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Advances in human papillomavirus detection and molecular understanding in head and neck cancers: Implications for clinical management

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Abstract

Head and neck cancers (HNCs), primarily head and neck squamous cell carcinoma (HNSCC), are associated with high-risk human papillomavirus (HR HPV), notably HPV16 and HPV18. HPV status guides treatment and predicts outcomes, with distinct molecular pathways in HPV-driven HNSCC influencing survival rates. HNC incidence is rising globally, with regional variations reflecting diverse risk factors, including tobacco, alcohol, and HPV infection. Oropharyngeal cancers attributed to HPV have significantly increased, particularly in regions like the United States. The HPV16 genome, characterized by oncoproteins E6 and E7, disrupts crucial cell cycle regulators, including tumor protein p53 (TP53) and retinoblastoma (Rb), contributing to HNSCC pathogenesis. P16 immunohistochemistry (IHC) is a reliable surrogate marker for HPV16 positivity, while in situ hybridization and polymerase chain reaction (PCR) techniques, notably reverse transcription-quantitative PCR (RT-qPCR), offer sensitive HPV detection. Liquid-based RT-qPCR, especially in saliva, shows promise for noninvasive HPV detection, offering simplicity, cost-effectiveness, and patient compliance. These molecular advancements enhance diagnostic accuracy, guide treatment decisions, and improve patient outcomes in HNC management. In conclusion, advances in HPV detection and molecular understanding have significant clinical management implications. Integrating these advancements into routine practice could ultimately improve patient outcomes.

KEYWORDS

early detection, head and neck cancers, HPV16, human papillomavirus, liquid biopsy

Abbreviations: HNC, head and neck cancers; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; OPC, oropharyngeal cancers; OPSCC, oropharyngeal squamous cell carcinoma.

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1 | HUMAN PAPILLOMAVIRUS (HPV)-DRIVEN HEAD AND NECK CANCERS (HNCs)

1.1 | Epidemiology

HNCs are a group of malignancies affecting the upper region of the respiratory and digestive tracts,[1] with the most common type being head and neck squamous cell carcinoma (HNSCC).[2,3] HNC has gained clinical attention due to its relationship with the high-risk HPV (HR HPV), with HPV16 and 18 collectively accounting for 85% of HNC cases worldwide.[4]

It is now understood that HPV16 is the predominant causal type that presents in 69.2% of laryngeal cancers and 68.2% of oral cavity cancers.[5] HPV status is a critical factor in determining the most appropriate treatment approach and predicting patient prognosis.[1,2,6–8] Studies have shown that patients with HPV-driven HNSCC are a distinct clinical subtype that exhibits unique cancer-associated pathways, which can impact and improve overall survival (OS) rates.

The genetic landscape of HNSCC carcinogenesis demonstrates two distinct oncogenic pathways. HNSCC can be stratified in HPV-negative driven by chemical carcinogens such as nicotine and HPV-positive cancers, driven by HPV infection.[9,10] Due to these casual differences (Table 1), the accurate diagnoses of HPV16 positive and negative cases may influence treatment options.[6]

1.2 | HNC incidence and global burden

Globally, HNC ranks as the seventh most common cancer, with over 660 000 new cases, 325 000 deaths reported annually,[11] and approximately 38 000 cases of HNC are attributable to HPV.[4] The incidence rate of HNC has increased by 36.5% over the past decade[12,13] and is anticipated to increase by 30% by 2030 globally.[8,12,14] Upon closer inspection, the global incidence of HNC demonstrated a significant overall increase from 2005 to 2015 of 36.5% in lip and oral cavity cancer, 16.5% in nasopharyngeal cancer, and 23.1% in laryngeal cancer.[15]

According to reported cases of oral cancer worldwide and across all Global Burden of Disease regions, in 2019, the highest Age-Standardized Incidence Rate was identified in South Asia at 9.65 per 100 000 individuals, a rate approximately double that recorded in North America.[16] In regions like Southeast Asia and Australia, the elevated prevalence of HNSCC is linked to the consumption of products containing carcinogens such as tobacco, alcohol, and betel quid. Conversely, in the USA and Western Europe, it is attributed to a rise in HPV infections[17–19] (Table 2).

The overall incidence of HPV-related oropharyngeal cancers was estimated to be 4.8 cases per 100 000 in 2013–2014, in contrast to the incidence of HPV-related non-oropharyngeal HNCs, which stood at 0.62 cases per 100 000.[20] In the United States, where extensive studies have been conducted, approximately 70% of all oropharyngeal cancers are attributed to HPV, a markedly higher percentage compared to the global average. The overall prevalence of HPV in oropharyngeal cancer rose from 40.5% before 2000% to 64.3% between 2000 and 2004, and further to 72.2% between 2005 and 2009. However, there has not been a significant increase in HPV prevalence in non-oropharyngeal cancers over the same time period.[19]

Overall, HNC contributes to a significant number of cancer-related deaths worldwide. Trends in incidence-based mortality amongst both genders varied in types of HNC due to related risk factors. The mortality for women compared with men having nasopharynx cancers is approximately 1:3, but it worsens to 1:4 and 1:5 in oropharyngeal and hypopharyngeal cancers, respectively.[21]

The gender gap in HNSCC incidence has been narrowing in recent years because of changing patterns of tobacco and alcohol use, an increase in HPV-related HNSCC, and sexual behaviors in both men and women.[22,23] A systemic review revealed a distinct global pattern between genders, indicating a more notable increase in HPV-related HNC subsites among males compared to females.[24]

The average age of diagnosis is reported at 50–70 years old.[25] HNC patient survival varies depending on several factors, such as the stage of cancer, tumor location, the patient's overall health, and the specific treatment provided. When examining data from the Surveillance, Epidemiology, and End Results registry, the 5-year survival rate rose from 55% between 1992 and 1996 to 66% between 2002 and 2006.[26] This increasing survival rate is due to the emergence of

TABLE 1 Clinical differences between HPV16-positive and HPV16-negative head and neck cancers.[10]

	HPV-positive HNSCC	HPV-negative HNSCC
Age (mean)	Younger (40–60)	Older (>60)
Main risk factors	Sexual contact, HPV16 and HPV18	Alcohol and smoking
Mutation burden	Lower	Higher
Main carcinogenic factor	Viral protein E6 and E7 action	DNA damage and inaccurate DNA repair promoted by alcohol catabolism and smoke carcinogen components action
Responsiveness to chemoradiation	Better than HPV-negative HNSCC	Worse than HPV-positive HNSCC
Prognosis	Better than HPV-negative HNSCC	Worse than HPV-positive HNSCC

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus.

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TABLE 2 The incidence cases (x103) of oral cancer in regions from 1990 to 2019.[16]

Year		1990 Incident cases (×10³)	2019 Incident cases (×10³)
	Global	175.63 (167.52–184.91)	373.10 (340.88–403.87)
Sex			
	Male	120.66 (112.73–129.49)	243.19 (218.65–268.26)
	Female	54.97 (51.32–58.50)	129.91 (117.07–142.96)
Region			
	Andean Latin America	0.31 (0.27–0.35)	0.87 (0.70–1.05)
	Australasia	1.53 (1.46–1.60)	1.84 (1.50–2.25)
	Caribbean	1.09 (1.02–1.16)	2.10 (1.79–2.45)
	Central Asia	1.35 (1.26–1.52)	2.41 (2.18–2.67)
	Central Europe	6.96 (6.77–7.14)	10.34 (8.98–11.72)
	Central Latin America	1.66 (1.60–1.70)	4.25 (3.63–4.93)
	Central Sub-Saharan Africa	0.64 (0.49–0.81)	1.52 (1.15–1.93)
	East Asia	13.86 (12.34–15.53)	52.04 (44.27–61.05)
	Eastern Europe	9.20 (8.68–9.79)	12.96 (11.61–14.51)
	Eastern Sub-Saharan Africa	2.47 (2.09–2.92)	6.12 (5.13–7.09)
	High-Income Asian Pacific	3.57 (3.44–3.67)	8.48 (7.21–9.69)
	High-Income North America	2.34 (2.27–2.40)	33.20 (28.79–38.39
	North Africa and Middle East	2.42 (2.03–2.79)	6.65 (5.81–7.69)
	Oceania	0.10 (0.08–0.13)	0.25 (0.19–0.34)
	South Asia	54.22 (47.97–61.09)	143.20 (120.85–166.17)
	Southeast Asia	11.13 (10.02–12.07)	28.95 (24.16–34.65)
	Southern Latin America	1.29 (1.23–1.35)	2.14 (1.68–2.68)
	Southern Sub-Saharan Africa	1.18 (1.05–1.39)	2.21 (2.01–2.44)
	Tropical Latin America	3.95 (3.81–4.08)	9.74 (9.16–10.24)
	Western Europe	34.03 (32.99–34.96)	40.62 (35.16–46.78)
	Western Sub-Saharan Africa	1.24 (1.05–1.42)	3.21 (2.71–3.75)

Note: Incident cases (95% uncertainty interval).

HPV-associated HNSCC, a group of patients with a better prognosis, rather than being solely the result of advancements in multimodal treatment.[27] A clinical trial (NCT00047008),[28] led by Ang et al. concluded that tumor HPV status serves as a strong and independent prognostic factor for survival in patients with oropharyngeal cancer.

Patients with HPV-positive cancer had higher OS and progression-free survival compared to patients with HPV-negative cancer. The 3-year rates of OS were 82.4% in the HPV-positive subgroup and 57.1% in the HPV-negative subgroup.[28] However, according to data about the burden of oral cancer and its attributable risk factors, in 2019, the number of deaths attributed to oral cancer surpassed more than twice the figure recorded in 1990. South Asia registered the highest age-standardized mortality rate for oral cancer, with a rate of 6.36 deaths per 100 000 individuals.[16]

1.3 | The HPV16 genome and viral oncogenes

HPV is a non-enveloped virus with a circular double-stranded DNA genome with 8000 base pairs. HPV can infect the basal cell layer of human epithelium, inducing benign hyperproliferations or promoting premalignant lesions.[3] HPV16, the HR variant, accounts for a large number of HNSCC cases.[29] All papillomaviruses share a common genomic structure characterized by eight open reading frames, including early genes (E1, E2, E4, E5, E6, and E7), late genes (L1 and L2), and the long control region (LCR) or upstream regulatory region (URR).[30]

The E1 viral helicase and E2 DNA-binding protein directly contribute to viral genome replication, while accessory proteins (E4, E5, E6, and E7) play roles in genome amplification and virulence.[3] In the later stages of the viral life cycle, the late genes L1 and L2 encode crucial viral capsid proteins necessary for the final assembly of virions and facilitating the entry of the virus into host cells. The LCR is pivotal for regulating viral replication and transcription[3,6] (Figure 1).

1.4 | HPV oncoproteins in HNCs

The HPV oncoproteins E6 and E7 promote cell replication by inhibiting key cell cycle regulators, including tumor protein p53 (TP53) and retinoblastoma (Rb)[31] (Figure 2). Both Rb (pRb) and p53 were shown to be elevated in 41.9% and 33.5% of oropharyngeal squamous cell carcinoma (OPSCC), respectively.[32] Disruption of the TP53 and Rb pathways is almost universal in HNSCC.

TP53 encodes a transcription factor that is responsible for maintaining genomic stability, DNA repair, regulating the cell cycle, triggering senescence, and controlling apoptosis. In normal cells, when DNA damage occurs, p53 accumulates, leading to a halt in the cell cycle before DNA replication, allowing time for DNA repair.[33] If the damage is extensive and irreparable, p53 initiates apoptosis to prevent the proliferation of cancerous cells. In HPV-infected cells, the degradation of p53 by E6 prevents cell cycle arrest and apoptosis, leading to chromosomal instability and contributing to

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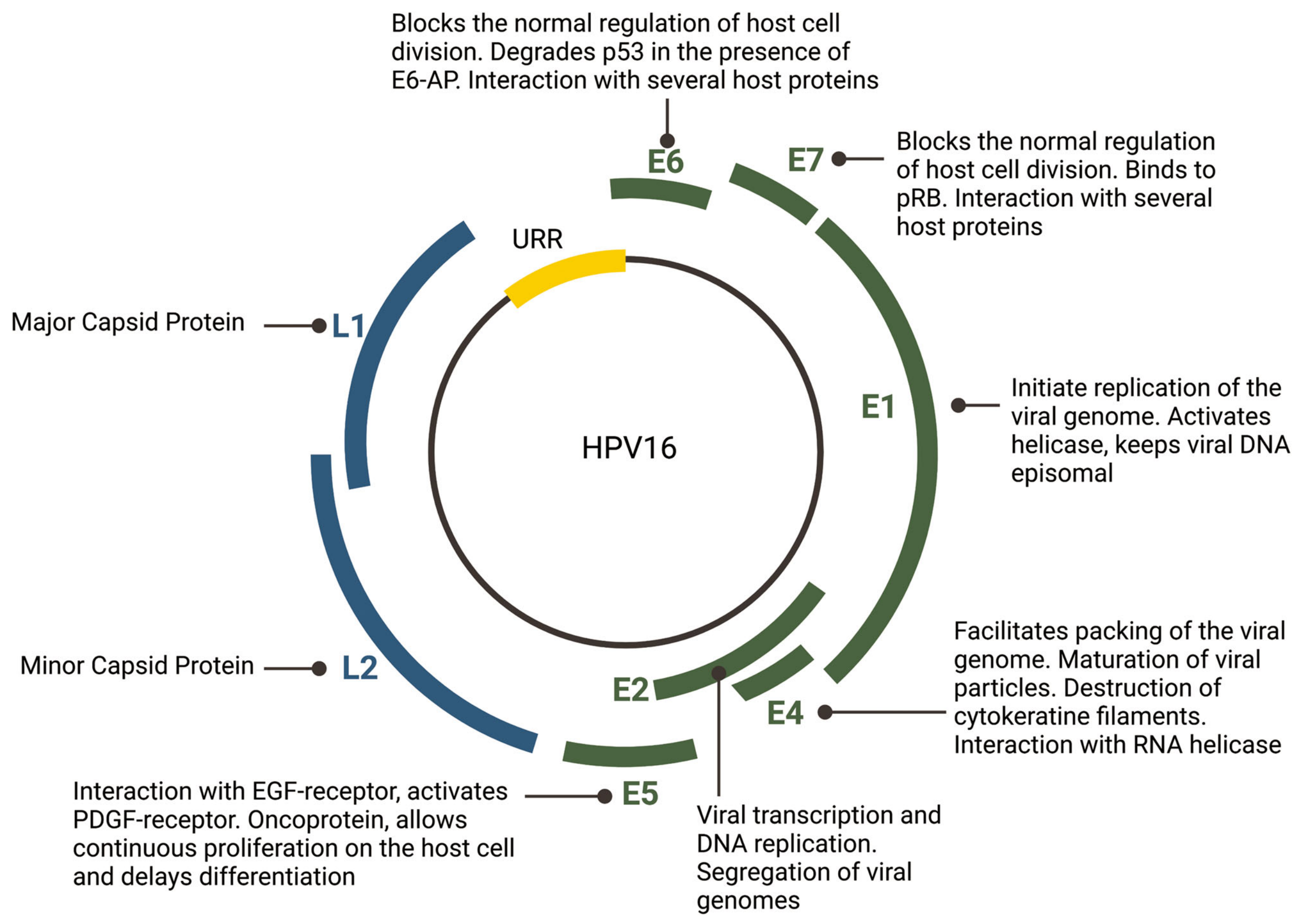

FIGURE 1 The human papillomavirus (HPV) genome and functions of open reading frames. URR, upstream regulatory region.[29]

carcinogenesis. The degradation of p53 induced by HPV16 E6 relies on the formation of a ternary complex involving E6, the ubiquitin E3 ligase E6-associated protein (E6AP). Once this ternary complex is formed, p53 undergoes rapid ubiquitination by E6AP and is subsequently degraded.[6] This is in contrast to mutations in the p53 gene, observed in around 80% of HNSCC cases,[33] which have been associated with tobacco smoking. Loss-of-function TP53 mutations are shown frequently in smoking-related HNSCCs.[34]

The Rb tumor suppressor gene plays a pivotal role in controlling the cell cycle and differentiation. This tumor suppressor protein serves as a regulator at the G1-S restriction point, capable of halting cell growth in the mid-G1-S phase. The functionality of pRb depends on its phosphorylation state throughout the cell cycle and its interactions with other proteins. Mutations lead to the inactivation of functional pRb, resulting in the loss of its growth-controlling and tumors-suppressing properties.[35]

The interaction between pRb and E2F is an essential checkpoint regulating the G1-S phase transition in cells. When cells are unprepared to progress into the S-phase, the pRb protein remains bound to the E2F family of transcription factors, inhibiting their ability to transcribe genes necessary for the S-phase.

In HPV-infected cells, E7 specifically targets pRb for ubiquitination. This process results in the release of E2F transcription factors, thereby disrupting the normal regulatory mechanism and facilitating the progression of cells into the S-phase. Additionally, the degradation of Rb results in the expression of the CDKN2A gene, encoding the tumor suppressors p14ARF and p16INK4A. p16INK4A serves as a surrogate marker of HPV-driven OPSCC by preventing cells from entering the cell cycle through the inhibition of cyclin-dependent kinases. Notably, loss of heterozygosity at the chromosomal region 9p21, where p16INK4A is located, is observed in up to 80% of HNSCCs.[8]

In host epithelial cells, the HPV life cycle involves multiple stages. The L1 capsid protein interacts with the basement membrane, causing structural changes in L1 and L2 that facilitate the transfer of virion particles. Oncoproteins E6 and E7 are then expressed, inducing cell cycle entry and proliferation, and their expression increases with neoplasia progression, contributing to malignancy. E1 and E2 regulate viral transcription, with E1 recognizing and unwinding the replication origin, and E2 controlling the promoter for E6 and E7 expression. Basal epithelial cells divide into transit amplifying cells that differentiate and migrate upwards. HPV DNA amplification is followed by the assembly of E4 and E5 proteins and the encapsidation of L1 and L2. New viral particles are synthesized and assembled in the nucleus, moving to the cytoplasm with the help of E4 and E5. New virions are formed in the nucleus and released to infect neighboring basal epithelial cells, restarting the cycle.

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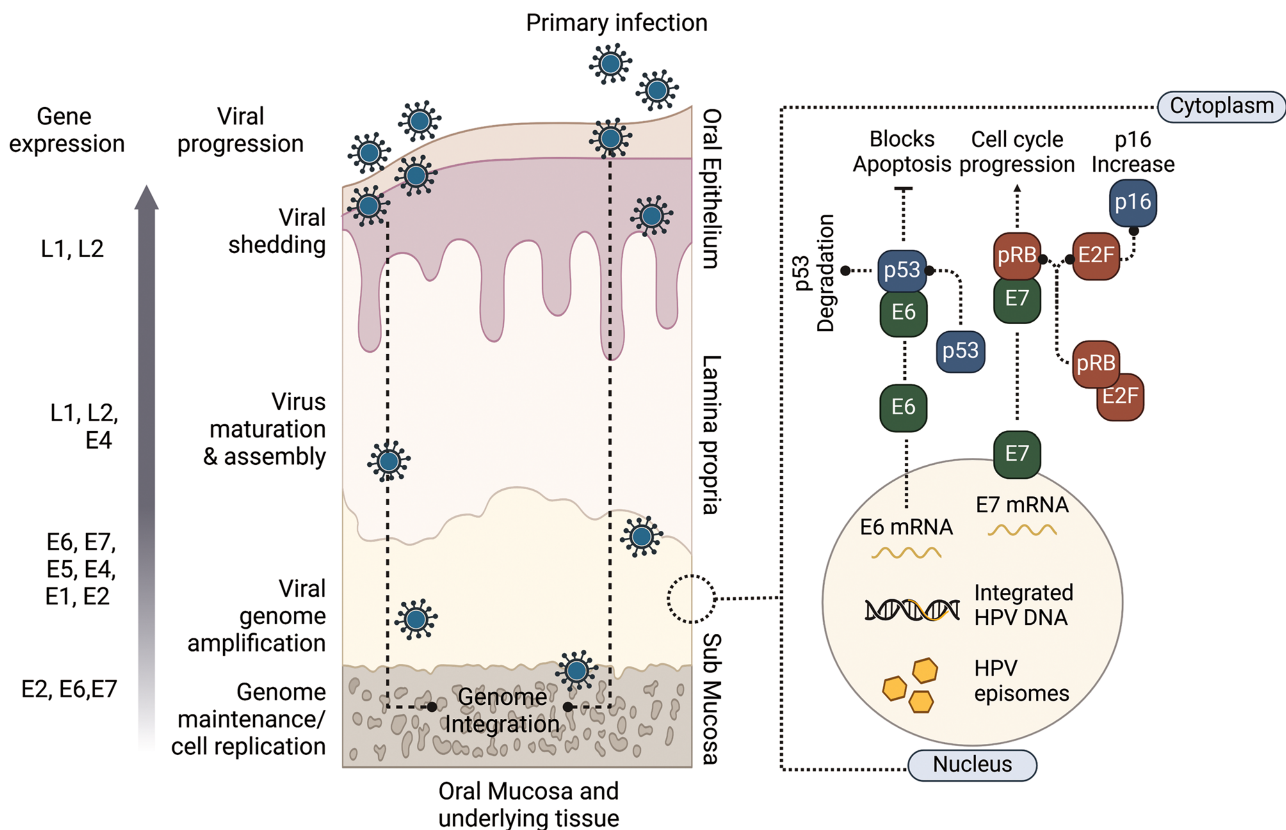

FIGURE 2 Cell-cycle progression in human papillomavirus (HPV) infection.

1.5 | The mysterious URR and its functions

The URR of HPV refers to the non-transcribe region located upstream of the viral early genes. This region plays a crucial role in regulating viral gene expression and replication. The E2 protein interacts with URR, influencing transcription. Moreover, E2, in collaboration with E1, is indispensable for viral DNA replication. E2 has a main part in various aspects of the viral life cycle, including DNA replication, the partitioning of the viral genome into daughter cells, and the regulation of transcription. Numerous in vitro studies have demonstrated the binding and transcription-modulating capabilities of the E2 protein from high-risk papillomaviruses on URR.[36] However, when the E2 gene integrates into the host genome, its activity is disrupted, while the URR remains intact. This phenomenon has led to using the URR as a potential target for HPV diagnosis.[37]

1.6 | Tumor microenvironment (TME) and applied immunotherapy in HPV HNSCCs

Standard treatment modalities for HNSCCs include surgical intervention, radiotherapy, and systemic therapy. However, despite the utilization of multiple treatment approaches, both response rates (RRs) and recurrence rates are still at high levels, typically around 40%–50%.[38] A persistently low treatment response rate among patients still poses a threat even when clinical factors, such as tumor subsites and disease stages, are known. Recently, the TME has been identified as a site for cancer-targeted therapy. For instance, the EGFR antibody cetuximab is used to treat patients, however, many studies mentioned the inferior efficacy of cetuximab plus radiotherapy when compared with chemoradiation with cisplatin in HPV-positive OPSCC patients.[38] The discovery of immune checkpoint inhibitors including pembrolizumab, nivolumab, and avelumab, designed to disrupt the interplay between programmed cell death protein 1 (PD-1) and its ligands PD-L1 and PD-L2, has heralded a new era for HNSCCs clinical treatment.[38,39]

There are studies which show that patients with HPV-positive HNSCCs contain a TME that differs from those with HPV-negative tumors.[40] It noted that HPV-positive tumors exhibit higher levels of NK/T-cells and GC TIL-B cells while having reduced levels of Tregs, fibroblasts, and macrophages compared to HPV-negative tumors.[38]

Recent advancements in technologies like single-cell RNA sequencing and spatial transcriptomics have highlighted the TME's complexity. These techniques reveal distinct gene expression patterns and spatial distributions of immune cells in HPV-positive and HPV-negative tumors, suggesting that viral infection plays a crucial role in shaping the TME and influencing tumor properties. Investigation into spatial organization and composition of tertiary lymphoid structures (TLSs) has shown that TLS in HNSCCs plays a role in the

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antitumour response.[41] Moreover, spatial transcriptomics analysis of the distribution of immune cells by HPV infection status revealed that a high proportion of lymphoid cells was detected in HPV-positive patients, while a higher representation of epithelial and endothelial cells was found in HPV-negative patients.[42] Also, HPV-positive and HPV-negative HNSCCs show distinct metabolic profiles. HPV-negative tumors prioritize metabolite production, energy generation, and protein synthesis, crucial for tumor growth, while HPV-positive tumors focus on DNA repair and replication, potentially leading to genomic instability.[42] Recognizing these differences is vital for personalized treatment strategies in HNSCCs management.

It is widely known that the immune system plays a key role in cancer cell regulation.[43] We know HPV alters the TME by modulating cytotoxic T cells, NK cells, and regulatory T cells.[44] Specifically, the presence of cytotoxic T lymphocytes (CTLs) targeting HPV16 E6 and E7 enhances immune recognition of tumor cells.[45,46] Immunotherapy has significantly extended OS and RRs in cancer patients, particularly those with HPV-positive HNCs.[47] HPV-positive tumors, especially PD-L1-positive, exhibit better RRs and OS with immunotherapy due to higher cytotoxic T-cell responses.[47] Immunotherapy significantly improved the OS (with a hazard ratio = 0.77, $p < 0.0001$) and RR (with a risk ratio = 1.41, $p = 0.02$) among all patients with HNSCCs. Patients with HPV-positive HNSCCs experienced notably improved RRs (risk ratio = 1.29, $p = 0.24$) and extended OS from 6.3 months to 11.5 months. Additionally, tumors positive for PD-L1 exhibited prolonged OS (9.9 vs. 6.5 months). Furthermore, immunotherapy demonstrated a lower incidence of adverse effects compared to standard therapy.[47]

1.7 | HPV diagnosis in HNCs

Detecting HPV in head and neck regions requires a combination of clinical, histopathological, and various molecular methods.

1.7.1 | P16 IHC: A surrogate for HPV16 positivity

p16 IHC staining stands out as the most reliable and widely accepted surrogate marker for detecting HPV in HNSCC. The most recent guidelines provided by both the American Society of Clinical Oncology and the College of American Pathologists emphasize the importance of evaluating HR HPV status in all oropharyngeal tissue specimens. These guidelines recommend p16 testing before proceeding to HPV-specific testing[48,49] and propose that p16 positivity should be characterized by the presence of more than 70% of tumor cells showing moderate to strong nuclear and cytoplasmic staining.

p16 plays a central role in the regulation of the cell cycle, primarily functioning as a negative regulator within the previously described pRb/E2F pathway. The CDKN2A gene encodes two key proteins: p14ARF and p16INK4A. The p14ARF protein exerts its regulatory function by inhibiting MDM2, which in turn activates p53, which promotes p21, resulting in halted progression through the G2/M checkpoint and preventing entry into mitosis. On the other hand, p16INK4A plays a crucial role by inhibiting the CyclinD1/CDK4 and CyclinD1/CDK6 complexes, which are responsible for the phosphorylation of pRb. This inhibition results in the formation of dysfunctional pRb/E2F complexes, blocking the progression of the cell cycle field[2,50]. Interestingly, the phosphorylation of pRb also triggers a feedback mechanism, resulting in the inhibition of p16INK4A expression. This intricate regulatory network contributes to the precise regulation of cell cycle progression. The degradation of pRb by E7 not only leads to the loss of feedback inhibition but also triggers the overexpression of p16INK4A. Thus, p16 can serve as a surrogate marker for HPV-driven OPSCC.[29]

Previous studies have shown approximately 80%–98% sensitivity and specificity range for p16 IHC in detecting HR HPV.[51] A systematic review found that the sensitivity and specificity of p16 IHC among OPSCC patients was 94% and 83%, respectively.[52] Regarding the sampling errors, 5.5% of samples did not allow for adequate diagnosis. Notably, 96.2% of benign tumor cases were accurately diagnosed, with only 3.8% of specimens being nondiagnostic. In cases involving malignant tumors, correct identification was achieved in 86.4% of instances. These results indicate that p16 IHC testing on solid samples faces several difficulties. The main sources of errors are identified as nonuniformity in lesions, incorrect placement of the needle, and a lack of adequate aspirated material.[53]

Recently, Singhi and Westra presented findings assessing p16 IHC in conjunction with HPV16 in situ hybridization (ISH) in HNSCC patients. All HPV16-positive cases exhibited positive p16 staining. In contrast, p16-positivity was observed in 24% HPV16-negative tumors.[54] This finding is supported by other recently published series, where p16 overexpression was identified in 93.2% of cases of HPV-positive OPSCC, while among HPV-negative patients, p16 overexpression was observed in 18.8% of cases.[28]

With these instances where overexpression of p16I is seen in HPV-negative HNSCC cases, it is not exclusively specific to active HPV.[25] This overexpression might also be due to other factors such as inflammation, regeneration, and mutations in the p53 gene.[55] Although p16 IHC is generally effective, its main drawback is that it serves as an indirect indicator of HPV presence.[56]

1.7.2 | ISH for HPV detection

ISH is a method enabling the identification of viral nucleic acids (DNA or RNA) within histological sections. Synthetic DNA/RNA oligonucleotides, labeled with fluorophore-coupled nucleotides, serve as probes to bind with complementary sequences in the cells and tissues, known as fluorescent ISH. The resulting hybridization can be observed through a fluorescence microscope.[57] ISH stands out as the only molecular method capable of reliably identifying HPV in relation to the topography of pathological lesions.[58]

Clinical laboratories have two primary approaches for direct HPV testing: DNA ISH (DISH) identifies integrated HPV DNA, and RNA ISH (RISH) detects actively transcribed HR HPV E6 and E7 mRNA. Although HPV DISH can detect the virus, RNA ISH is the preferred

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approach.[51] The latter approach provides evidence of a transcriptionally active virus rather than simply the presence of the virus, which is biologically more relevant.

Notably, the majority of p16-positive oropharyngeal tumors that are negative with DISH tend to test positive when using RISH.[59] In a study led by Rooper et al.[59] comparing HNSCC cases previously identified to p16 IHC and HPV DISH, ISH successfully detected E6/E7 mRNA in 88% of p16 + /DISH− cases, 100% of p16 + /DISH+ cases, and 0% of p16 − /DISH− cases. Of note, RISH signals were visualized at low to medium magnification in 97% of positive cases. Over the last decade, studies on RISH have shown excellent diagnostic performance, with sensitivities ranging from 87% to 100% and specificities from 88% to 100%.[55]

There are still some limitations regarding ISH. The current understanding of interpreting ISH signals lacks uniform agreement on established thresholds, which adds complexity to their interpretation compared to the more straightforward p16 IHC.[58] In addition, its main drawback is high cost, the technical ability to perform the test can limit its availability in pathology laboratories.[58] Another is its inability to identify the specific HR HPV types, information that could be valuable for determining the prognosis of HPV-positive HNC.

1.7.3 | Polymerase chain reaction (PCR) for HPV detection

For a long time, PCR has served as a highly sensitive, widely accessible, and cost-effective means of detecting HPV and other viruses. Theoretically, PCR can identify a single copy of any gene and can be applied to solid tissue or liquid samples.

The historical perspective in using PCR for detection of HPV originated in the late 1980s period. Snijders et al.[60] explored the capabilities of PCR in HPV detection using two pairs of general primers based on short, conserved regions among HPV genotypes, specifically targeting the L1 region. Matrix comparison of sequenced HPV types la, 6b, 16, and 18 unveiled the most conserved regions within the E1 and L1 regions (open reading frames, ORFs).[60] This study marked the beginning of primer-based detection of specific regions of HPV DNA, opening the era of employing PCR for the identification of distinct regions of HPV DNA and RNA.

In 1990, a study used general primer (GP)-mediated PCR on cervical scrapes, employing both L1-specific general primers (GP L1) and a novel E1-specific primer set (GP E1) to identify a wide range of HPV genotypes. Notably, differences in HPV positivity rates between GP E1 and GP L1 primers were attributed to potential variations in primer affinity for different HPV targets, the degree of homology of probes used with amplified DNA, or partial deletions in the HPV genome.[61] This led to the design and use of the general primers GP5 and GP6 on purified HPV DNA to identify mucosotropic HPV types 6, 11, 16, 18, 31, and 33. This approach enabled the identification of HPV types through the amplification of approximately 150 bp of the L-region.

Fast forward to modern-day practices, reverse transcription-quantitative PCR (RT-qPCR) has become a mainstream PCR technique for detecting and quantifying viral copies.[51] Gao et al.[62] have pioneered real-time RT-qPCR assays to analyze E6 and E7 mRNA expressions in 13 high-risk HPV types, identifying HPV16 as the most prevalent, representing 74.7% of cases in their study. Among 150 examined tumors, 122 were HPV positive (81.3%), with each case showing both E6 and E7 transcripts from the same HPV type. In contrast, the 28 HPV-negative cases showed no E6 or E7 transcripts. A significant finding was that p16 reliably indicated HPV's transcriptional activity in oropharyngeal cancer.

Pan et al. aimed to develop a novel one-step multiplex reverse transcript real-time PCR to detect E6/E7 mRNA from 14 high-risk HPV genotypes. This method showed 88.9% sensitivity and 71.6% specificity, compared to histopathological evaluations. Another study tested the RT-qPCR TaqMan assay for HPV16 E6, using RNA from two HPV16-positive cell lines, achieving 95% sensitivity and 97% specificity.[63] These studies highlight the precision of qPCR in detecting HPV mRNA E6/E7 and confirm its utility in identifying HPV presence and activity.

Recently, there have been concerns regarding formalin fixation and paraffin embedding (FFPE) samples in PCR. Normally, solid tumor samples are either collected as FFPE or fresh frozen tissue. Compared with fresh tissue, FFPE preserves the morphology and cellular details with the possibility of storage at room temperature for several years. In a study to compare real-time PCR signal-amplified ISH (using FFPE samples) and conventional PCR (using fresh tissue) for detection and quantification of HPV in archival cervical cancer tissue,[64] an inhibitory effect of FFPE on real-time qPCR evaluation was observed. This led to an underestimation of the viral copy number. While real-time PCR is increasingly available and holds the potential for time-saving high-throughput analysis, it remains a costly test.

Specifically, its application to FFPE specimens requires dilution of extracted DNA ranging from 1:10 to 1:1,000, with each specimen to be tested three times, thereby increasing costs. Additionally, interpreting certain PCR curves becomes challenging due to nonspecific amplifications, often requiring additional procedures to resolve these issues.[64] Fresh frozen tissue is recommended due to its superior diagnostic performance. However, due to the cost associated with the collection of fresh tissue, it is not suitable for routine screening of HPV infection.[51]

1.8 | Clinical applications of liquid-based RT-qPCR

Tissue biopsies used in RT-qPCR testing face challenges due to their invasive procedures, associated patient risks, complex sample preparation needs, high costs, and limited sensitivity in tumor detection. Recently, there has been a shift towards liquid biopsies, potentially marking a new era in HPV detection. The ideal characteristics of these methods include being noninvasive,

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cost-effective, quick, accurate, and capable of identifying transcriptionally active high-risk HPV infections. Among the various body fluids available for testing, blood and saliva stand out as the most relevant for HNC diagnostics.

1.8.1 | HPV16 assays using blood

Blood is unique as it is the only fluid that directly interacts with every organ in the body, making it an appealing option for less invasive early detection methods. Numerous studies have focused on identifying and analyzing tumor by-products, including circulating tumor cells (CTCs) and fragments of tumor DNA found in the bloodstream (circulating tumor DNA [ctDNA]).

CTCs are cancer cells that migrate from the primary tumor into the bloodstream or lymphatic system, allowing them to spread throughout the body.[65] CTCs enumeration has been linked to various aspects of cancer progression and patient outcomes, such as tumor stage and survival rates. CTCs can be used to predict treatment response and detect resistance. However, isolating CTCs from whole blood samples poses a significant technical challenge. Recent advancements[65–67] in cell isolation and purification techniques, including efforts to culture CTCs ex vivo for immunotherapy, have shown promise. Economopoulou et al. examined 22 OPSCC patients before and after treatment, isolating CTCs to evaluate HPV E6/E7+ expression.[68] Notably, CTCs from HPV16-negative cases did not show positive expression of HPV16 E6/E7 mRNA, and there was no significant correlation found between E6/E7 expression in CTCs after treatment, progression-free survival or OS.

The primary target for ctDNA detection is typically the HPV E6/E7 open reading frame. However, there is an assumption that ctHPVDNA is not observed in patients with noninvasive HPV-associated lesions or HPV infection without carcinogenesis.[69] It was shown that very low or undetectable initial levels of ctDNA are linked to a low tumor HPV copy number.[70] The sensitivity rates of most methods used for detecting ctDNA in HPV-driven OPSCC range from 70% to 80%, and this can be enhanced by incorporating additional targets such as E2, E4, and E5. However, this may potentially create false positives, thereby decreasing the specificity rate.[69]

In a study led by Ahn et al., patients with oropharyngeal and unknown primary squamous cell carcinoma were utilised to identify HPV16 E6 and E7 DNA in pretreatment plasma. The sensitivity was quite low, reported as 67.3%.[71] Similar to this data, Cao and colleagues demonstrated that HPV DNA was detectable in 65% of pretreatment plasma samples from HPV-positive OPC patients using E6/7 qPCR. None of the HPV-negative HNC patients or noncancer controls exhibited detectable HPV DNA.[72] In addition to PCR, next-generation sequencing (NGS) has been employed for the sequencing of HPV ctDNA, aiming to enhance the identification of minimal disease volumes. A recent investigation revealed a test sensitivity of 95% and specificity of 98.1% for plasma NGS-based testing.[73]

1.8.2 | HPV16 assays using saliva

Saliva, comprising a complex mix of secretions with a predominant water content and minor percentages of proteins, lipids, carbohydrates, and other substances, proves advantageous for liquid biopsy. This composition ensures lower levels of inhibitory substances, making saliva particularly suitable for detecting biomarkers such as proteins, DNA, mRNA, and metabolites. Its direct exposure to the oral cavity makes saliva especially promising for identifying viral particles and genes associated with oral cancers.

Saliva's benefits include cost-effectiveness, ease of collection, the possibility for frequent samplings, and a lower risk of transmitting infections.[74] Unlike other methods, saliva collection is nondisruptive, avoiding patient discomfort, pain, or toxicity. Grewal et al.'s study[75] indicated saliva could serve as an alternative to solid specimens for HPV detection in OPSCC patients, with no significant difference in the prevalence of HPV16/18 E6/E7 proteins and DNA levels between saliva and solid specimens.

Salivary PCR-based HPV DNA testing, especially targeting the L1, E6, and E7 regions, has been extensively studied. Fakhry et al.[76] reported 80.6% sensitivity and 100% specificity in detecting HPV in saliva. Similarly, Nordfors et al.[77] demonstrated a sensitivity of 82% for tonsil cancer and 50% for base of tongue cancer. Saito and others[78] improved the detection of salivary HPV16 DNA, achieving 96% sensitivity and 100% specificity.

Ekanayake Weeramange et al.[79] found HPV16 in 92% of saliva samples, indicating its prevalence in OPC patients. Wasserman et al.[80] observed 47% of saliva samples from their study participants tested positive for HPV DNA, predominantly HR subtypes. The positive HR HPV saliva assay in Wasserman's study showed 100% specificity and positive predictive value for identifying p16-positive tumors in the oropharynx.

In contrast, a negative HR HPV assay had a 96% negative predictive value for non-oropharyngeal sites. The study highlighted that saliva testing had a 77% sensitivity and 94% specificity for detecting p16-positive tumors, regardless of their site. Another significant study employed real-time qPCR to assess HPV16 E6 and E7 DNA levels in primary tumors and salivary rinses.[81] It found that 45.6% of primary HNSCC tumors and 32.6% of saliva samples from HNSCC patients had detectable HPV16 DNA. Qureishi et al.[82] reported a 72.2% sensitivity and 90% specificity in saliva testing for HPV in OPSCC, compared to p16 IHC and ISH. The importance of assessing mRNA expression from integrated HPV DNA in clinical practice is evident, as it serves as an indicator of the virus's active status. Chai et al.'s pilot study[83] demonstrated the effectiveness of saliva-based qPCR for HPV mRNA E6/E7 in early detection of HPV16 in HNSCC, with high specificity and correlation with p16 positive tumors.

Taken together, these studies suggest there is potential in using saliva as a diagnostic medium for HPV-related cancers, reflecting its utility for early detection and screening. The findings of saliva-based qPCR to detect HPV in HNCs are summarized in (Table 3). Studies comparing blood and saliva have suggested that saliva offers higher accuracy and better advantages than blood.

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TABLE 3 Summary of findings about saliva-based qPCR to detect HPV16.

Targets	Findings	References
HPV16 DNA E6/E7	se = 80.6%, sp = 100%	Fakhry et al.[76]
	se = 82% in tonsil cancer, se = 50% in base of tongue cancer	Nordfors et al.[77]
	se = 96%, sp = 100%	Saito et al.[78]
	92% of cases: HPV16 (+) 72.4% of OPC cases: HR HPV DNA (+) 89.3% of cases: p16 (+)	Ekanayake Weeramange et al.[79]
	se=77%, sp = 94% overall 47% of samples: HPV DNA (+), with 79% is HR subtypes sp = 100%, positive predictive value = 100% for identifying p16 (+) tumors	Wasserman et al.[80]
	45.6% of HNSCC tumors and 32.6% of saliva samples: HPV16 DNA (+)	Zhao et al.[81]
	se = 72.2%, sp = 90% in comparison to p16 IHC and ISH positive predictive value = 96.3%, negative predictive value = 47.4%	Qureishi et al.[82]
HPV mRNA E6/E7	39/42 patients with p16 (+) tumors had HPV16 DNA (+); 0/40 patients with p16 (−) tumors showed HPV16 DNA (+); sp = 100%. 22/40 patients with p16 (+) tumors had HPV16 mRNA (+); 0/40 patients with p16 (−) tumors showed HPV16 mRNA (+); sp = 100%.	Chai et al.[83]

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; HR, high-risk; IHC, immunohistochemistry; ISH, in situ hybridization; OPC, oropharyngeal cancer; qPCR, quantitative polymerase chain reaction.

Saliva-based diagnostics offer numerous advantages compared to blood. Collecting saliva is simple, convenient for storage and transportation, and does not require specialized training. Saliva collection is cost-effective, is scalable to a larger population, and allows for repeated or multiple samplings when needed. The noninvasive nature of saliva collection is an appealing option for collecting clinical samples.

An important advantage of saliva diagnostics is that patients can conveniently collect samples at home, reducing healthcare costs. In this regard, saliva analysis could enhance patient compliance, especially for those requiring frequent surveillance. This is crucial not just for diagnosing but also for monitoring posttreatment conditions. Cancer patients often undergo treatments such as surgery and radiotherapy. However, posttreatment monitoring is crucial to ensure continued progress. Tracking biological markers of HPV in saliva and monitoring viral replication would allow healthcare providers to detect the risk of recurrence early and assess treatment effectiveness. A gradual decrease in HPV RNA indicates successful elimination of the virus from the body. Conversely, increased viral replication and activity suggest ineffective treatment, prompting healthcare providers to adopt a more proactive approach. Additionally, regular hospital visits for check-ups can be challenging for patients. Collecting saliva samples at home is more convenient and preemptive, making patients more likely to comply with the treatment process.

2 | CONCLUSION

HNCs encompass a diverse group of malignancies, with HNSCC being the most common form. These cancers are of particular interest due to their association with HRHPV, specifically HPV16, which is responsible for a substantial number of cases worldwide. HPV-driven HNSCC is recognized as a distinct clinical entity, characterized by its epidemiological features, molecular biology, and response to treatment. The clinical relevance of HPV in HNSCC cannot be overstated, as HPV-positive cancers tend to occur in younger patients, often linked to sexual behaviors rather than the traditional risk factors of alcohol and tobacco. The prevalence of HPV-positive oropharyngeal cancers, particularly in regions like North America and Western Europe, reflects changing patterns in risk factors, including rising HPV infections.

Patients with HPV-positive HNSCC generally have a better prognosis, attributed to the tumor's enhanced responsiveness to chemoradiation therapies. For instance, the mean age of patients with HPV-positive HNSCC ranges between 40 and 60 years, contrasting with those over 60 years for HPV-negative cases. Additionally, HPV-positive tumors have a lower mutation burden and are primarily driven by the oncogenic action of viral proteins E6 and E7, which disrupt the functions of tumor suppressors p53 and Rb.

Diagnosis of HPV-driven HNSCC has evolved, with p16 IHC serving as a reliable surrogate marker for HPV positivity. The sensitivity and specificity of p16 IHC have made it a cornerstone in diagnosing HPV-associated cancers. Molecular techniques such as ISH and PCR have also been instrumental in detecting HPV DNA and mRNA, offering insights into the virus's role in cancer development.

In assessing the diagnostic precision of HPV detection methods, recent studies emphasize the viability of both blood and saliva specimens. Sensitivity rates for ctDNA approaches targeting the HPV E6/E7 gene span from 70% to 80%. Conversely, saliva-based PCR diagnostics demonstrate enhanced performance, with reported

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sensitivities up to 96% and a specificity consistently at 100%, indicating a promising avenue for noninvasive oncological screenings. Furthermore, while NGS exhibits superior sensitivity and specificity (95% and 98.1%, respectively) for ctDNA, its application remains impractical for large-scale screening due to its substantial financial and infrastructural demands. Collectively, these findings advocate for integrating saliva-based methodologies in clinical settings, given their high accuracy, patient compliance benefits, and the practicality of sample collection for the early detection and ongoing monitoring of HPV-associated malignancies.

3 | CONCLUDING REMARKS

The epidemiological and molecular characterization of HNSCC highlights the critical role of HPV, especially HPV16, in the disease's etiology and progression. HPV status could serve as a key determinant in clinical decision-making, influencing treatment approaches and prognosis. The differential outcomes between HPV-positive and HPV-negative HNSCC suggest the need for tailored therapeutic strategies. Advances in diagnostic technologies, particularly the use of liquid biopsies, offer promising avenues for early detection, monitoring, and personalized management of HPV-associated HNSCC. Ongoing research into the molecular mechanisms of HPV and its interactions with host cells is required for developing targeted interventions. Given the rising global burden of HNCs, comprehensive public health initiatives are necessary. These initiatives should emphasize HPV vaccination, prevention, and early detection modalities to address this significant health issue effectively.

AUTHOR CONTRIBUTIONS

All authors critically read, edited, and approved the manuscript. Ngoc Ha Tran drafted the manuscript. Ngoc Ha Tran and Dayna Sais wrote and drew figures and tables. Nham Tran reviewed and revised the final version of manuscript.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study. All references to the original literature sources have been provided in the references section. No additional data are provided as the authors did not create new data or original experimental data for this review.

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