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Head and neck squamous cell carcinoma: Ambiguous human papillomavirus status, elevated p16, and deleted retinoblastoma 1

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Abstract

Background—Head and neck squamous cell carcinoma (HNSCC) is potentially curable, but treatment planning remains a challenge. Oncogenic human papillomavirus (HPV)-positive disease is often associated with a good prognosis compared with HPV-negative disease. However, some HPV-positive HNSCC recurs, often with distant metastases and significant treatment resistance.

Methods and Results—We performed p16 immunohistochemistry (IHC), in situ hybridization (ISH) for high-risk HPV, and comprehensive genomic profiling on oropharyngeal HNSCC with basaloid features and particularly aggressive disease course, noting a rare genetic event: a deleting mutation (exons 5–17) of the tumor suppressor and dominant cell cycle regulator retinoblastoma 1 (RB1). Genomic and transcriptomic data available through FoundationOne and The Cancer Genome Atlas (TCGA) were reviewed for additional HNSCC cases with RB1 alterations.

Conclusion—RB1 alterations may have important prognostic implications, particularly in the context of high p16 expression, in both HPV-positive and HPV-negative HNSCC.

Keywords

head and neck squamous cell carcinoma; retinoblastoma 1 (RB1); The Cancer Genome Atlas (TCGA); p16; human papillomavirus

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INTRODUCTION

The 5-year survival rate for human papillomavirus (HPV)-positive cancer is 70% to 80% versus 25% to 40% for HPV-negative head and neck squamous cell carcinoma (HNSCC).^{1–4} A possible link between HPV-positive head and neck cancer and basaloid squamous cell carcinoma (BSCC), a rare variant of squamous cell carcinoma (SCC; approximately 2% of all SCCs)⁵ usually diagnosed in patients with more advanced-stage disease, has been suggested; it has also been proposed that HPV-positive and HPV-negative BSCC are 2 distinct subtypes.^{5,6} HPV-positive BSCC presents with features similar to those generally seen in HPV-positive HNSCC: afflicts a younger population; almost exclusively occurs in the oropharynx; and has a more favorable clinical outcome compared with its HPV-negative counterpart.⁷ A retrospective review of a large dataset ($n > 34,000$) indicated that BSCC histology did not independently alter overall survival.⁶

In HPV-positive disease, oncogenic viral infection results in E6/E7 inactivation of p53 (*TP53*) and retinoblastoma 1 (RB1), respectively.⁸ Immunohistochemical (IHC) staining of p16 (*CDKN2A*) is considered a reliable proxy for HPV status in cancers of the tongue base and tonsils.^{9,10} For other anatomic sites, p16 IHC staining falls below 50% in terms of true-positive rates.^{9,11} The accuracy of in situ hybridization (ISH) is similarly associated with anatomic sites, but the results are generally more difficult to interpret than p16 IHC staining.^{12,13} Polymerase chain reaction is considered the gold standard for HPV detection; however, polymerase chain reaction analysis is less practical than IHC or ISH for clinical purposes, both of which are more readily applied to formalin-fixed paraffin-embedded tissue.¹²

HPV-positive oropharyngeal cancer is highly treatment responsive, and cure rates are now reported to be over 90% for the best prognostic subsets.^{14,15} Nonetheless, recurrences are possible and have been reported up to 5 years after initial treatment.¹⁶ A greater understanding of molecular and clinical determinants of prognosis is needed. The following case report demonstrates this point with supplements from The Cancer Genome Atlas (TCGA) and FoundationOne database analyses.

CASE REPORT

A 65-year-old white man with a medical history of asbestosis, deep vein thrombosis, hyperlipidemia, atrial fibrillation, and pacemaker placement sought clinical care after developing a palpable neck mass. He had a 24-pack-year smoking history and previously worked as a steam-fitter. Fine-needle aspiration of the cervical lymph node revealed poorly differentiated carcinoma, classified as SCC. A CT scan revealed a heterogeneous soft-tissue mass along the tongue base, extending inferiorly to the epiglottis, and abutting the hyoid bone. A centrally necrotic left level IIa lymph node was also noted, measuring 2.4 cm. Significant ¹⁸F-fluorodeoxyglucose uptake was detected in the base of the tongue, involving the epiglottis and supraglottic space, as well as in the left and right level II cervical nodes (Figure 1A). Direct laryngoscopy was confirmatory. Hematoxylin-eosin staining of the tumor revealed SCC with basaloid features (Figure 1B) and IHC showed high expression of p16, interpreted as indicative of HPV infection (Figure 1C).

Accordingly, the patient was treated with concurrent chemoradiotherapy for stage IVA (T2N2c) HPV-positive BSCC of the base of the tongue, using weekly cisplatin (40 mg/m²) and 70 Gy of radiation over 7 weeks. Restaging positron emission tomography/CT revealed complete initial response above the clavicles. However, metastatic disease was noted with multiple bone and liver metastases. A biopsy confirmed SCC consistent with the tongue base primary. Concurrent ISH analysis of the primary tumor did not confirm HPV-positive status, as originally suggested by the elevated p16 levels. Subsequent comprehensive genomic profiling of the patient's tumor revealed mutated *RB1* – a finding potentially associated with worse prognosis¹⁷ – and wild-type *TP53* and *CDKN2A*. Interestingly, comprehensive genomic profiling also identified low levels of HPV viral DNA, in support of the original diagnosis of HPV-positive BSCC. The disease advanced despite treatment with a programmed cell death protein 1 targeting antibody on an investigational protocol.¹⁸ Restaging scans after 3 cycles of immunotherapy revealed progression of hepatic disease and new osseous metastatic disease.

Immunohistochemistry

IHC was performed for p16 on a representative 4-μm cut from a formalin-fixed paraffin-embedded tissue block using a purified mouse antihuman monoclonal antibody to p16INK4A (G175-405, 1:400 dilution; BD Pharmingen, Franklin Lakes, NJ) on a Ventana Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ). A positive test was defined as intermediate/strong nuclear and cytoplasmic p16 staining in 70% of cells and results were compared with both positive and negative controls.

In situ hybridization

The patient's tumor was sent to Integrated Oncology for ISH of HPV 6/11, 16/18, and 31/33.

Comprehensive genomic profiling

In brief, next-generation sequencing (hybrid capture-based) was performed by a Clinical Laboratory Improvement Amendment-certified laboratory (Foundation Medicine, Cambridge, MA) using DNA extracted from formalin-fixed paraffin-embedded sections, as previously described.¹⁹ The coding regions of 315 cancer-related genes as well as introns from 28 frequently rearranged cancer genes were sequenced using 49 bp reads (Illumina HiSeq 2500, Hayward, CA) with a median exon covered of 555X. Reads were mapped to human genome and analyzed for genomic alterations.

The Cancer Genome Atlas data analysis

Results for the TCGA cohort²⁰ shown in this study are based on data generated by the TCGA Research Network (<http://cancergenome.nih.gov/>). TCGA datasets were downloaded from cBioPortal^{21,22} or <http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>. Cutoff Finder²³ was used to identify cutoff points (for high vs low) for the TCGA cohort based on RNA sequencing expression data for p16 and/or RB1. Kaplan–Meier curves were generated and statistical significances were calculated using GraphPad Prism version 6.00 for Mac (GraphPad Software; La Jolla, CA).

Clinical pathology

At recurrence, biopsy of a liver metastasis confirmed SCC, as had been determined for the primary tumor (Figure 1B). ISH of the primary tumor was performed to ascertain whether HPV 16, 18, 31, or 33 were detectable. In distinction from the IHC and comprehensive genomic profiling results, ISH results were negative for HPV. ISH has been shown to have a false-negative rate of 13% to 41%.²⁴

Molecular analysis

Comprehensive genomic profiling of a specimen from a metastatic liver site revealed a deletion of *RB1* exons 5 to 17, predicted to inactivate RB1 function by disrupting the E2F transcription factor 1 (E2F1) binding pocket (Figure 1D).^{25–28} Comprehensive genomic profiling detected HPV 16 viral DNA, supporting the initial IHC-based diagnosis of HPV-positive HNSCC. *TP53* and *CDK2NA* were found to be wild type.

The Cancer Genome Atlas and FoundationOne cohort analysis

The TCGA (accessed using cBioportal)^{21,22} network has published data on 279 HNSCC samples (243 HPV-negative and 36 HPV-positive) for which HPV status was determined by HPV RNA-sequencing, HPV whole exome and whole genome sequencing, HPV MassArray, p16 staining, and HPV ISH.²⁰ *RB1* mutations (missense and truncating) are uncommon in HNSCC, occurring in 3% to 4% of cases (8/279, TCGA; 35/772 FoundationOne). However, inclusion of deep (likely homozygous) and shallow (likely heterozygous) *RB1* deletions increased the percentage of altered cases to 43% (Figure 2A). Presence of *RB1* mutations or deletions did not alter survival probability (data not shown), similar to what has been described for RB1 on the protein level.¹⁷ There was significant co-occurrence ($p = .003$) of p16 mRNA upregulation (RNA-sequencing; z-score >0.2) with *RB1* deletions/mutations (Figure 2A).

In the TCGA dataset, HPV-negative cases with upregulated p16 (HPV-negative/p16 high) had significantly better survival outcomes than HPV-negative cases (Figure 2B). A similar analysis stratifying cases with high p16 expression (HPV-positive and HPV-negative) by *RB1* expression also revealed significant survival differences, with high p16/low RB1 — the molecular profile described in the case report — correlating with significantly reduced survival (Figure 2C).

DISCUSSION

The aggressive natural history of the HPV-positive oropharyngeal carcinoma described in this case report prompts analysis of the tumor's molecular alterations. Cell cycle dysregulation is commonly seen in HNSCC, regardless of HPV status.²⁰ The most frequently altered cell cycle regulators in HNSCC are cyclin D (*CCND1*), p16 (*CDKN2A*), cyclin-dependent kinase 6 (*CDK6*), *RB1*, *E2F1*, and *TP53*.^{29,30} In normally cycling cells, interactions among *CCND1*, *CDK4*, and *CDK6* impose inactivating phosphorylation of RB1 (Figure 2D),¹⁷ dissociating RB1 from the transcription factor E2F, enabling G1 to S cell cycle progression.³¹ The p16 suppresses *CCND1*/*CDK4*/*CDK6* activity, and thereby indirectly reduces phosphorylation of RB1 (Figure 2D).¹⁷ In HPV-positive HNSCC, the

virally encoded oncoprotein E7²⁹ targets RB1 for degradation,³² likely to overcome the suppressive role of infection-related upregulation of p16 expression (potentially through induction of the histone 3 lysine 27-specific [H3K27] demethylases lysine [K]-specific demethylase 6A [KDM6A] and [KDM6B])^{33–35} in HPV-positive cells.³⁴ In contrast, HPV-negative cancer typically has lower p16 expression, but mutated TP53.^{20,36} It is unclear if cell cycle dysregulation affects response to immunotherapy; in this case report, resistance to pembrolizumab³⁷ may have been due to the absence of microsatellite instability and only an intermediate mutation burden (8 per megabase; FoundationOne), both factors reported to be response predictive in some cancers, if present or high, respectively.^{38,39}

The relevance of basaloid features in the presence or absence of HPV infection is not fully understood and warrants further study, particularly with concurrent consideration of smoking status.^{5,6} The patient's significant smoking history is a potentially confounding factor; genomically, the patient's tumor was TP53 and *CDKN2A* wild type, which is more consistent with HPV-positive disease, and for both genes to be wild type would be rare for HPV-negative tobacco-associated tumors.^{20,40} However, that does not eliminate the possibility that tobacco smoke was the causative agent in the described case, nor does it clarify the genomic impact of long-term exposure to tobacco on HPV-positive cancers, particularly in terms of RB1 status.

Previous clinical studies of HPV and p16 status suggested a 3 class model, in which class III (HPV-positive/p16-high) is associated with the most favorable survival outcome compared with classes I and II (HPV-negative/p16-low and HPV-positive/p16-low, respectively),⁴¹ a pattern loosely applicable to recurrent or metastatic head and neck cancer.⁴² Identification of HPV-negative cases with upregulated p16 raises the provocative possibility of an additional HNSCC class, “class IV” (Figure 2E), which, based on the TCGA dataset, has survival characteristics similar to HPV-positive cases and significantly better survival outcomes than HPV-negative cases (Figure 2B). Large-scale efforts are needed to study “class IV” cases and to determine if further classification of HNSCC cases is of clinical relevance. Chung et al⁴³ recently published clinical data for nonoropharyngeal SCC and compared it to previously published data for oropharyngeal SCC,^{15,44,45} highlighting prolonged survival of patients with p16-positive oropharyngeal SCC (including base of the tongue). The 5-year overall survival estimates were 79.8% (95% confidence interval [CI] = 76.0% to 83.7%) and 45.8% (95% CI = 38.1% to 53.5%) for patients with p16-positive and p16-negative oropharyngeal SCC, respectively.⁴³ The authors could not include genomic determination of HPV status in the analysis of oropharyngeal SCC; for cases of HPV-negative, nonoropharyngeal SCC (oral cavity, hypopharynx, or larynx), high levels of p16 based on IHC also predicted improved survival. Inaccurate determination of HPV status and a low number of cases for some of the groups represented significant limitations of the study.^{43,46}

Interestingly, further analysis of the integrating pattern of *RB1* expression in cases with high p16 expression (HPV-positive and HPV-negative) suggested significant survival differences. The overall survival for the p16-high/RB1-high group (median survival of 76.20 months [95% CI = 1.053–6.128]) was significantly better than for the p16-high/RB1-low group (median survival of 17.10 months [95% CI = 1.5–4.1]; $p = .0282$; Figure 2C). Without consideration of *RB1*, the reported patient's disease matches class III (HPV-positive/p16-

high); however, in view of the unexpected disease progression, we hypothesize that loss of RB1 warrants consideration and propose modification of classes III and IV by adding “RB1-high (A)” and “RB1-low (B)” to each class (Figure 2E). The patient’s disease now matches class IIIB (HPV-positive/p16-high/RB1-low) with a much worse prognosis than the originally defined class III,⁴¹ as also seen in the TCGA cohort (Figure 2C). Perhaps in the context of downregulated RB1, the survival benefit associated with high p16⁴³ is lost because of the nullification of p16’s suppressive role on cell cycle progression (Figure 2D); it is possible that alterations in other genes involved in cell cycle regulation also affect the survival of patient’s with HPV-positive/p16-high HNSCC.

Important limitations of this case report are: that the proposed model has not been validated using an independent patient cohort; lack of functional validation of the deletion of RB1 exons 5 to 17; and inherent limitations of the TCGA datasets (discussed here^{47,48}). Particularly functional studies using HPV-negative models of HNSCC are needed to validate the precise impact of a deletion of RB1 exons 5 to 17 in terms of E2F1 binding and in terms of additional known functions of RB1.^{49,50}

In summary, specific consideration of “class IV” HNSCC cases and cell cycle regulators, particularly RB1 for “class IV” and “class III” (Figure 2E),¹⁷ which may influence response to therapy and prognosis regardless of HPV status, could facilitate appropriate stratification of patients in clinical trials, assessment of response to therapy, and a more accurate projection of prognosis. Large-scale clinical studies are needed to evaluate the proposed role and value of considering RB1 functional status in HNSCC.

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FIGURE 1. Pertinent findings. (A) ^{18}F -fluorodeoxyglucose uptake displayed via positron emission tomography/CT. (B) Hematoxylin-eosin (H&E) staining of the patient's tumor. (C) The p16 immunohistochemical (IHC) staining of the patient's tumor. (D) Schematic representation of the detected retinoblastoma 1 (RB1) deletion. [Color figure can be viewed at wileyonlinelibrary.com].

FIGURE 2.

Analysis of The Cancer Genome Atlas head and neck squamous cell carcinoma (HNSCC) datasets. (A) Alterations in p16 and retinoblastoma 1 (RB1) for 279 HNSCC cases (human papillomavirus [HPV]-negative and HPV-positive). (B) Kaplan–Meier survival plots comparing HPV-positive, HPV-negative, and HPV-negative/p16-high (RNA sequencing) cases (C) Kaplan–Meier survival plots comparing cases with p16-high/RB1-high and p16-high/RB1-low expression (RNA sequencing). (D) Schematic representation of RB1-associated cell cycle regulation. (E) Previously defined classes of HNSCC based on HPV status and p16⁴¹ with the addition of HPV-negative/p16-high as a new class, and “A” RB1-high and “B” RB1-low as subcategories of classes III and IV. CDK, cyclin-dependent kinase; CCND1, cyclin D1. [Color figure can be viewed at wileyonlinelibrary.com].