Emerging Trends in the Pathological Research of Human Papillomavirus-positive Oropharyngeal Squamous Cell Carcinoma

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Abstract

Oropharyngeal squamous cell carcinomas (OPSCCs) have shown an alarming rate of increase in incidence over the past several decades, markedly in men. In the United States, transcriptionally-active human papillomavirus (HPV), particularly HPV 16, has become the highest contributive agent of OPSCCs, affecting approximately 16,000 people a year. Compared to patients with HPV-negative OPSCCs, patients with HPV-positive OPSCCs exhibit better health responses to chemoradiotherapy and an overall increase in long-term survival. Despite promising treatment options, many OPSCCs are discovered at an advanced stage, and ~20% of cases will recur after definitive treatment. Therefore, extensive research is ongoing to identify new targets for precision treatment and to stratify tumor prognosis. The aim of this review is to capture the most updated research on HPV-positive OPSCCs, emphasizing their relevance as potential new targets for precision medicine and survival prognosis.

Keywords

Tumor microenvironment; APOBEC3; DNA methylation; Novel therapeutics

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Author contributions

Study design (HW, QS, JL), analysis and interpretation of data (JC, HW, YX, KP), manuscript writing (JC, QS, YX, JL, KP, HW), critical revision (JC, QS, YX, JL, KP, HW), critical funding (HW). All authors have made a significant contribution to this study and have approved the final manuscript.

Conflict of interest

JL has been an editorial board member since January 1, 2022, and HW has been a Deputy Editor-in-Chief of Journal of Clinical and Translational Pathology since May 2021. The authors have no other conflicts of interest related to this publication.

Ethical statement

This is a review article, no Institutional Review Board approval was required. However, the written informed consent was obtained from the patient for publication of the images.
Introduction

Approximately 80% of oropharyngeal squamous cell carcinomas (OPSCCs), which include oropharyngeal wall, tonsillar, and base-of-tongue tumors, are induced by high-risk human papillomavirus (HPV) (Fig. 1).1–3 HPV-positive OPSCC patients are typically younger, male (approximately 75%), and usually nonsmokers.4,5 HPV-positive OPSCCs show distinctive biology and have better prognoses than HPV-negative OPSCCs, which has initiated the development of de-escalation treatment clinical trials that aim to promote positive results while reducing treatment-associated comorbidities.6,7 Despite the better prognosis, about 20% of OPSCC patients will recur after 5 years of initial treatment, with an additional percentage of fatal cases.6,8 Nevertheless, recent progress in OPSCC research has identified potential novel targets for precision medicine and early tumor detection.

Tumor microenvironment

The tumor microenvironment is the system around a tumor, including the blood vessels, immune cells, fibroblasts, signaling molecules, and the extracellular matrix. Most high-risk HPV infections are eliminated by the body’s robust immune system, which recognizes viral antigens by T lymphocytes.9 However, viral infections in HPV-positive OPSCCs persist by manipulating the immune system and surrounding micro-system. The tumor microenvironment (TME) includes various stromal cells such as rich lymphocytes and myeloid cells, fibroblasts, and endothelial cells, which interact with tumor cells.9–12 The TME of OPSCCs is highly immunosuppressive by presenting immune checkpoint ligands, down-regulating human leukocyte antigen expression, inactivating the nuclear factor-kappa B pathway, causing cytotoxic T lymphocytes to malfunction, and activating immunosuppressive cell types, such as regulatory T cells, tumor-associated macrophages, and myeloid-derived suppressor cells (Fig. 2).10–15 The immunomodulatory effect of HPV-related OPSCCs is also related to the HPV integration status. Compared to HPV-integrated OPSCCs, integration-negative tumors demonstrate significant elevation of genes expressed in natural killer cells, T-cells (CD4+, regulatory, CD3+, and CD8+), and B cells. Integration-negative tumors are associated with better prognoses.16 In HPV-positive OPSCCs, tumor-infiltrating lymphocytes (TILs) are reported to exert a protective function by way of an adaptive host immune response that targets viral antigens, leading to the identification of a specific subpopulation of lymphocytes that fights against HPV-associated cancer (HPV-16 E7 T cells).17

A series of efforts have been made to employ biomarkers from the TME, including TILs, to guide prognosis and precision immunotherapies for OPSCCs. Using the enrichment scores of 33 immune cell types based on the gene expression data of OPSCC tissues and the surrounding benign tissues, Mito et al.18 identified three types of immune signatures—cold, lymphocyte, and myeloid/dendritic cell. Most HPV-positive OPSCCs exhibit lymphocyte signatures, with several immune cell types, including CD4+ T cells, CD8+ T cells, B cells, and plasma cells, showing the highest scores. HPV-positive OPSCCs also demonstrate the longest overall survival.18 Faraji et al.19 also have revealed that an increased TIL density correlates with a low risk of recurrence in low-stage HPV-positive OPSCC. In contrast, decreased TIL infiltration, associated with cigarette exposure, has been linked to a higher
The spatial architecture patterns of TILs and the surrounding nucleated cells in hematoxylin and eosin-stained images of HPV-positive OPSCC patients can be characterized using P-TIL, an imaging biomarker, which can help differentiate stage I HPV-positive OPSCC patients into low- and high-risk subgroups and help patient triage for de-escalation.

**Aberrant DNA methylation and prognosis**

HPV E6 and E7 regulate DNA methylation of the host genome in addition to inactivating p53 and retinoblastoma protein, respectively.\textsuperscript{22–24} Earlier studies indicate that HPV-positive OPSCCs tend to contain higher amounts of aberrantly methylated DNA in the individual genes involved in cell-cycle regulation, cellular adhesion, cellular migration, apoptosis, and differentiation.\textsuperscript{25,26} Recent comprehensive DNA methylation studies at the whole-genome level also have revealed an HPV-positive OPSCC subtype with DNA hypermethylation. Moreover, Ando et al.\textsuperscript{27} have investigated the methylation profiles of HPV-positive head and neck squamous cell carcinomas (HNSCCs) and healthy mucosal samples and identified a group of 59 genes with a negative correlation between DNA methylation and RNA expression; furthermore, unsupervised hierarchical clustering analysis of the genes revealed a high-DNA-methylation phenotype in HPV-positive cases. Additional analysis of the 59 genes in The Cancer Genome Atlas (TCGA) OPSCC samples demonstrated the high-DNA-methylation phenotype. Likewise, Nakagawa et al.\textsuperscript{28} found a high-DNA-methylation subtype in HPV-positive OPSCCs, which was positively correlated with an improved prognosis. After performing Infinium 450 k array analysis on 170 OPSCC samples, unsupervised hierarchical clustering with >1,000 probes showed that HPV-positive OPSCCs were stratified into two epigenotypes with distinct clinicopathological features. The HPV-positive, high-DNA-methylation phenotype had the best outcome among the HPV-positive OPSCC cases. It is known that HPV-positive HNSCCs have two different types of HPV infection patterns—HPV integration-positive and HPV integration-negative (episomal). In addition, Ren et al.\textsuperscript{29} have shown that infection patterns correlate with HPV gene expression patterns. In HPV-positive HNSCC cases from the TCGA dataset, those with HPV integration-positive tumors showed a high expression of E6/E7 and a low expression of E2/E4/E5. In contrast, those with HPV integration-negative tumors showed an increased expression of E2/E4/E5 and a low expression of E6/E7. These findings were validated using an independent HPV-related OPSCC set and a TCGA cervical cancer cohort. When the HPV genome integrates into the human genome, E2 is usually disrupted, resulting in the upregulation of E6/E7.\textsuperscript{30–32} Although further analysis is needed, the upregulated E6 and E7 genes may be associated with the different HNSCC subtype DNA methylation patterns.

**Apolipoprotein B mRNA editing enzyme catalytic sub-unit-like protein 3**

Whole-genome sequencing studies of various cancers over the last decade have led to an important conclusion: almost all HPV-positive head/neck cancer (HNC), in addition to many HPV-negative HNC and cancers from several other organ systems, have a high percentage of somatic mutations linked to members of the apolipoprotein B mRNA editing enzyme catalytic subunit-like protein 3 (APOBEC3, A3) family.\textsuperscript{33–38} APOBEC3-induced mutations include cancer driver mutations like the activating mutations in PIK3CA.\textsuperscript{39,40}
APOBEC-mediated mutational signatures are found, with striking similarity, in viral and cancer genomes. The APOBEC3 mutation signature is defined by C-to-T and C-to-G changes in 5′-TCA and 5′-TCT trinucleotide motifs. The human APOBEC3 family includes seven enzymes, A3A-D and A3F-H. In HPV-positive and HPV-negative HNCs, current evidence points to APOBEC3A (A3A) and APOBEC3B (A3B) as the most likely sources of the overall APOBEC mutation signature. As part of the innate immune response following high-risk HPV infections, both A3A and A3B are upregulated by HPV oncoproteins (E6 and E7) and by interferon, potentially to promote viral genetic diversification, while collateral host genomic DNA damage contributes to carcinogenesis. Law et al. have demonstrated that in a murine adenomatous polyposis coli multiple intestinal neoplasia model, transgenic overexpression of human A3A, but not A3G, resulted in an increased incidence of polyp formation and induced C-to-T mutations in APOBEC-signature trinucleotide motifs. All seven human A3 enzymes in the murine fumaryl-acetoacetate hydrolase model for hepatocellular carcinoma, including A3A, A3B, and A3H, were tested sequentially. However, only human A3A significantly increased the frequencies of hepatocellular tumors to above control levels. The most substantial evidence that APOBEC3 enzymes drive tumor evolution in humans is their positive associations with poor clinical outcomes and significant increases in the proportion of APOBEC3 signature mutations from primary to metastatic disease.

One remaining question is whether viral editing and host genome mutations are concurrently linked events mediated by the same A3. In one recent study, Faden et al. quantified the APOBEC mutational burden and activity in both the host and virus by sequencing the host somatic exomes, transcriptomes, and HPV16 genomes from 79 HPV-positive human OPSCC specimens. They concluded that the primary mutational signature in somatic exomes is APOBEC. While there is a mean of five (range: 0–29) mutations per genome in viral genomes, APOBEC mutations have a mean of one (range: 0–5). Compared to non-APOBEC mutations, viral APOBEC mutations are more often low-variant allele fraction mutations, indicating that APOBEC mutagenesis actively occurs in viral genomes during infection. HPV/APOBEC-induced mutation patterns in OPSCCs are similar to the mutation patterns observed in cervical tumors. Additionally, paired host and viral analyses reveal that APOBEC-enriched tumor samples have higher viral APOBEC mutation rates (p=0.028) and APOBEC-associated RNA editing (p=0.008), which further indicates that APOBEC mutagenesis in host and viral genomes is directly linked and likely occurs during infection. The above observation supports the general hypothesis that HPV induces A3 activity, possibly to generate variation in viral progeny. However, albeit infrequently, mutations in cancer-causing genes such as PIK3CA can result from other viral activities such as the induction of replication stress, which can cause A3 activity against the host genome. Although cells with these mutations gain a selective survival advantage, they can be suppressed for years by the host immune system. Therefore, tumor development, even in the beginning stages of HPV infection, can result from A3 activity.

**Novel therapeutic approaches for HPV-positive OPSCCs**

By promoting de novo or potentiating pre-existing antitumor immune responses, immune therapy for HPV-positive OPSCC has been developed based on our expanding knowledge.
of tumor immunology and the TME. As mentioned earlier, HPV-positive OPSCC belongs to the most immune-rich category of tumors, and it is associated with infiltration of type I macrophages, natural killer cells, CD4+ CD25+ Tregs, CD8+ PD-1+ T cells, and high expression of PD-1, CTLA-4, and TIM-3 on the T cells. Not surprisingly, HPV-positive OPSCC has repeatedly shown favorable responses to immune checkpoint inhibitors in animal models and clinical trials. For example, one clinical trial (Keynote-012) that evaluated pembrolizumab (an anti-PD-1 antibody) in refractory/metastatic HNSCC patients showed that although the overall objective response rate was 18%, subgroup analyses illustrated that the response rate was 25% in HPV-positive patients versus 14% in HPV-negative HNSCC patients. In another multi-institutional, international durvalumab trial with an anti-PD-L1 antibody, the trial NCT02207530 showed an objective response rate of 16.2%, which translates into 29.4% in HPV-positive patients and 10.8% in HPV-negative patients. Although immunotherapies do not directly target driver oncogenic mutations, they do affect immune cells in tumor nests and can influence tumor responses to treatments. Various immune checkpoint inhibitors, including anti-PD1, anti-PDL1, and CTLA-4 antibodies, have been tested to treat HNSCC; however, thus far, only anti-PD-1/ PD-L1 antibodies have been approved for clinical use. The anti-PD-1 antibodies nivolumab and pembrolizumab were granted Federal Drug Administration approval in 2016 to treat patients with metastatic, platinum-refractory HNSCC, with supporting data from the CheckMate-141 and KEYNOTE-040 trials, respectively. In 2019, pembrolizumab was further approved as a first-line therapy for patients with PD-L1-positive metastatic or unresectable HNSCC, with supporting data from the KEYNOTE-048 trial.

Therapeutic vaccines transfer antigens to antigen-presenting cells and trigger cytotoxic T-cell and/or helper T-cell responses to get rid of existing tumors. A variety of therapeutic vaccines have been developed, including live vector (bacterial or viral vector), peptide, DNA/RNA, and whole cell-based vaccines. In recent years, a series of therapeutic vaccines have been evaluated in clinical trials to treat patients with HPV-positive OPSCC. In addition, many studies are now evaluating the therapeutic effect of vaccines in combination with an immune checkpoint inhibitor or other immunomodulatory agent. Early results from a few trials show promising results. For example, an objective response rate of 36% and a median overall duration of 17.5 months were observed in 22 HPV-positive OPSCC patients in a trial combining nivolumab with an HPV-16 E6/E7 peptide vaccine, and these results were superior to those from trials evaluating nivolumab alone. In addition, in the MEDI0457 trial, a DNA vaccine encoding the E6 and E7 antigens was administered with DNA encoding interleukin-12, which induced lasting HPV-specific immune activity in 18 of 21 patients with locally advanced p16-positive HNSCC. Another ongoing clinical trial is testing an E7-targeting mRNA vaccine delivered in combination with an agonistic anti-CD40 antibody. Moreover, a novel E6/E7-targeting vaccine, with or without immune checkpoint inhibitors, is being tested. The results of these vaccine trials will be the next cornerstone of immunotherapies for HPV-positive OPSCC patients.

DNA methylation is widespread in OPSCCs and substantially impacts tumor prognosis, so methylation seems to be a natural therapeutic target for HPV-positive OPSCC treatment. Epigenetic therapies of 5-azacytidine and 5-aza-20-deoxycytidine, which are cytidine analogs incorporated into DNA, lead to covalent adduct formation and work as DNA
methyltransferase inhibitors. The Federal Drug Administration has approved these therapies to treat certain myelodysplastic syndromes and chronic myelomonocytic leukemia cases, but their efficacy for solid cancers is still under consideration. One ongoing clinical trial of 5-azacytidine aims to treat HPV-positive and HPV-negative HNSCCs (NCT02178072).

One critical question before making A3 a therapeutic target for HPV-positive OPSCC patients is whether A3 activity against the host genome is occurring at diagnosis and during treatment. More and more evidence suggests that A3 activity continues to generate mutations during treatment. For example, a recent study in which suppression of A3B expression by inducible RNA interference delayed the acquisition of tamoxifen resistance in an \textit{in vivo} xenografted breast cancer model supports the concept that A3 activity contributes to the evolution of therapeutic resistance. This observation suggests a therapeutic benefit of inhibiting A3B and other A3 enzymes as an adjuvant to chemotherapy, a notion actively pursued in academia and industry alike.

Conclusions

Significant progress has been made over the last five years in understanding the pathogenesis of HPV-positive OPSCCs, especially in the TME, APOBEC3-induced somatic mutations, aberrant DNA methylation, as well as novel therapeutics. Biomarkers based on the in-depth understanding of the abovementioned pathological processes, such as the density of tumor-infiltrating lymphocytes and high-DNA-methylation profiles, serve as new and refined prognostic markers for OPSCC patient survival, thus guiding the treatment strategy. In addition, emerging therapies based on the latest discoveries, including immune checkpoint inhibitors, APOBEC3B inhibitors, and DNA methyltransferase inhibitors, will provide additional precision treatments for OPSCC patients.

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Abbreviations:

- \textbf{APOBEC3} \quad A3, apolipoprotein B mRNA editing enzyme catalytic subunit-like protein 3
- \textbf{HNC} \quad head/neck cancer
- \textbf{HNSCCs} \quad head and neck squamous cell carcinomas
- \textbf{HPV} \quad human papillomavirus
- \textbf{OPSCC} \quad oropharyngeal squamous cell carcinoma
- \textbf{TCGA} \quad The Cancer Genome Atlas
- \textbf{TILs} \quad tumor-infiltrating lymphocytes

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Fig. 1. Oropharyngeal squamous cell carcinoma.
(a) A 66-year-old male with a 4-centimeter mass (T) involving the left base of the tongue and oropharynx with focal ulceration. (b–d) The computed tomography scan (b, axial), magnetic resonance image (c, T1, axial), and positron emission tomography/computed tomography scan (d, axial) showing a hyperintense base of the tongue lesion that extends into the floor of the mouth (arrows) and left neck level 2 lymph node metastasis (arrow heads).
Fig. 2. Oropharyngeal squamous cell carcinoma.
(a, b) Biopsy of the base of the tongue mass (stained with hematoxylin and eosin), shown in Figure 1, demonstrating the morphology of human papillomavirus-related squamous cell carcinoma with tumor-infiltrating lymphocytes (b, inset). (c, d) Immunohistochemistry showing that the tumor cells are positive for p40 (c) and p16 (d). (a, 100× magnification; b–d, 400× magnification).