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**BIOLOGY CONTRIBUTION**

# THE RELATIONSHIP BETWEEN HUMAN PAPILLOMAVIRUS STATUS AND OTHER MOLECULAR PROGNOSTIC MARKERS IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

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**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 $p16^{INK4a}$ staining and treatment outcomes. It was correlated with tumor hypoxia (tumor $pO_2$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ staining was associated with better TTP ($p = 0.014$) and OS ($p = 0.00002$). There was no relationship between HPV status and tumor $pO_2$ 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. 

**HPV, Head and neck cancer, $p16^{INK4a}$, EGFR, Hypoxia, CD3.**

## 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 $p16^{INK4A}$, which acts by binding to cell-cycle regulators. Loss of $p16^{INK4A}$ is associated with poor prognosis in HNSCC and the converse is true for its overexpression (8, 9). In tobacco-related HNSCC, $p16^{INK4a}$ is often absent because of homologous deletion or promoter hypermethylation. However, in HPV(+) HNSCC tumors, $p16^{INK4a}$ is overexpressed 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 $p16^{INK4a}$ may explain the

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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 $p16^{INK4a}$ staining, evaluated the prognostic significance of HPV and $p16^{INK4a}$ in these tumors, explored the relationship between HPV and tumor hypoxia (by tumor $pO_2$ 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°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 exACTGGene 50-bp Mini DNA Ladder (Fisher Scientific, Pittsburgh, PA) was used to measure DNA concentration of HPV-fragment amplicons. Amplicon concentration <100 ng/10 $\mu$L PCR was scored as ''weak'' and ≥100 ng/10 $\mu$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 uL annealing buffer containing 0.4 pmol sequencing primer at 95°C for 2 min, 50°C for 5 min, and 25°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

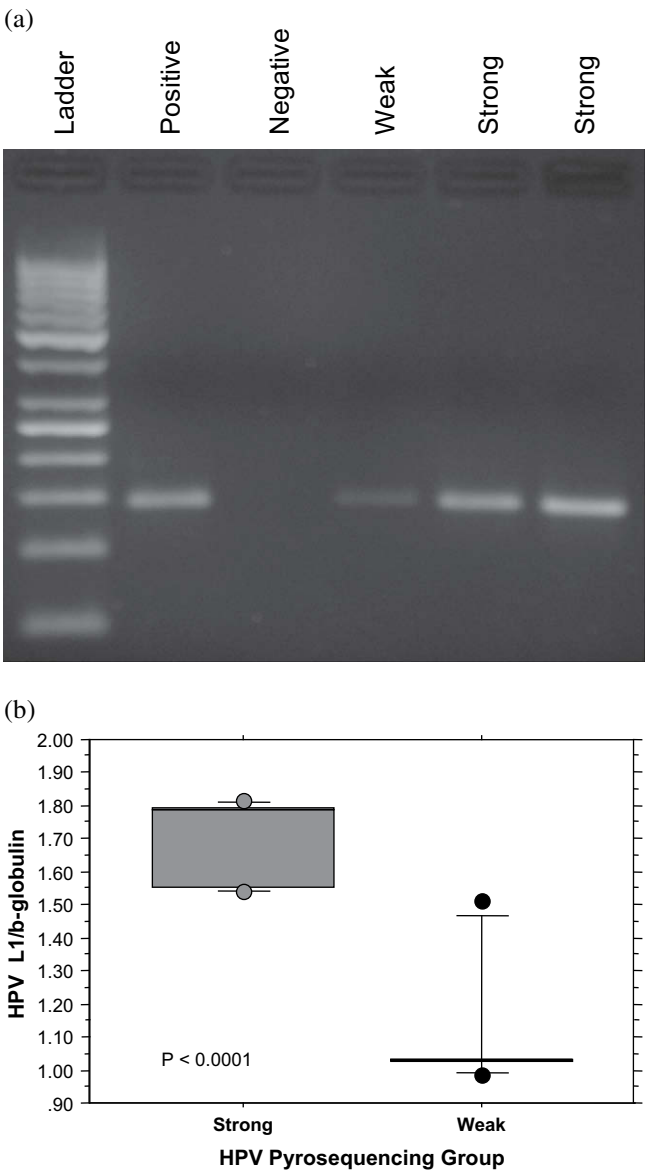


Fig. 1. (a) Representative agarose gel stained with ethidium bromide for fragment size determination (at expected size of ~185 base pairs) and polymerase chain reaction (PCR) amplification yield for the L1 region of human papillomavirus (HPV) genome. The gel shows PCR results for samples with strong, weak, and negative signal intensity based on measured amplicon concentration (undectable level as negative, <100 ng/10 $\mu$L as weak and ≥100 ng/10 $\mu$L strong). (b) Box and whisker plot, showing the quantitative real-time PCR results for HPV L1 signals normalized against $\beta$-globulin in 12 randomly selected samples: 6 from the HPV pyrosequencing strong group and 6 from the HPV pyrosequencing weak group.