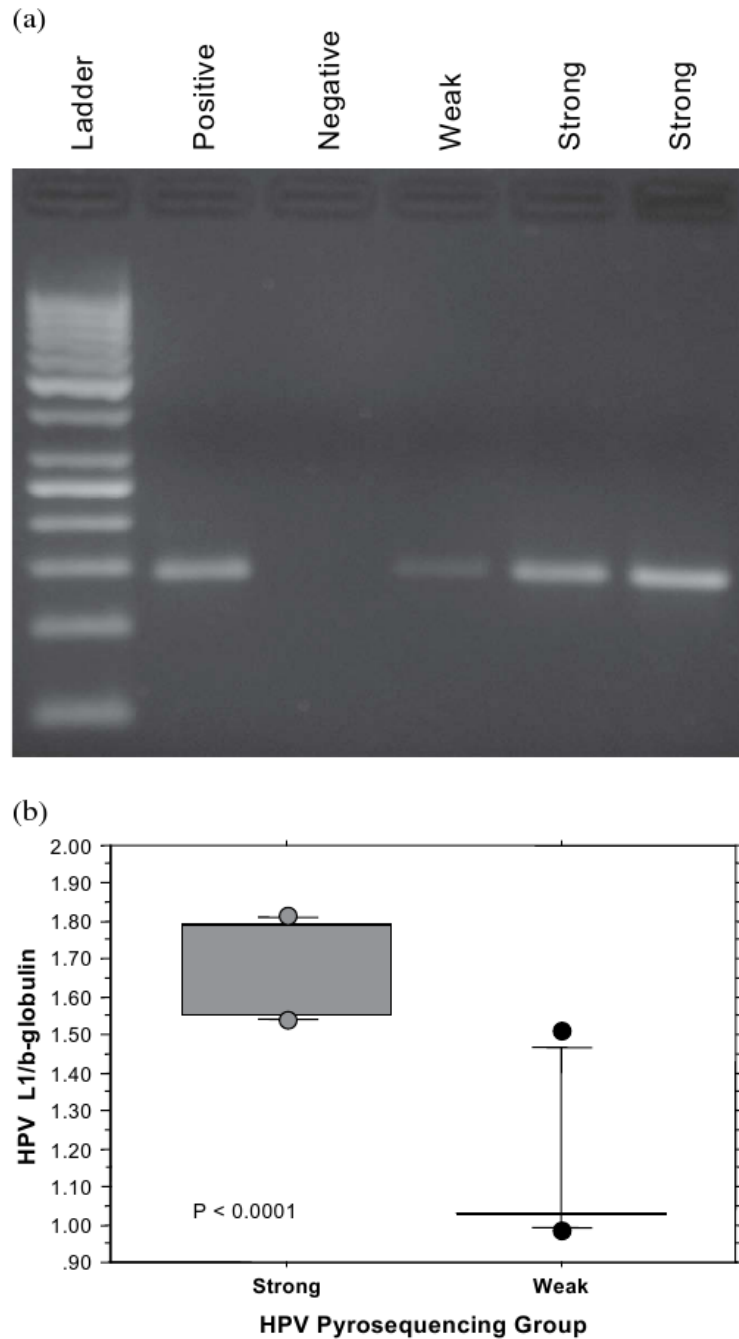
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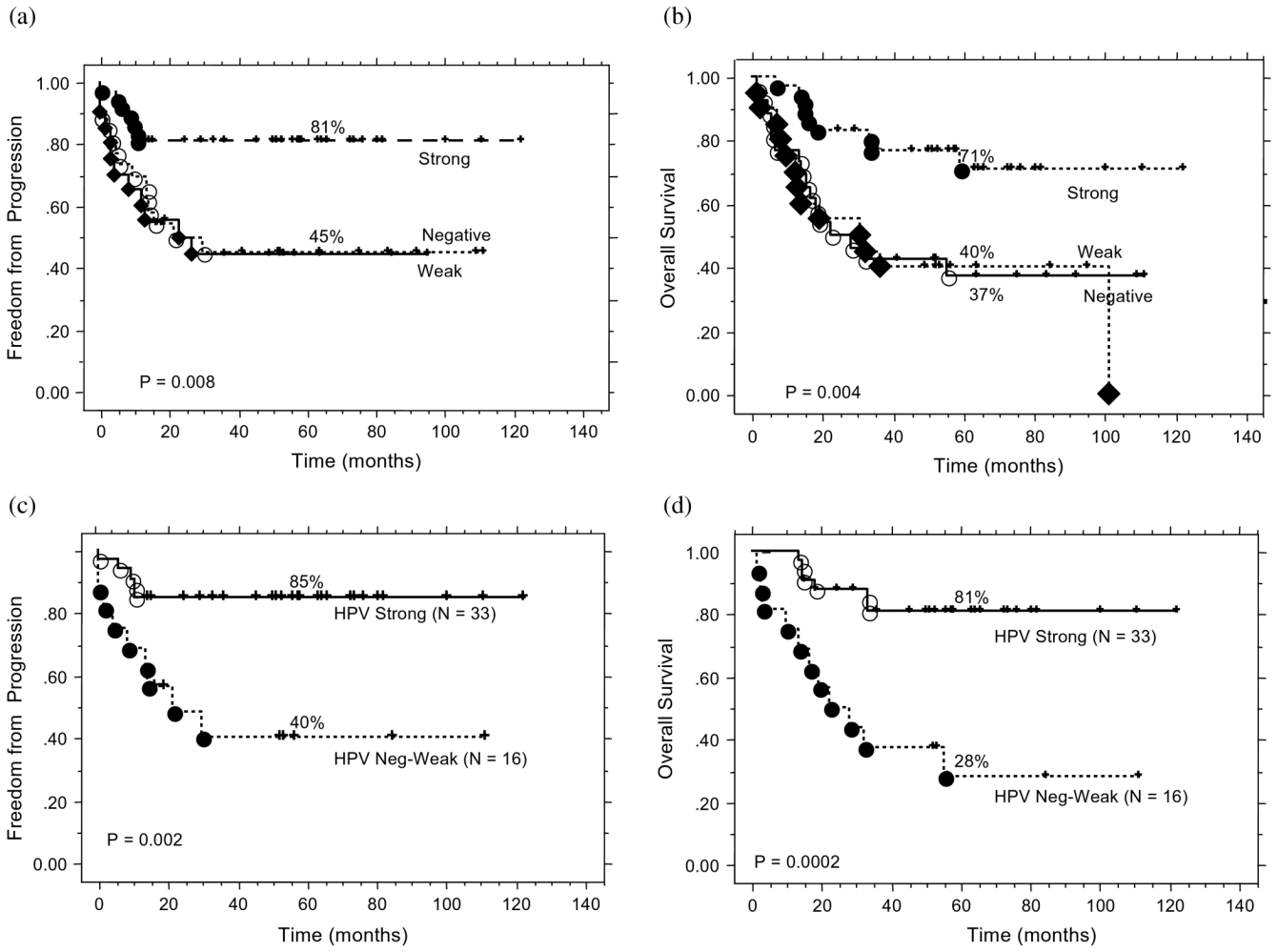
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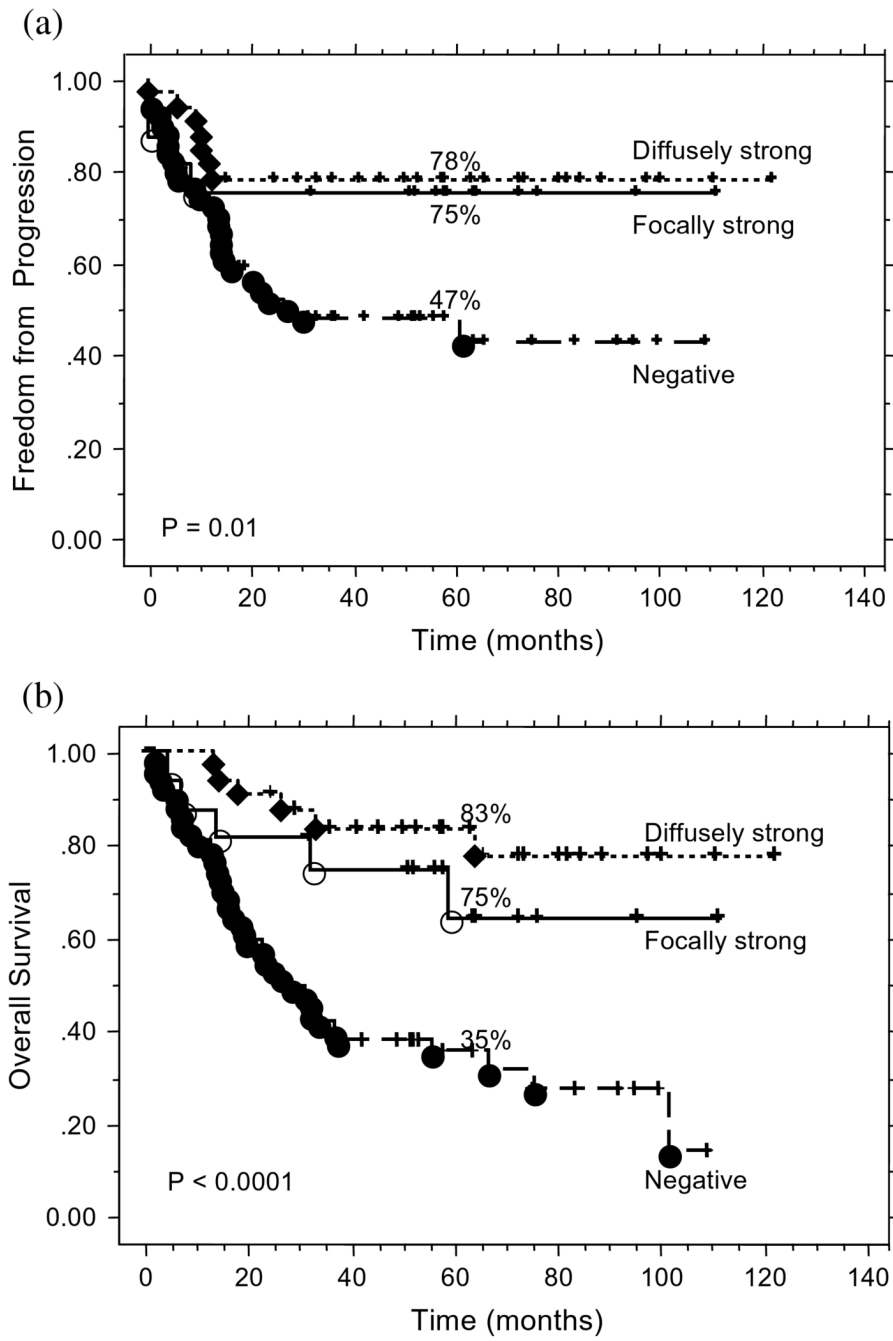


**Fig. 1.**

(a) Representative agarose gel stained with ethidium bromide for fragment size determination (at expected size of ~185 base pairs) and polymerase chain reaction (PCR) amplification yield for the L1 region of human papillomavirus (HPV) genome. The gel shows PCR results for samples with strong, weak, and negative signal intensity based on measured amplicon concentration (undetectable level as negative, <math><100\text{ ng}/10\ \mu\text{L}</math> as weak and \beta-globulin in 12 randomly selected samples: 6 from the HPV pyrosequencing strong group and 6 from the HPV pyrosequencing weak group.

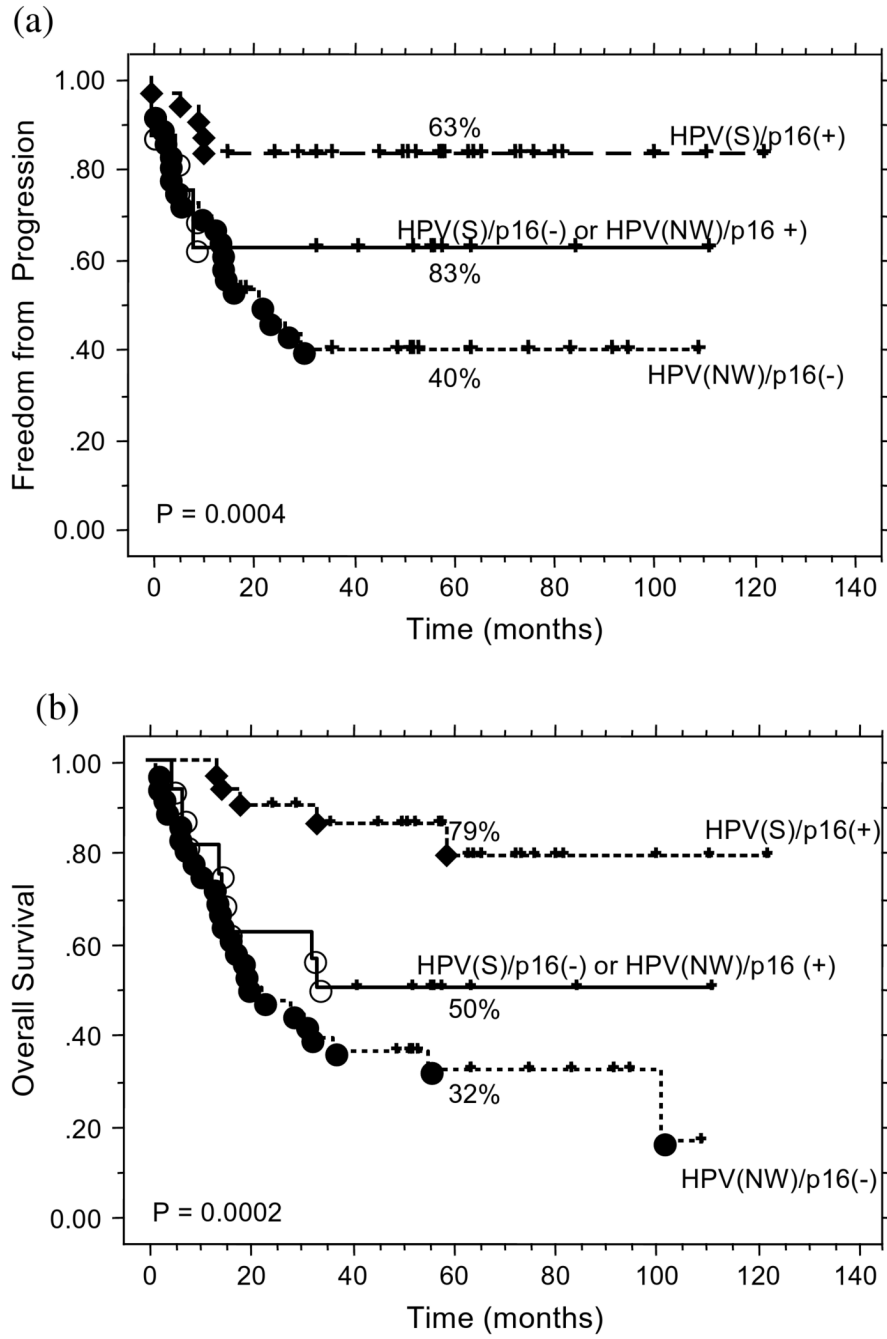


**Fig. 2.**  
 (a) Time to progression by human papillomavirus (HPV) pyrosequencing status for all patients.  
 (b) Overall survival by HPV pyrosequencing status for all patients. (c) Time to progression by HPV pyrosequencing status for the subset of patients with oropharyngeal carcinoma ( $n = 49$ ).  
 (d) Overall survival by HPV pyrosequencing status for the subset of patients with oropharyngeal carcinoma ( $n = 49$ )



**Fig. 3.** (a) Time to progression by p16<sup>INK4a</sup> status for all patients. (b) Overall survival by p16<sup>INK4a</sup> status for all patients.





**Fig. 4.** (a) Time to progression by human papillomavirus (HPV) and p16<sup>INK4a</sup> status combined for all patients. (b) Overall survival by HPV and p16<sup>INK4a</sup> status combined for all patients. HPV(S) = strong HPV signal; HPV(NW) = negative or weak HPV signal.

**Table 1**Relationship between HPV pyrosequencing status and p16<sup>INK4a</sup> staining intensity\*

p16 <sup>INK4a</sup> staining intensity	HPV pyrosequencing status			Total
	Negative	Weak	Strong	
Negative	20 (24.4%)	16 (19.5%)	6 (7.3%)	42 (51.2%)
Focally strong	5 (6.1%)	3 (3.7%)	6 (7.3%)	14 (17.1%)
Diffusely strong	1 (1.2%)	1 (1.2%)	24 (29.3%)	26 (31.7%)
Total	26 (31.7%)	20 (24.4%)	36 (43.9%)	82 (100%)

Abbreviation: HPV = human papillomavirus.

\*  $p < 0.0001$ ,  $\chi^2$  test.

**Table 2**

Relationship between HPV pyrosequencing status and clinical parameters

Parameter	HPV Neg/wk (n = 46)	HPV Strong (n = 36)	p value
Age			
Mean ( $\pm$ SE)	61 ( $\pm$ 2.1)	55 ( $\pm$ 1.6)	0.03
<60	20 (24.4%)	26 (31.7%)	0.01
$\geq$ 60	26 (31.7%)	10 (12.2%)	
Gender			
Male	36 (43.9%)	33 (40.2%)	0.13
Female	10 (12.2%)	3 (3.7%)	
Site			
Oropharynx	16 (19.5%)	33 (40.2%)	<0.0001
Oral cavity	12 (14.6%)	1 (1.2%)	
Larynx	6 (7.3%)	1 (1.2%)	
Hypopharynx	6 (7.3%)	1 (1.2%)	
Nasopharynx	3 (3.7%)	0 (1.2%)	
Other*	3 (3.7%)	0	
Cigarette pack-year			
Mean ( $\pm$ SE)	40.0 ( $\pm$ 5.5)	28.3 ( $\pm$ 4.5)	0.12
Differentiation			
Well	8 (9.8%)	4 (4.9%)	0.52
Moderate	29 (35.4%)	26 (31.7%)	
Poor	9 (11.0%)	6 (7.3%)	
T-classification			
0-2	20 (24.4%)	22 (26.8%)	0.12
3-4	26 (31.7%)	14 (17.1%)	
N-classification			
0-1	10 (12.2%)	1 (1.2%)	0.008
2	23 (28.0%)	29 (35.4%)	
3	13 (15.9%)	6 (7.3%)	
Stage			
II-III	8 (9.8%)	1 (1.2%)	0.07
IV	38 (46.3%)	35 (42.7%)	
Treatment			
Nonsurgical therapy <sup>†</sup>	31 (37.8%)	33 (40.2%)	0.01
Surgical therapy <sup>‡</sup>	15 (18.3%)	3 (3.7%)	
Hemoglobin			
Mean ( $\pm$ SE)	13.5 ( $\pm$ 0.28)	14.3 ( $\pm$ 0.23)	0.06
$\leq$ 12 g/dL	13 (15.9%)	3 (3.7%)	0.05
$>$ 12 g/dL	33 (40.2%)	31 (37.8%)	
Unknown	0	2 (2.4%)	

Abbreviations: CRT = chemoradiation; HPV = human papillomavirus; Neg = negative; OP = Oropharynx; RT = radiation; S = surgery; SE = standard error; Wk = weak.

\* Others included two paranasal sinus and one unknown squamous cell carcinomas.

<sup>†</sup> Primary nonsurgical therapy included radiation alone (3 patients) and chemoradiation (61 patients).

<sup>‡</sup> Primary surgical therapy included surgery alone (2 patients), surgery + postoperative radiation (9 patients), and surgery + postoperative chemoradiation (7 patients)

**Table 3**

Pattern of failure by HPV signal intensity

	HPV Status		<i>p</i> value	$\chi^2$
	Strong ( <i>n</i> = 36)	Negative-weak ( <i>n</i> = 46)		
% 5-year local control	94.4 ± 3.9	66.4 ± 7.5	0.004	8.3
% 5-year regional control	88.7 ± 5.3	67.1 ± 7.0	0.012	6.3
% 5-year freedom from distant metastasis	94.4 ± 3.8	73.6 ± 7.8	0.032	4.6

*Abbreviation:* HPV = human papillomavirus.

Table 4

Results of univariate and multivariate analyses

Parameter	TTP			DSS			OS		
	Univariate p value	Multivariate HR (95% CI)	p value	Univariate p value	Multivariate HR (95% CI)	p value	Univariate p value	Multivariate HR (95% CI)	p value
Age (cont, favor younger age)	0.16	NI	NI	0.06	NI	NI	0.017	NI*	NI*
Sex (F vs. M)	0.31	NI	NI	0.25	NI	NI	0.76	NI	NI
Smoking status (cont, favor less PY)	0.35	NI	NI	0.2	NI	NI	0.0031	1.01 (1.00–1.02)	0.014
HPV/p16 <sup>INK4a</sup> score									
(1)HPV(NW)/p16 <sup>INK4a</sup> (-)	—	—	—	—	—	—	—	—	—
(2)HPV(S)/p16 <sup>INK4a</sup> (-) or HPV(NW)/p16 <sup>INK4a</sup> (+)	0.2	0.55 (0.22–1.37)	.21	0.12	0.42 (0.16–1.12)	0.08	0.24	0.66 (0.29–1.51)	0.33
(3)HPV(S)/p16 <sup>INK4a</sup> (+) Favor group 20,001 and 3	20,001	0.23 (0.08–0.69)	0.008	0.0007	0.11 (0.03–0.50)	0.004	0.0002	0.20 (0.07–0.61)	0.004
N-Classification (N0–1 vs. N2–3)	0.62	NI	NI	0.73	NI	NI	0.43	NI	NI
Hb (Cont, g/dL, favor higher level)	0.02	0.86 (0.69–1.06)	0.15	0.005	0.82 (0.66–1.02)	0.073	0.0015	0.79 (0.66–0.96)	0.017
Treatment (CRT vs. S+RT, favor CRT)	0.007	2.31 (1.09–4.88)	0.028	0.002	2.64 (1.20–5.84)	0.016	0.032	2.42 (1.11–5.28)	0.027

Abbreviations: CI = confidence interval; Cont = on a continuum; CRT = chemoradiation; DSS = disease-specific survival; F = female; Hb = hemoglobin; HPV = human papillomavirus; HR = hazard ratio; M = male; NI = not included; OS = overall survival; PY = pack year of cigarette use; RT = radiation; S = surgery; TTP = time to progression.

\* Age not included because it highly correlated with pack-year of cigarette use.



**Table 5**

Relationship between HPV pyrosequencing signal intensity and other molecular markers

Parameters	All (%)	HPV negative-weak	HPV strong	<i>p</i> value
Median tumor pO <sub>2</sub> (mm Hg)				
Mean value	14.0	13.3	15.5	0.43
CAIX				
Negative	38 (46.3%)	19 (23.3%)	19 (23.2%)	0.44
Weak	30 (36.6%)	18 (22.0%)	12 (14.6%)	
Strong	13 (15.8%)	9 (11.0%)	4 (4.9%)	
Inevaluable	1 (1.2%)	0	1 (1.2%)	
EGFR staining intensity				
Negative	45 (54.9%)	17 (20.7%)	28 (34.1%)	0.0006
Weak	3 (3.7%)	1 (1.2%)	2 (2.4%)	
Strong	29 (35.4%)	24 (29.3%)	5 (6.1%)	
Inevaluable	5 (6.1%)	4 (4.9%)	1 (1.2%)	
% cells staining positive for EGFR				
0%–10%	47 (57.3%)	17 (20.7%)	30 (36.6%)	0.0002
11%–80%	16 (19.5%)	12 (14.6%)	4 (4.9%)	
81%–100%	14 (17.0%)	13 (15.8%)	1 (1.2%)	
Inevaluable	5 (6.1%)	4 (4.9%)	1 (1.2%)	
CD3 staining intensity				
Negative	4 (4.9%)	3 (3.7%)	1 (1.2%)	0.03
Weak	35 (42.7%)	24 (29.3%)	11 (13.4%)	
Strong	40 (48.9%)	16 (19.5%)	24 (29.3%)	
Inevaluable	3 (3.7%)	3 (3.7%)	0	

Abbreviation: HPV = human papillomavirus.

Table 6

Five-year overall survival by different biomarker categories, stratified by HPV status

Marker	Staining intensity	All patients	HPV neg-weak		HPV strong	
			p value	HPV neg-weak	p value	HPV strong
CAIX	Neg-weak	61% (n = 68)	0.007	42% (n = 37)	0.09	79% (n = 31)
	Strong	31% (n = 13)		22% (n = 9)		50% (n = 4)
EGFR	Neg-weak	72% (n = 48)	<0.0001	52% (n = 18)	0.03	83% (n = 30)
	Strong	19% (n = 29)		17% (n = 24)		30% (n = 5)
CD3	Neg-weak	34% (n = 39)	0.005	22% (n = 27)	0.04	64% (n = 12)
	Strong	70% (n = 40)		60% (n = 16)		75% (n = 24)

Abbreviations: HPV = human papillomavirus; neg = negative.