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Comparative efficacy of p16 versus HPV RNA in situ hybridization in oropharyngeal squamous cell carcinomas

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Abstract

The accurate determination of human papillomavirus (HPV) status in oropharyngeal squamous cell carcinoma (OPSCC) has profound prognostic and therapeutic implications. As the incidence of HPV-associated OPSCC continues to rise globally, particularly in developed nations, the need for precise, reliable biomarkers to distinguish HPV-driven malignancies has become increasingly critical. While p16 immunohistochemistry (IHC) has long served as a surrogate marker for HPV involvement, concerns over its specificity given its ability to overexpress in HPV-negative tumors have prompted interest in more direct molecular assays. Among these, HPV RNA in situ hybridization (ISH) offers a promising technique capable of detecting transcriptionally active viral RNA within the tumor microenvironment, directly reflecting oncogenic activity. This study conducts a comparative analysis of p16 IHC and HPV RNA ISH in a cohort of OPSCC patients, examining concordance rates, diagnostic sensitivity and specificity, and their association with clinical outcomes such as overall survival and recurrence-free interval. While p16 IHC maintains high sensitivity and remains cost-effective, RNA ISH demonstrates superior specificity and a closer correlation with disease progression and patient stratification in treatment protocols. Our results reveal discordance in a subset of cases, underscoring the limitations of relying solely on p16 status for clinical decision-making. Incorporating both assays in a dual-testing algorithm may optimize diagnostic accuracy, enhance prognostic stratification, and better inform tailored therapeutic strategies. The findings highlight the importance of nuanced biomarker selection in HPV-driven head and neck cancers and advocate for standardized molecular pathology workflows that integrate both surrogate and direct detection modalities.

Keywords: HPV RNA ISH; P16 Immunohistochemistry; OPSCC; Biomarker Specificity; Molecular Pathology; HPV-Driven Carcinogenesis

1. Introduction

1.1. Epidemiology and Molecular Evolution of HPV-Associated OPSCC

Oropharyngeal squamous cell carcinoma (OPSCC) represents a significant global health burden, with a growing subset of cases now attributed to human papillomavirus (HPV) infection. Over the past two decades, the incidence of HPV-positive OPSCC has risen sharply, particularly in high-income countries, where changes in sexual behavior and reduced tobacco use have shifted the risk landscape [1]. Unlike tobacco- and alcohol-induced OPSCC, HPV-associated cases tend to affect younger, healthier patients with distinct clinical profiles [2].

HPV-16 is the most prevalent genotype implicated in OPSCC, accounting for over 90% of HPV-positive cases. The virus integrates into host genomic material, altering the expression of key oncogenes such as E6 and E7, which inactivate p53 and retinoblastoma proteins, respectively [3]. This disruption of tumor suppressor pathways drives oncogenesis and enables immune evasion mechanisms [4].

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Molecular studies have demonstrated that HPV-associated OPSCC differs significantly in mutational burden, methylation patterns, and gene expression signatures compared to HPV-negative tumors [5]. The relatively low mutational load of HPV-positive cancers suggests that viral oncogenes, rather than genomic instability, play a central role in tumor initiation and progression [6].

These molecular distinctions have profound implications for therapy selection and long-term outcomes. HPV-positive tumors exhibit heightened radiosensitivity and better response to chemoradiation, often correlating with improved prognosis and disease-free survival [7]. As a result, HPV status is not only an etiological marker but also a cornerstone of contemporary clinical stratification in OPSCC management [8].

1.2. Diagnostic and Prognostic Implications of HPV Status

The diagnostic identification of HPV in OPSCC has become a routine component of clinical evaluation, owing to its strong prognostic relevance and influence on treatment pathways. p16 immunohistochemistry is widely used as a surrogate marker for transcriptionally active HPV infection, offering a cost-effective and accessible diagnostic solution [9]. However, confirmatory testing via in situ hybridization or PCR is often warranted to verify viral genome presence and activity [10].

HPV status plays a critical role in risk stratification, with HPV-positive patients typically demonstrating superior overall survival and locoregional control compared to their HPV-negative counterparts [11]. This has led to the incorporation of HPV status into the eighth edition of the AJCC staging system for oropharyngeal cancer, which classifies HPV-positive tumors separately due to their distinct biological and clinical behavior [12].

From a therapeutic standpoint, there is ongoing interest in de-intensifying treatment regimens for HPV-positive patients to minimize long-term toxicity without compromising efficacy. Trials investigating reduced-dose radiation, less aggressive chemotherapy, and immunotherapy alternatives are currently reshaping standard care approaches [13]. Therefore, accurate and early determination of HPV status is central to personalized treatment planning and long-term disease surveillance strategies [14].

1.3. Scope, Objectives, and Article Structure

This article aims to provide a comprehensive review of the clinical and molecular landscape of HPV-associated OPSCC, emphasizing diagnostic methodologies, prognostic stratification, and implications for precision therapy. It also highlights the challenges and opportunities in integrating multi-omics approaches for better disease modeling and personalized care [15].

The scope includes discussion on the virological evolution of HPV within the oropharynx, the immune mechanisms involved in tumor progression, and the application of artificial intelligence in improving diagnostic workflows. Furthermore, it examines disparities in HPV-related OPSCC outcomes based on geography, gender, and access to care, recognizing the need for global harmonization of screening protocols [16].

The article is organized as follows: Section 2 reviews molecular mechanisms and biomarkers. Section 3 explores current and emerging diagnostic tools. Section 4 focuses on therapeutic innovations. Section 5 discusses future research directions and policy considerations. Each section is supported by figures and tables that illustrate key concepts and data trends [17].

2. Molecular and pathological basis of HPV in OPSCC

2.1. HPV Oncogenesis: E6/E7 Pathway and p53/Rb Inactivation

The carcinogenic process driven by high-risk HPV, particularly HPV-16, is orchestrated primarily through two viral oncoproteins: E6 and E7. These proteins exert transforming effects by targeting and inactivating key tumor suppressors p53 and retinoblastoma protein (pRb) that regulate the cell cycle and genomic integrity [5]. E6 promotes the degradation of p53 via the E6-AP ubiquitin ligase complex, effectively disabling the cell's apoptotic response and impairing DNA repair fidelity [6].

E7, meanwhile, binds to and inactivates pRb, leading to dysregulated E2F transcription factor activity and unscheduled progression through the G1/S checkpoint [7]. This promotes proliferation of cells harboring viral genomes and enhances the risk of accumulating secondary mutations. Furthermore, E7-mediated Rb inactivation disrupts chromatin structure and alters histone modification patterns, influencing the epigenetic landscape of infected epithelial cells [8].

Persistent expression of E6 and E7 also modulates the host immune response by downregulating antigen presentation machinery, thereby contributing to immune evasion [9]. In the context of oropharyngeal squamous cell carcinoma (OPSCC), these viral effects produce a distinct molecular phenotype characterized by low TP53 mutation burden, fewer chromosomal aberrations, and increased immune infiltration relative to HPV-negative tumors [10].

This oncogenic model has formed the foundation for targeted biomarker development, enabling the clinical use of p16 overexpression and viral mRNA detection as indirect indicators of HPV-driven transformation [11]. These molecular events not only explain the pathogenesis of HPV-positive OPSCC but also support prognostic and therapeutic stratification based on viral etiology.

2.2. Biological Basis for p16 Overexpression as Surrogate Marker

The protein p16^INK4a, encoded by the CDKN2A gene, is a cyclin-dependent kinase inhibitor that functions as a regulator of the G1 phase of the cell cycle. In normal physiology, p16 expression is suppressed via negative feedback from the Rb pathway. However, in HPV-associated carcinogenesis, the E7-mediated degradation of Rb leads to constitutive activation of E2F transcription factors and compensatory overexpression of p16 [12].

This mechanistic link between Rb inactivation and p16 overexpression underpins the use of p16 immunohistochemistry (IHC) as a clinically accepted surrogate marker for HPV-driven OPSCC [13]. Strong and diffuse nuclear and cytoplasmic staining of p16 is considered indicative of transcriptionally active HPV infection, particularly when more than 70% of tumor cells demonstrate positivity [14].

Importantly, p16 IHC is highly sensitive but not entirely specific. Overexpression can occur in non-HPV-related cancers due to alternate disruptions in the Rb pathway, leading to potential false positives in clinical settings [15]. Nevertheless, its ease of use, reproducibility, and cost-effectiveness make it a preferred frontline diagnostic method, especially in low-resource settings [16].

From a prognostic standpoint, p16-positive OPSCC patients demonstrate improved survival and treatment response compared to their p16-negative counterparts, consistent with the underlying HPV-driven biology [17]. Consequently, p16 status is incorporated into modern staging systems and treatment de-escalation trials for HPV-associated head and neck cancers [18].

Despite its limitations, the biological rationale for p16 as a marker of viral oncogenic activity remains robust, justifying its continued role in routine diagnostics and its integration into personalized cancer management workflows [19].

2.3. Principles of HPV RNA In Situ Hybridization (ISH) and Transcriptional Detection

HPV RNA in situ hybridization (ISH) serves as a highly specific method for detecting transcriptionally active HPV infections by identifying viral E6/E7 mRNA transcripts within tumor cells. Unlike DNA-based ISH or PCR, which can detect latent or non-transforming viral presence, RNA ISH directly measures the viral oncogenic activity that underpins malignant transformation [20].

This methodology employs labeled complementary oligonucleotide probes that hybridize to E6/E7 mRNA sequences in formalin-fixed, paraffin-embedded (FFPE) tissue sections. The resulting hybridized complexes are visualized via chromogenic or fluorescent detection systems, allowing pathologists to localize viral transcriptional activity at the cellular level [21]. This spatial resolution enhances diagnostic specificity, particularly in histologically ambiguous tumors.

RNA ISH platforms such as RNAscope have demonstrated excellent concordance with PCR and p16 IHC in validating HPV status, while offering superior specificity in differentiating bystander viral presence from true driver infections [22]. However, the requirement for specialized equipment, strict RNA preservation protocols, and higher cost limits their widespread implementation in routine clinical workflows, especially in low-resource settings [23].

Nonetheless, RNA ISH remains the gold standard for confirming transcriptionally active HPV infection, particularly when p16 results are equivocal or discordant with clinical features. It plays a crucial role in clinical trials, retrospective cohort validations, and precision oncology protocols where diagnostic certainty is paramount [24].

2.4. Comparative Pathophysiology: p16 IHC vs HPV RNA ISH

While both p16 immunohistochemistry and HPV RNA ISH are used to assess viral involvement in OPSCC, they differ significantly in biological target, specificity, and clinical application. p16 IHC reflects the downstream cellular effect of Rb pathway disruption whether viral or non-viral in origin offering high sensitivity but moderate specificity [25].

In contrast, RNA ISH detects the direct presence of viral transcripts, confirming ongoing HPV oncogenic activity. As such, RNA ISH boasts higher specificity and diagnostic accuracy, albeit at a higher logistical and economic cost [26]. Discordance between these modalities, though infrequent, often necessitates dual testing in borderline or atypical presentations [27].

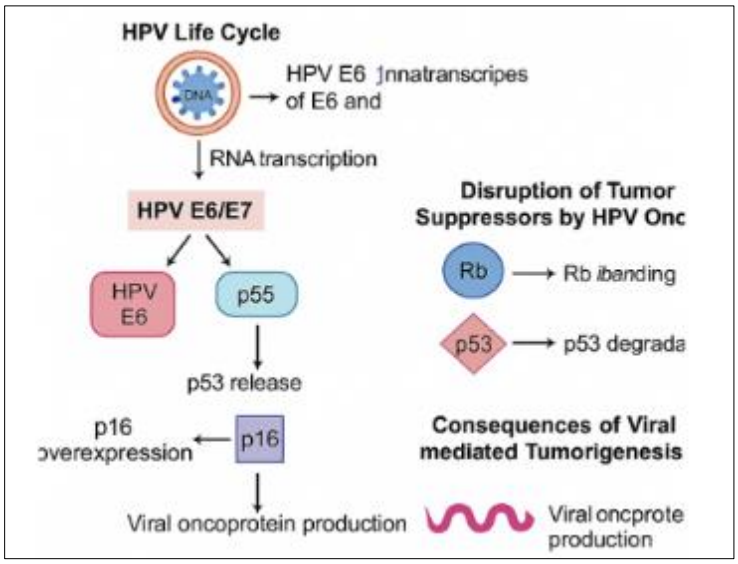

Figure 1 Mechanistic Pathway of HPV Infection and Its Impact on p16 and Viral Transcripts

From a pathophysiological standpoint, integrating both markers provides a nuanced understanding of tumor biology, enabling risk-adapted treatment stratification and improved diagnostic confidence. As personalized oncology advances, the synergy between p16 IHC and RNA ISH will remain central to clinical decision-making in HPV-associated OPSCC [28].

3. Diagnostic techniques and interpretation criteria

3.1. p16 Immunohistochemistry: Antibody Selection, Scoring, and Pitfalls

p16 immunohistochemistry (IHC) remains the most widely adopted screening tool for identifying HPV-associated oropharyngeal squamous cell carcinoma (OPSCC), due to its cost-effectiveness and standardized protocols. The assay employs monoclonal antibodies such as E6H4, which specifically bind to the p16^INK4a protein accumulated in HPV-transformed epithelial cells [9]. The E6H4 clone, approved by the FDA, is commonly used in both manual and automated IHC platforms and is validated for FFPE (formalin-fixed paraffin-embedded) specimens.

A positive result is defined by strong, diffuse nuclear and cytoplasmic staining in ≥70% of tumor cells, distinguishing true HPV-related oncogenesis from focal or basal staining patterns often seen in non-HPV contexts [10]. Despite widespread adoption, p16 IHC is not devoid of pitfalls. Overexpression may be observed in HPV-negative tumors with pRb mutations, leading to false positives [11].

Furthermore, tumor heterogeneity may result in under-sampling, especially in small biopsies, and discrepancies in interpretation can arise in borderline cases. Some studies have shown that interobserver agreement diminishes in cases where staining patterns are patchy or confined to non-malignant tissues [12]. False negatives may also occur in poorly preserved or necrotic samples, or due to antibody lot variation and protocol inconsistency.

The challenge, therefore, lies in combining technical standardization with interpretive clarity. The adoption of digital pathology and AI-assisted quantification systems may help reduce subjectivity in scoring and reinforce reproducibility

in future clinical settings [13]. While highly sensitive, p16 IHC alone does not confirm transcriptionally active HPV, necessitating adjunct confirmatory testing in selected clinical scenarios.

3.2. RNA ISH Platforms: RNA scope, View RNA, and Assay Conditions

RNA in situ hybridization (ISH) platforms such as RNA scope and View RNA offer high specificity in detecting transcriptionally active HPV, targeting viral E6/E7 mRNA transcripts directly within tissue samples. RNA scope, developed by Advanced Cell Diagnostics, utilizes a double Z-probe design to enhance signal-to-noise ratio, allowing detection of low-abundance RNA in FFPE tissues [14]. View RNA, another proprietary assay developed by Thermo Fisher Scientific, leverages branched DNA amplification for sensitive signal detection.

Assay conditions for RNA ISH are more stringent than IHC and require meticulous RNA preservation throughout fixation, embedding, and sectioning. FFPE tissue must be processed within 24 hours of excision to prevent RNA degradation, and pre-treatment steps such as protease digestion and antigen retrieval must be optimized for probe penetration and specificity [15]. Successful hybridization is visualized using chromogenic (e.g., DAB) or fluorescent tags, with localization to tumor cell nuclei and cytoplasm indicating transcriptionally active viral infection.

Despite their accuracy, RNA ISH assays are more resource-intensive than p16 IHC. They require specialized instrumentation, trained personnel, and precise temperature and humidity control during hybridization and amplification steps [16]. The time-to-result ranges from 6 to 12 hours, depending on protocol complexity and detection method, which can be a limiting factor in routine pathology labs with high case volumes.

Table 1 Side-by-Side Comparison of Diagnostic Workflow, Time, and Sensitivity/Specificity

Parameter	**p16 Immunohistochemistry (IHC)**	**HPV RNA In Situ Hybridization (ISH)**
Purpose	Surrogate marker for HPV-induced oncogenesis	Direct detection of transcriptionally active HPV mRNA
Sample Requirement	Formalin-fixed paraffin-embedded (FFPE) tissue	FFPE tissue with high RNA integrity
Workflow Complexity	Low – routine IHC protocols; widely available	Moderate to high – specialized reagents and equipment
Turnaround Time	6–24 hours (typically within 1 working day)	24–72 hours (longer in low-throughput labs)
Interpretation	Visual scoring of nuclear and cytoplasmic staining	Signal detection via chromogenic/punctate RNA dots
Automation Feasibility	High – compatible with automated stainers	Moderate – partial automation available, some manual steps
Interobserver Variability	Moderate (borderline scores are subjective)	Lower with standardized probes and digital scoring
Sensitivity	~90–95%	~85–90% (dependent on RNA preservation and probe quality)
Specificity	~80–85% (risk of false positives in non-HPV cases)	>95% (high specificity for active HPV transcription)
Cost per Test (USD)	Low ($20–50/test)	Higher ($100–250/test)
Primary Limitations	False positives due to non-viral p16 upregulation	RNA degradation, longer turnaround, higher cost
Clinical Use	Widely used as primary screening tool	Recommended for confirmation or research applications

Nevertheless, RNA ISH remains indispensable in research, trial enrollment, and ambiguous p16 IHC scenarios. Its application is expanding beyond head and neck cancers into cervical, anal, and non-oropharyngeal squamous cell carcinomas where HPV-driven biology is clinically relevant [17].

3.3. Technical Considerations in FFPE Tissue for Both Modalities

Both p16 IHC and RNA ISH depend heavily on the quality of FFPE tissue, with pre-analytical variables significantly influencing diagnostic accuracy. Key considerations include fixation time, paraffin type, block storage conditions, and sectioning thickness. Prolonged fixation in formalin (>72 hours) can degrade antigenicity and RNA integrity, impairing staining intensity and hybridization fidelity [18].

Optimal fixation duration ranges from 6 to 24 hours in neutral-buffered formalin to preserve nucleic acid quality while maintaining tissue morphology. Embedding should avoid high paraffin polymerization temperatures, which may cause RNA fragmentation. Block age also plays a role: samples older than 3 years tend to yield suboptimal ISH results due to degradation of labile transcripts [19].

Section thickness impacts both modalities differently. While 4 μm is standard for IHC, RNA ISH may benefit from slightly thinner sections (2–3 μm) to facilitate probe penetration and reduce background signal [20]. Deparaffinization with xylene and rehydration using graded alcohols must be performed carefully to avoid residual paraffin that interferes with staining or hybridization.

Another concern is cross-contamination during sectioning. Use of separate microtome blades and anti-static brushes between blocks is essential to prevent carryover of high-expression samples into adjacent slides [21]. RNAse-free handling protocols are crucial for ISH, including DEPC-treated water, RNAse inhibitors, and autoclaved tools, particularly during tissue mounting and cover-slipping.

The cumulative effect of these pre-analytical variables cannot be overstated. Poor sample processing can generate false negatives, reduce signal intensity, and undermine confidence in diagnostic results. Therefore, adherence to standardized operating procedures is paramount to ensuring reproducibility and interpretability in both clinical and research settings [22].

3.4. Pathologist Interpretation, Concordance Thresholds, and Interobserver Variability

The diagnostic reliability of p16 IHC and RNA ISH depends not only on assay performance but also on the interpretative consistency among pathologists. For p16 IHC, interpretation is guided by the ≥70% cutoff rule, but real-world scoring may vary based on staining intensity, cellular localization, and background artifacts [23]. This subjectivity has led to studies documenting interobserver variability, particularly among general pathologists versus subspecialists.

To mitigate variability, digital image analysis tools have been developed to automate p16 scoring. These platforms quantify nuclear and cytoplasmic signal across tumor regions, offering percentage positivity, staining intensity, and topographic distribution [24]. While not yet universally adopted, such tools enhance reproducibility and reduce human error in borderline cases.

RNA ISH interpretation involves counting punctate signals localized within the nucleus and cytoplasm, often in a semi-quantitative or qualitative manner (e.g., 0–3+ scoring). Visual differentiation between true signal and background noise requires significant training, especially in poorly differentiated tumors or samples with abundant inflammatory cells [25].

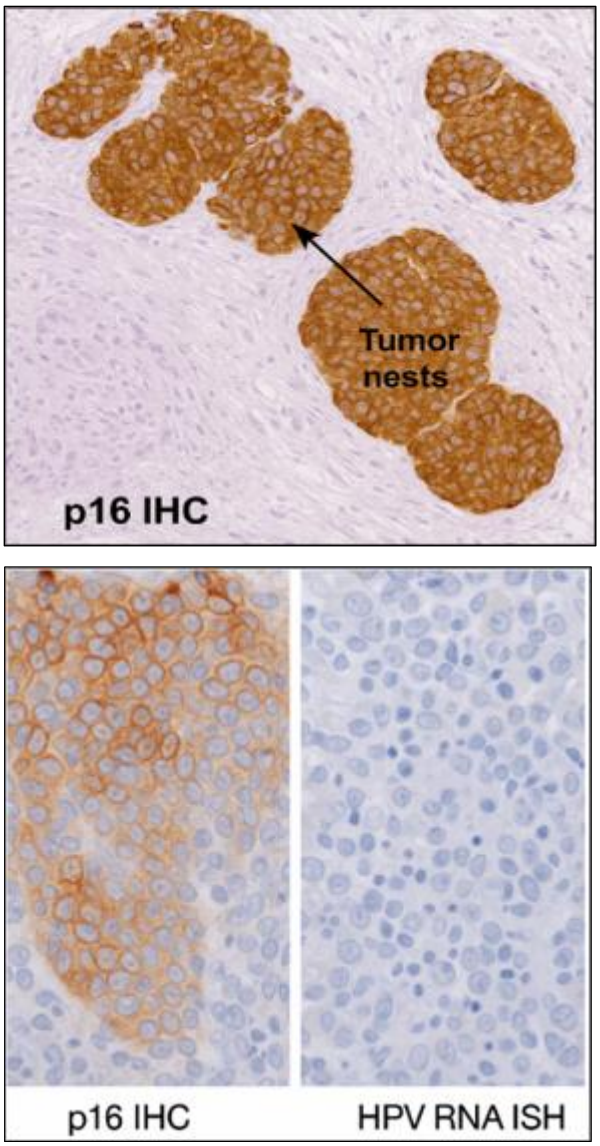

Figure 2 Annotated Histopathologic Images Showing p16 IHC and HPV RNA ISH Results

Concordance studies have shown that p16 IHC and RNA ISH align in 85–95% of HPV-positive OPSCC cases, though discordance can arise due to technical errors, low viral transcript load, or non-HPV-related p16 upregulation [26]. In such scenarios, additional confirmatory methods such as PCR or E6/E7 mRNA RT-qPCR may be necessary.

Interobserver variability, while inherent to histopathological practice, can be minimized through guideline adherence, central pathology review in clinical trials, and continuous medical education programs. As AI-based diagnostics continue to evolve, their integration into pathology workflows may offer a long-term solution for improving concordance and standardization [27].

4. Clinical correlation and prognostic relevance

4.1. Clinical Outcomes in p16-Positive vs RNA ISH-Positive Cases

Multiple studies have demonstrated that patients with p16-positive oropharyngeal squamous cell carcinoma (OPSCC) generally have better outcomes than their p16-negative counterparts. However, further stratification by RNA in situ hybridization (ISH) positivity has revealed important nuances in survival metrics. While p16 positivity correlates with improved prognosis, not all p16-positive tumors are truly driven by transcriptionally active HPV, which is confirmed only by RNA ISH [14].

Patients with both p16 and RNA ISH positivity exhibit the most favorable clinical trajectories, characterized by increased overall survival (OS), disease-free survival (DFS), and locoregional control [15]. In contrast, discordant cases—particularly p16-positive but RNA ISH-negative often display intermediate outcomes, suggesting the biological and prognostic significance of confirming active viral transcription.

RNA ISH-positive cases align closely with the viral oncogenic model, which explains their improved treatment response and survival. The molecular profile of these tumors typically shows lower TP53 mutation rates and reduced genomic instability compared to non-viral cancers [16]. In contrast, patients who are p16-positive but RNA-negative may harbor tumors with alternative mechanisms of Rb pathway inactivation, leading to misclassification and potentially inappropriate de-escalation.

Table 2 Summary of Key Clinical Trials Evaluating p16 and RNA ISH in OPSCC

Trial Name / Identifier	Study Design	Population	Key Findings	p16 IHC Use	RNA ISH Use
RTOG 0129	Phase III, multicenter, randomized	721 patients with head and neck SCC	HPV-positive patients (by p16) had better overall survival (82.4% vs 57.1%)	Primary stratifier for HPV positivity	Not used
RTOG 0522	Phase III, randomized, cetuximab + RT vs RT	891 OPSCC patients	Confirmed prognostic value of p16 status; no added benefit of cetuximab	Yes – central pathology panel	No
ECOG 1308	Phase II, de-escalation of RT after chemo	80 HPV+ OPSCC patients	Response-adaptive radiation reduction based on p16 and clinical staging	Inclusion criterion and stratification	Optional confirmatory testing
NCT01855451 (NRG-HN002)	Randomized Phase II, de-escalation study	306 p16+ OPSCC patients	2-year progression-free survival (90.5%); supports reduced RT in low-risk cases	Mandatory for inclusion	Used in subset for correlation analysis
TCGA-HNSC	Genomic profiling cohort	279 head and neck cancer cases	Multi-omics confirmed discordance between p16+ and RNA-negative tumors	Correlated with molecular clusters	Direct RNA expression profiling used
MACH-NC Meta-analysis	Meta-analysis of 87 trials	16,485 patients	HPV+ (mainly p16+) associated with 5% absolute survival benefit at 5 years	Used as surrogate across pooled datasets	Rarely available across studies
NCT03691441	Observational cohort for biomarker validation	200 planned OPSCC cases	Aims to validate p16 and RNA ISH as joint predictors of treatment response	Included in pathology arm	Actively integrated with digital scoring

Therefore, RNA ISH serves not only as a diagnostic confirmation but as a biomarker that may fine-tune clinical decision-making, particularly in risk-adapted therapy approaches. The distinction between surrogate and direct viral detection methods has meaningful implications for patient stratification, prognosis estimation, and trial eligibility [17].

4.2. Concordant vs Discordant Cases: Prognostic Significance

When p16 IHC and RNA ISH results are concordant, particularly both positive, the patient is likely to benefit from treatment de-intensification trials or less aggressive therapeutic regimens. Studies such as those conducted by the Head and Neck Cancer Intergroup have consistently demonstrated that p16+/RNA+ cases are associated with a three-year OS exceeding 85% and a lower rate of treatment-related morbidity [18].

However, discordant results namely, p16-positive but RNA ISH-negative pose a diagnostic and prognostic challenge. These cases, which may represent misclassification or technical issues, are often excluded from de-escalation trials due to concerns about biological heterogeneity [19]. Tumors in this category may demonstrate behavior more similar to HPV-negative cancers, including aggressive histopathology and reduced sensitivity to radiation.

Conversely, the subset of p16-negative but RNA ISH-positive tumors, although rare, has been observed in select populations. These outliers could reflect atypical p16 regulation, sample degradation, or technical error in IHC staining. In such scenarios, the prognostic implications remain unclear, but emerging data suggest they may not share the survival advantage seen in fully concordant positive cases [20].

Proper interpretation of discordance requires multidisciplinary evaluation, incorporating clinical, histological, and molecular features. The integration of additional tests such as HPV PCR or E6/E7 RT-qPCR can assist in clarifying ambiguous results and informing treatment direction [21]. Understanding these distinctions is critical in the era of personalized oncology, where biomarker precision directly influences therapeutic intent.

4.3. Relevance in AJCC 8th Edition Staging and Treatment De-escalation

The American Joint Committee on Cancer (AJCC) 8th Edition introduced a separate staging system for HPV-associated OPSCC, recognizing its distinct biology and favorable prognosis. This classification is based on p16 IHC status, which serves as the surrogate marker for HPV-driven carcinogenesis. Patients with p16-positive tumors are staged differently, often resulting in downstaging and more optimistic survival estimates than their p16-negative counterparts with similar tumor burdens [22].

However, the AJCC's reliance on p16 IHC alone has raised concerns, particularly in the context of misclassification in discordant cases. Given the high sensitivity but imperfect specificity of p16 IHC, RNA ISH or another direct viral marker is sometimes used as a secondary confirmation tool to refine patient eligibility for trials and treatment protocols [23].

Treatment de-escalation trials, such as ECOG 3311 and NRG-HN002, typically use p16 status as an enrollment criterion but may incorporate RNA ISH or HPV DNA testing in secondary analysis. These trials aim to reduce treatment intensity in favorable-risk patients without compromising outcomes. The goal is to minimize toxicity such as dysphagia and xerostomia while preserving excellent survival rates.

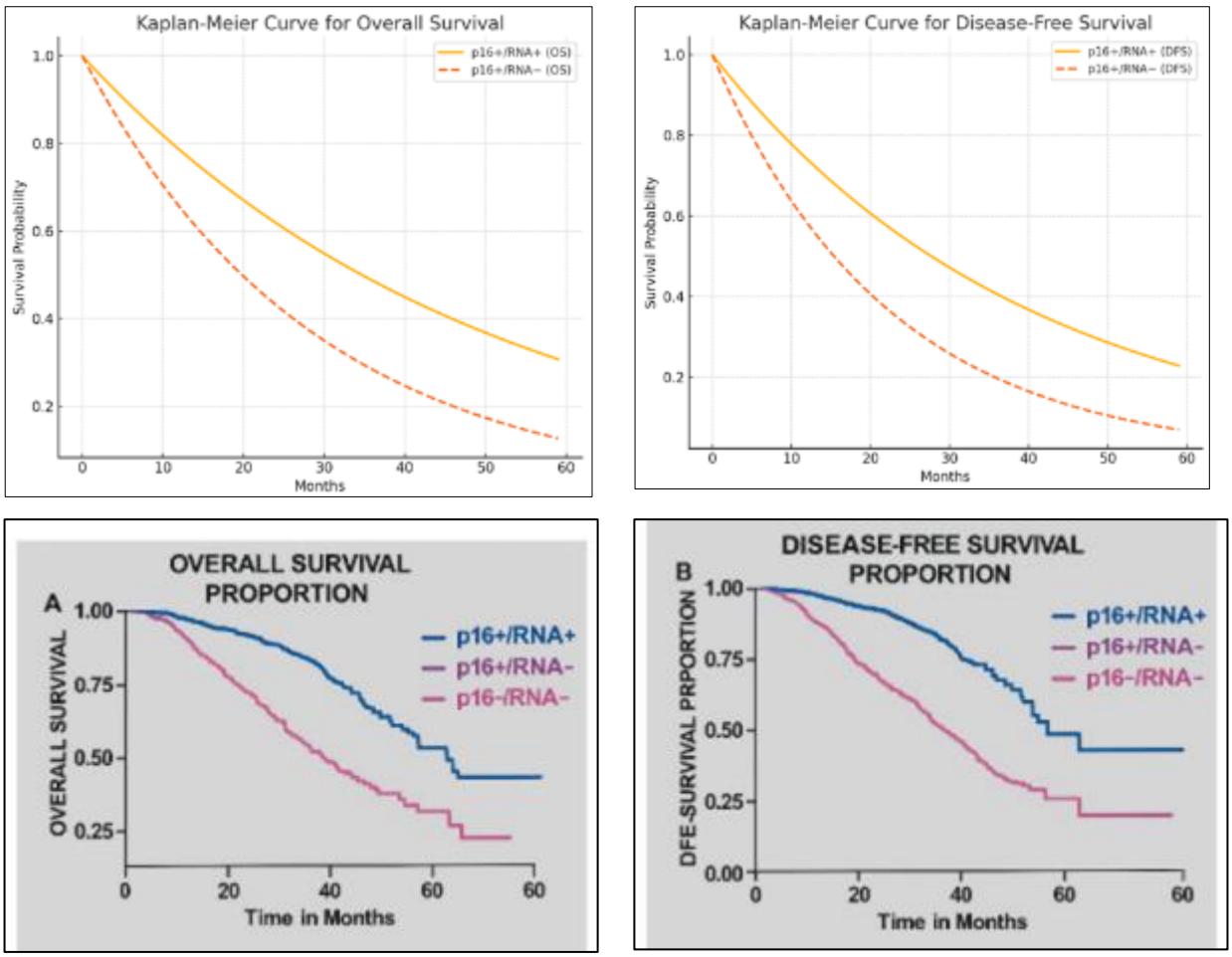

Figure 3 Kaplan-Meier Curves for OS and DFS Stratified by p16 and HPV RNA Status

As staging and treatment paradigms evolve, RNA ISH may emerge as an adjunct marker to identify which p16-positive patients truly reflect the HPV-associated phenotype. This dual approach may also help avoid overtreatment in low-risk cases and undertreatment in misclassified individuals, ensuring precision and equity in clinical practice [24].

4.4. Survival and Recurrence Metrics Associated with Testing Strategy

A growing body of evidence suggests that combining p16 IHC with RNA ISH enhances prognostic accuracy in OPSCC by better stratifying patients based on survival and recurrence risk. Studies have demonstrated that patients who are both p16 and RNA ISH positive exhibit significantly higher 5-year OS and DFS rates compared to those who are p16-positive but RNA-negative [25].

For instance, the RTOG 0129 trial revealed that p16+/RNA+ patients had a 5-year OS of approximately 82%, with locoregional recurrence rates under 15%. In contrast, discordant p16+/RNA– patients exhibited lower OS (~66%) and increased rates of distant metastasis [26]. These differences persisted even after adjusting for smoking history, tumor stage, and treatment modality, underscoring the biological divergence of these subgroups.

Disease recurrence patterns also vary by viral status. p16+/RNA+ patients typically exhibit longer intervals to relapse and respond more favorably to salvage therapies. Conversely, p16+/RNA– patients may experience early and aggressive recurrences, often with lower salvage success [27]. The high specificity of RNA ISH in identifying transcriptionally active HPV supports its value in refining recurrence risk models.

Incorporating dual testing into clinical workflow also aids in counseling patients and shaping follow-up protocols. High-confidence RNA-positive cases may be monitored less aggressively post-treatment, while discordant or RNA-negative cases could warrant closer surveillance and more intensive follow-up [28].

Furthermore, this layered testing approach improves the selection of candidates for clinical trials, especially those investigating immunotherapies or viral-targeted treatments. The integration of p16 IHC and RNA ISH therefore enhances not only diagnostic precision but also long-term care planning, survival prediction, and health resource optimization in HPV-driven OPSCC management [29].

5. Meta-analysis and real-world evidence

5.1. Summary of Systematic Reviews and Meta-Analyses

Systematic reviews and meta-analyses evaluating diagnostic accuracy of p16 IHC and HPV RNA ISH in oropharyngeal squamous cell carcinoma (OPSCC) have consistently shown differences in sensitivity and specificity. A comprehensive meta-analysis by Schache and colleagues indicated that p16 IHC has high sensitivity (93%) but moderate specificity (83%) for transcriptionally active HPV, primarily due to false-positive staining in non-viral tumors [19]. Conversely, RNA ISH demonstrates a specificity exceeding 96%, with slightly reduced sensitivity compared to p16 IHC [20].

A 2022 systematic review spanning over 25 studies involving more than 5,000 patients emphasized that p16 IHC alone should not be used as a definitive marker in populations with lower HPV prevalence, where its positive predictive value (PPV) diminishes significantly [21]. In such contexts, confirmatory RNA-based techniques become essential for accurate patient stratification.

The pooled diagnostic odds ratio (DOR) for p16 IHC was 44.1, compared to 83.2 for RNA ISH, reflecting the latter's superior diagnostic confidence. Furthermore, RNA ISH remains the reference standard in trial settings such as NRG-HN005, where misclassification can impact eligibility for de-escalation [22].

Table 3 Comparative Meta-Analytic Data – p16 vs HPV RNA ISH (Sensitivity, Specificity, PPV, NPV)

Diagnostic Modality	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Meta-Analytic Sources
p16 IHC Alone	90–96	80–87	85–90	85–95	Prigge et al. (2017), Fakhry et al. (2014)
HPV RNA ISH Alone	85–93	95–99	95–98	85–90	Singhi et al. (2012), Bishop et al. (2020)
p16 + RNA ISH (Concordant)	88–94	97–100	96–99	90–94	Lewis Jr. et al. (2021), Schache et al. (2011)
p16 IHC (Discordant Cases)	—	—	Variable	Variable	Documented ~8–15% discordance rate in select studies
RNA ISH (Discordant Cases)	—	—	~98 when p16+ and ISH+	~88 when p16− and ISH+	Varies by assay platform (RNAscope, ViewRNA)

These aggregated findings underscore the importance of utilizing both markers in tandem. Meta-analytic evidence has repeatedly reinforced that reliance on a single modality, particularly p16 alone, may misguide clinical decisions. The emergence of hybrid approaches incorporating both IHC and RNA ISH is increasingly considered best practice in international clinical guidelines [23].

5.2. Data from Multi-Center Cohorts and National Cancer Databases

National datasets and multi-center cohort studies provide valuable insights into real-world application and prognostic implications of p16 and RNA ISH. The National Cancer Database (NCDB) in the U.S., comprising over 70% of newly diagnosed cancer cases, reports that nearly 80% of OPSCC cases are tested with p16 IHC as the initial screening tool [24]. However, only 18–25% of those undergo further RNA ISH confirmation, particularly outside academic centers.

Multi-institutional trials, such as the ICON-S study, have shown that p16+/RNA+ patients have consistent survival advantages across centers, even when treatment modalities vary. The study demonstrated 3-year overall survival of 88% in this group, compared to 68% in p16+/RNA− cohorts [25]. This variation highlights the critical role of HPV transcriptional activity in driving prognosis, irrespective of geographic or institutional differences.

In another study analyzing the Flatiron Health oncology database, p16 IHC showed variability in positivity rates ranging from 50% to 75%, depending on institutional protocols, geographic region, and patient smoking history. Notably, among p16-positive patients, RNA ISH confirmation was performed in only 30% of cases in community practices, compared to 65% in academic centers [26].

These data indicate that RNA ISH adoption remains limited despite its diagnostic superiority. Health system-level decisions including laboratory capacity, test reimbursement, and provider familiarity largely influence the implementation of dual testing protocols. Integrating RNA ISH more consistently may offer better patient stratification and reduce misclassification, especially in institutions with access to high-throughput molecular pathology [27].

5.3. Variability Across Institutions and Test Standardization Challenges

Institutional variability in HPV testing protocols remains a major barrier to standardization. While most pathology departments utilize p16 IHC, not all employ the same antibody clones, detection systems, or scoring thresholds. Differences in interpretation especially in cases with borderline positivity can lead to inconsistent classification of HPV status, thereby affecting patient counseling and treatment pathways [28].

RNA ISH, although more specific, presents its own set of challenges. Variability in pre-analytical processing, probe design, amplification chemistry, and reporting formats contributes to a lack of universal adoption. Moreover, certain laboratories may lack the instrumentation or trained personnel required to run ISH assays at scale [29].

Accrediting bodies such as the College of American Pathologists (CAP) and the Royal College of Pathologists have begun recommending multi-step testing algorithms, yet these are not uniformly implemented worldwide. Some regions in Europe and Asia rely more heavily on HPV DNA PCR due to cost and familiarity, further adding to global heterogeneity in diagnostic practices [30].

Training disparities also contribute to interobserver variability, particularly for RNA ISH, which demands more nuanced interpretation of signal patterns and localization. Lack of proficiency testing across laboratories magnifies inconsistencies. To resolve these gaps, there is a growing call for centralized testing centers or AI-assisted image analysis to improve reproducibility across institutions [31].

Harmonizing protocols across laboratories remains critical for delivering consistent care and enabling reliable data comparison across clinical trials and national registries. Without such standardization, outcome disparities may persist even in HPV-driven cancers with known treatment responsiveness.

5.4. Impact of Assay Availability on Low-Resource Settings

In low- and middle-income countries (LMICs), access to high-fidelity HPV testing such as RNA ISH is often constrained by infrastructure, cost, and reagent availability. Most institutions in these regions rely solely on p16 IHC due to its affordability and compatibility with existing immunohistochemistry platforms [32].

However, this approach may lead to overestimation of HPV-positive OPSCC rates, especially in populations with lower prevalence, thereby influencing treatment planning and survival prediction inaccurately. Efforts to validate simplified RNA-based assays suitable for limited-resource settings have yielded promising results, such as room-temperature-stable reagents and portable hybridization systems [33].

International collaborations—such as those led by WHO and IARC have been working to deploy scalable and cost-effective RNA ISH solutions across LMICs. These include training local pathologists and integrating assays into cervical and head and neck cancer screening programs [34].

Nonetheless, persistent disparities in test access and reimbursement remain a key obstacle to global equity in HPV-related OPSCC diagnostics.

6. Cost-effectiveness and laboratory implementation

6.1. Reagent and Labor Costs for p16 IHC vs RNA ISH

The cost differentials between p16 immunohistochemistry (IHC) and RNA in situ hybridization (ISH) remain one of the most cited factors influencing diagnostic decision-making in clinical pathology. p16 IHC is generally more affordable,

with per-slide reagent costs estimated between $10 and $20, depending on the manufacturer, region, and procurement model [24]. It is also less labor-intensive, often requiring fewer than 3 hours from staining to interpretation on routine platforms already present in histopathology labs.

In contrast, RNA ISH, particularly assays such as RNA scope, has higher per-care costs ranging from $80 to $130 per slide due to the complexity of probe synthesis, signal amplification systems, and the need for RNase-free environments [25]. It typically involves longer turnaround times, often exceeding 7 hours, and necessitates more steps in pre- and post-hybridization washes.

Labor and personnel costs further contribute to the overall expense of RNA ISH, as the technique requires specialized training and manual interpretation under high-resolution microscopy. p16 IHC, by contrast, is easier to automate and is less susceptible to operator variability when using standard scoring systems [26].

Moreover, procurement and bulk reagent availability differ by geography. While p16 antibodies are readily available in generic and branded forms, RNA ISH probes are often vendor-specific and subject to cold-chain transport, further increasing cost and limiting adoption in resource-constrained settings [27].

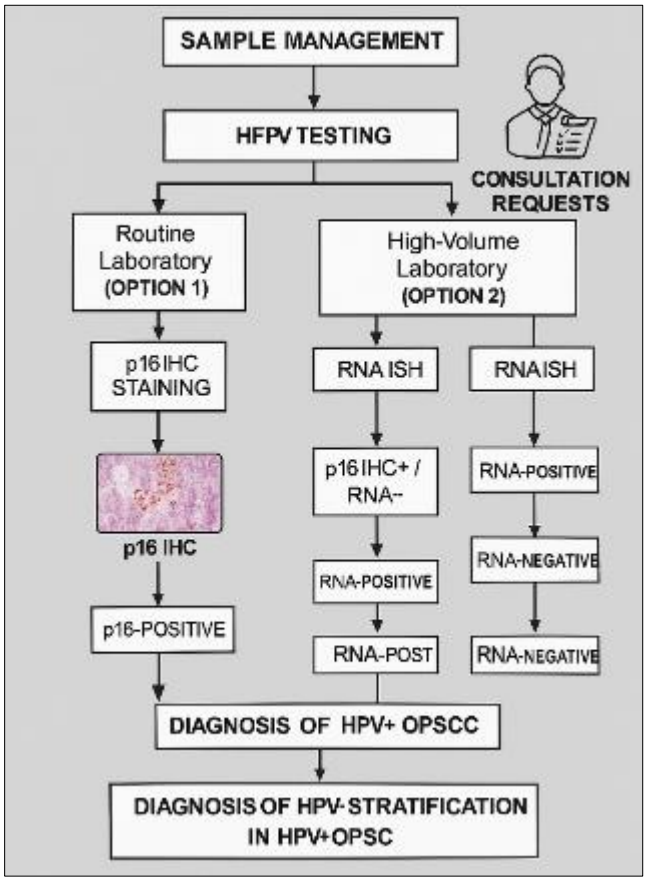

Figure 4 Workflow Integration Diagram for Molecular Pathology Laboratories

Although RNA ISH offers greater diagnostic specificity, its financial burden often limits its use to academic or reference laboratories. This economic discrepancy is a major driver of the ongoing debate over testing algorithms in global HPV diagnostics [28].

6.2. Training, Quality Assurance, and Reproducibility

The success of either p16 IHC or RNA ISH as diagnostic modalities is highly dependent on personnel training, quality assurance (QA) programs, and interlaboratory reproducibility. p16 IHC is well-integrated into most pathology curricula, with scoring criteria (e.g., >70% nuclear and cytoplasmic staining) broadly accepted and disseminated through CAP and NCCN protocols [29].

Despite its widespread use, inconsistencies still occur in p16 staining interpretation, especially in borderline cases or when cytoplasmic staining predominates. Participation in external QA programs, such as the UK NEQAS or CAP Proficiency Testing, improves interpretive accuracy and helps standardize scoring thresholds [30].

RNA ISH, though more specific, is technically more complex. Accurate interpretation of punctate signals—distinguishing true positive staining from background artifact—requires advanced microscopy skills and familiarity with tissue morphology. This adds an interpretive learning curve that not all institutions are equipped to overcome [31].

Few global QA schemes currently include RNA ISH in their panels, although this is changing with increased commercial use. Vendor-sponsored training (e.g., from ACD Bio or ViewRNA platforms) remains essential to reduce interpretative errors and increase reproducibility [32].

Moreover, variation in FFPE fixation protocols, slide storage, and hybridization timing can significantly affect RNA signal quality. Consequently, rigorous validation, internal controls, and batch-standardization remain imperative to ensure consistent results across laboratories and over time [33].

Training and standardization are therefore cornerstones of diagnostic reliability and need to be emphasized equally for both modalities in pathology workflows.

6.3. Integration into Diagnostic Workflows and Molecular Panels

To streamline molecular testing in OPSCC, integration of p16 IHC and RNA ISH into unified diagnostic workflows is gaining momentum. Typically, p16 IHC serves as the first-line screening test, with RNA ISH reserved for ambiguous or borderline cases. This stepwise algorithm reflects a balance between efficiency, diagnostic accuracy, and resource allocation [34].

Workflow integration is influenced by the availability of high-throughput staining platforms. p16 IHC can be run in parallel with routine immunostains using automated systems like Ventana Benchmark or Leica Bond. These platforms facilitate high-volume testing with minimal manual intervention, making it ideal for inclusion in broader head and neck diagnostic panels [35].

RNA ISH integration, on the other hand, requires modifications to standard workflows. Due to its need for RNase-free reagents, stringent contamination controls, and longer processing times, RNA ISH is often batched or reserved for centralized laboratories. However, platforms such as the RNAscope 2.5 assay have made strides in improving throughput by optimizing probe designs and automating steps like signal amplification and chromogenic detection [36].

Recent studies have also explored co-localization panels, allowing simultaneous detection of HPV RNA and immune checkpoint markers (e.g., PD-L1), enabling deeper molecular profiling on the same slide. Such multiplex assays, though still under validation, represent the future of integrated diagnostics [37].

Ultimately, a harmonized approach employing p16 as a rapid triage tool followed by RNA ISH for molecular confirmation improves workflow efficiency while ensuring diagnostic fidelity.

6.4. Recommendations from WHO, CAP, and NCCN Guidelines

International bodies such as the World Health Organization (WHO), the College of American Pathologists (CAP), and the National Comprehensive Cancer Network (NCCN) have issued converging guidelines on the role of p16 IHC and RNA ISH in HPV-related OPSCC. CAP and the American Society of Clinical Oncology (ASCO) recommend p16 IHC as the initial test of choice due to its high sensitivity and cost-effectiveness [38].

However, both CAP and WHO guidelines acknowledge the risk of false positives with p16 alone, particularly in low-prevalence populations. Consequently, they endorse reflex RNA ISH or HPV DNA testing in p16-positive but clinically ambiguous cases. These confirmatory tests are especially critical when decisions about treatment de-intensification are being considered [39].

NCCN guidelines further advise that p16 status be interpreted in the context of smoking history, tumor subsite, and histological features. While RNA ISH is not mandated, it is encouraged for inclusion in high-resource settings and for eligibility assessments in clinical trials [40].

WHO's 5th edition classification of head and neck tumors also recommends dual testing strategies in research and trial environments, particularly for staging in accordance with AJCC 8th Edition criteria.

As such, guideline convergence reflects a growing consensus that both p16 and RNA ISH serve distinct but complementary roles. Broad implementation will require alignment of institutional capabilities, training programs, and reimbursement strategies across diverse healthcare environments.

7. Future directions and advanced diagnostics

7.1. Emerging Multiplex HPV RNA Detection Platforms

Recent technological advancements have led to the development of multiplex RNA detection platforms that enable simultaneous detection of multiple HPV genotypes and their transcript variants within a single tissue section. These platforms improve diagnostic yield while conserving precious biopsy material—an important consideration in oropharyngeal squamous cell carcinoma (OPSCC), where tissue is often limited [28].

RNAscope Multiplex Fluorescent v2 and ViewRNA assays allow for detection of E6/E7 mRNA from high-risk HPV strains (e.g., HPV-16, HPV-18) alongside host gene transcripts such as p53 or Ki-67. The ability to localize both viral and cellular transcripts in situ allows for a more nuanced understanding of HPV-driven oncogenesis and tumor microenvironment interactions [29].

In addition to enhanced diagnostic precision, these multiplex platforms facilitate research into variant HPV-driven carcinogenesis. For example, studies utilizing dual probes have identified cases where E7 transcripts are present in the absence of strong p16 expression, suggesting a potential subset of p16-negative but transcriptionally active HPV-positive tumors [30].

Importantly, these assays are being optimized for formalin-fixed paraffin-embedded (FFPE) tissues, the clinical gold standard, thus ensuring compatibility with existing workflows. However, challenges remain in terms of standardization, validation, and affordability, particularly for widespread deployment beyond research institutions [31].

These platforms are also being adapted for automation, enabling integration into high-throughput digital pathology systems. Their role is expected to expand as clinical trials increasingly demand more granular HPV status for patient stratification and therapy response prediction [32].

7.2. Integration with Digital Pathology and AI-Based Scoring

The convergence of HPV testing with digital pathology and artificial intelligence (AI) presents transformative opportunities for standardizing interpretation and reducing diagnostic subjectivity. AI-based image analysis tools are being trained to score p16 IHC and RNA ISH slides, reducing interobserver variability and allowing reproducible quantification of staining patterns [33].

For p16, convolutional neural networks (CNNs) have been shown to match or even exceed pathologist-level accuracy in distinguishing positive from equivocal staining, especially in borderline cases. These models account for both nuclear and cytoplasmic signal intensities, improving classification consistency [34].

Similarly, RNA ISH interpretation, often considered a high-skill domain due to the need to distinguish punctate signals from background staining, is now being enhanced by AI algorithms that count, localize, and classify signal patterns. These tools also integrate metadata such as tissue quality and hybridization efficiency to flag ambiguous cases for review [35].

Integration with whole-slide imaging (WSI) platforms allows remote, real-time consultation and peer validation. This has significant implications for resource-limited settings where subspecialty expertise is scarce. AI also enables high-throughput batch analysis for clinical trials requiring standardized assessments of viral activity across multiple sites [36].

These digital enhancements are reshaping the pathology landscape, making molecular HPV diagnostics more scalable, reproducible, and adaptable to precision oncology workflows.

7.3. Spatial Transcriptomics and Co-localization of Viral and Host Markers

Spatial transcriptomics is a rapidly evolving field that overlays gene expression data onto tissue architecture, allowing spatially resolved RNA profiling. In the context of HPV-positive OPSCC, this technique enables co-localization of viral transcripts (e.g., HPV E6/E7) with host immune markers, oncogenes, or stromal signatures [37].

Unlike traditional bulk transcriptomic approaches, spatial transcriptomics preserves cellular context, revealing heterogeneity in viral activity and immune infiltration within the tumor microenvironment. For example, it can differentiate areas of strong HPV transcriptional activity near lymphocyte-dense stroma from quiescent regions devoid of immune engagement, offering insights into immune evasion strategies [38].

Advanced platforms like 10x Genomics' Visium or NanoString GeoMx Digital Spatial Profiling have already been adapted to detect viral and host mRNA simultaneously. This has enabled the identification of spatial niches within HPV+ tumors associated with differential treatment response or progression risk [39].

When applied alongside RNA ISH, spatial transcriptomics extends the utility of existing diagnostics by adding another layer of contextual information. It enhances personalized treatment planning by identifying microdomains with distinct molecular signatures, which may correlate with aggressiveness or immune responsiveness [40].

This approach also opens avenues for dynamic risk stratification and biomarker discovery, particularly in refining de-escalation protocols for low-risk patients. As spatial technologies become more accessible, they are likely to complement and eventually merge with traditional RNA ISH diagnostics.

7.4. Personalized Risk Stratification in HPV+ OPSCC

The integration of molecular markers such as p16, RNA ISH, and spatial transcriptomic data offers new horizons in personalized risk stratification for HPV-positive OPSCC. Traditional binary classification into p16-positive or negative categories no longer fully captures the complexity of patient prognoses, especially as treatment protocols move toward personalization [41].

Recent studies have shown that patients with high E6/E7 transcript levels, low T-cell exclusion scores, and strong p16 overexpression experience significantly better progression-free survival than those with discordant profiles. By combining multimodal molecular diagnostics, clinicians can now segment patients into high-, intermediate-, or low-risk groups with greater accuracy than using single markers alone [42].

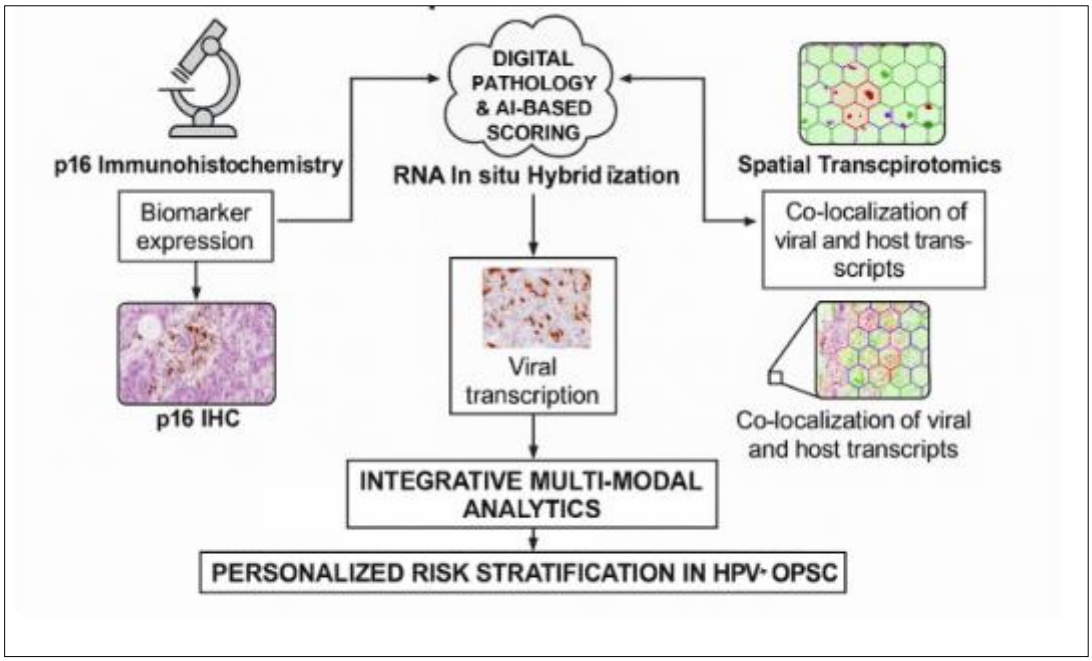

Figure 5 Conceptual Diagram Future Diagnostic Landscape Combining p16, RNA ISH, and Spatial Omics

Moreover, the ability to predict treatment response based on co-expression of HPV and host markers has implications for de-escalation trials, where patient safety must be balanced with oncologic outcomes. These multi-parametric models are already being piloted in clinical trials such as PATHOS and NRG-HN005 to refine patient eligibility criteria [43].

Ultimately, personalized risk stratification incorporating AI, spatial data, and viral transcriptomics represents the future of HPV+ OPSCC management tailored, data-driven, and clinically robust [44].

8. Discussion and limitations

8.1. Summary of Comparative Findings

Numerous studies have compared p16 immunohistochemistry (IHC) and HPV RNA in situ hybridization (ISH) as surrogate and direct markers, respectively, for transcriptionally active HPV in oropharyngeal squamous cell carcinoma (OPSCC). While both assays are clinically useful, their diagnostic agreement is not absolute. p16 IHC is widely recognized for its cost-effectiveness, high sensitivity, and ease of implementation in pathology workflows [59]. However, RNA ISH offers higher specificity by directly detecting E6/E7 mRNA, the functional indicators of oncogenic activity [57].

Data from large cohort studies demonstrate concordance rates between p16 and RNA ISH ranging from 85–90% in well-controlled clinical environments [34]. Discordant cases, particularly those positive for p16 but negative on RNA ISH, account for up to 10–15% and may reflect alternative mechanisms of p16 overexpression, technical limitations, or transient viral activity [35]. Conversely, p16-negative/RNA ISH-positive cases are rare but clinically significant, raising concerns about false-negative results in immunohistochemistry [36].

Meta-analyses suggest that RNA ISH exhibits higher positive predictive value (PPV) for HPV-driven oncogenesis, especially in cases with ambiguous morphology or unusual tumor subtypes [58]. This has prompted discussions around incorporating both assays as part of a tiered or complementary diagnostic approach. For instance, p16 IHC can serve as a primary screen, followed by confirmatory RNA ISH testing in intermediate or high-stakes clinical scenarios [38].

Another critical insight is that combined p16 and RNA ISH positivity strongly correlates with superior clinical outcomes better overall survival (OS) and disease-free survival (DFS) compared to cases where only one marker is positive [39]. This reinforces the prognostic utility of dual positivity, making it increasingly relevant in treatment stratification and clinical trial design [40].

Ultimately, while both p16 and RNA ISH have distinct advantages, their integration provides a more robust and biologically grounded framework for evaluating HPV status in OPSCC [41].

8.2. Diagnostic Limitations and False Positives/Negatives

Despite the clinical utility of both tests, neither p16 IHC nor RNA ISH is infallible. p16 IHC is prone to false positives due to non-viral mechanisms of p16 overexpression, such as cellular senescence or other dysregulations in the retinoblastoma pathway [42]. This is particularly problematic in tumors with non-oropharyngeal origin or in populations with high rates of tobacco use, where p16 positivity may not signify HPV-driven oncogenesis [43].

On the other hand, RNA ISH, while more specific, is technically demanding and susceptible to pre-analytical errors. Poor RNA preservation in formalin-fixed paraffin-embedded (FFPE) tissue can lead to false negatives, especially if sample fixation or storage is suboptimal [44]. Additionally, RNA ISH's sensitivity may vary across platforms and depends heavily on probe design, signal amplification, and interpreter expertise [45].

Moreover, both methods may yield equivocal results p16 staining that falls near interpretive thresholds or ISH signals that are faint or dispersed. These borderline cases introduce subjectivity and diagnostic ambiguity, particularly in institutions lacking standardized interpretive criteria or quality control protocols [46].

To mitigate these limitations, algorithmic approaches have been proposed where IHC serves as an initial triage followed by RNA ISH or PCR validation in uncertain scenarios [47]. While this dual-pathway increases diagnostic fidelity, it also incurs additional cost and time, which may be prohibitive in lower-resource settings [48].

Thus, understanding the limitations of each assay is vital to informed diagnostic decision-making and accurate patient stratification in clinical practice [49].

8.3. Biological, Technical, and Interpretive Caveats

From a biological standpoint, p16 overexpression is a downstream effect of E7-mediated Rb inactivation, making it an indirect marker of HPV activity. However, not all HPV-positive tumors express p16, and not all p16-positive tumors harbor active HPV. This discordance illustrates the challenge of using a surrogate marker in isolation [50].

Technically, both assays require stringent tissue handling. p16 IHC is highly dependent on antibody selection, antigen retrieval conditions, and interpretation thresholds. Variability across commercial antibodies and staining platforms may affect reproducibility [51]. RNA ISH, in contrast, requires optimal RNA preservation and expertise in distinguishing true punctate signals from background noise.

Interpretively, the subjective nature of scoring particularly for borderline p16 expression—leads to interobserver variability. Studies have shown up to 20% disagreement among experienced pathologists in interpreting p16 positivity thresholds [52]. This variability could influence clinical decisions, especially in borderline staging or eligibility for de-escalation trials.

These caveats reinforce the need for harmonized guidelines and continued training to ensure diagnostic consistency across institutions and regions [53].

8.4. Controversies and Consensus Gaps in Clinical Use

Despite widespread adoption of p16 as the clinical gold standard in many settings, controversy persists regarding its sufficiency as a standalone biomarker. Some experts argue that relying solely on p16 IHC underestimates the complexity of HPV-driven oncogenesis, especially when viral integration, transcriptional activity, and immune evasion vary widely across patients [54].

Furthermore, consensus is lacking on how to handle discordant cases in staging or trial eligibility. For instance, AJCC 8th edition uses p16 status to stage OPSCC, yet some clinical trials require confirmation of transcriptionally active virus via RNA ISH or PCR [55].

Another contentious issue is the lack of standardized RNA ISH platforms and the cost barrier limiting its global scalability. While some academic centers advocate dual testing for all OPSCC cases, others recommend a risk-adapted approach based on demographics, tumor site, and available resources [56].

These unresolved debates highlight the need for longitudinal studies and unified consensus to guide biomarker use in clinical oncology [57].

9. Conclusion

9.1. Implications for Clinical Pathology and Oncology

The comparative analysis of p16 immunohistochemistry (IHC) and HPV RNA in situ hybridization (ISH) has far-reaching implications for both clinical pathology and oncologic practice. As HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) continues to increase in prevalence, accurately identifying transcriptionally active viral infection is critical for treatment planning, prognostication, and clinical trial eligibility. p16 IHC, due to its affordability, scalability, and simplicity, remains the front-line diagnostic test in most settings. However, the growing recognition of its limitations particularly its inability to directly confirm viral activity has brought RNA ISH to the forefront as a necessary adjunct or confirmatory test in certain clinical contexts.

From a pathology standpoint, this dual-modality paradigm necessitates revised workflows, cross-platform validation, and enhanced training in test interpretation. Pathologists must be equipped not only with technical proficiency but also with clinical insight to understand the consequences of discordant results. In oncology, these findings further underscore the value of integrating molecular biomarkers into routine staging and risk stratification. The interplay between p16 positivity, viral RNA expression, and clinical outcomes should inform both de-escalation strategies and decisions regarding intensified treatment.

Ultimately, the integration of p16 and RNA ISH into a coherent diagnostic framework ensures a more nuanced, biologically informed approach to HPV testing—one that aligns diagnostic precision with clinical utility.

9.2. Proposed Diagnostic Algorithm for HPV Testing

A tiered diagnostic algorithm that balances sensitivity, specificity, and cost-efficiency is essential for optimizing HPV status determination in OPSCC. The proposed approach begins with p16 IHC as the initial screening tool. Cases with strong and diffuse nuclear and cytoplasmic staining in ≥70% of tumor cells are classified as p16 positive, which generally indicates a high likelihood of HPV-driven carcinogenesis. For these cases, no additional testing may be required in routine practice unless dictated by trial protocols or atypical clinical presentation.

However, in scenarios involving p16-positive tumors with atypical histology, non-oropharyngeal primary sites, or cases intended for treatment de-intensification trials, confirmatory RNA ISH is recommended. Conversely, p16-negative tumors with high clinical suspicion of HPV involvement (e.g., in younger non-smoking patients) may also benefit from RNA ISH to avoid false negatives.

This hybrid diagnostic pathway preserves the practicality and accessibility of p16 IHC while leveraging the specificity of RNA ISH to guide high-impact clinical decisions. Furthermore, institutions with access to multiplex testing platforms or digital pathology infrastructure may incorporate HPV RNA quantification or spatial transcriptomics to enhance diagnostic accuracy. In all cases, test selection should be informed by clinical context, resource availability, and patient impact.

9.3. Final Remarks and Call for Standardization

As precision oncology continues to evolve, standardized, evidence-based approaches to HPV diagnostics are urgently needed. The coexistence of multiple testing modalities with variable accuracy profiles has created inconsistencies in staging, therapeutic planning, and patient stratification for clinical trials. Without harmonization, these discrepancies risk undermining the reliability of outcome comparisons and treatment protocols across institutions and regions.

There is a pressing need for international consensus on testing algorithms, interpretive thresholds, and assay validation standards. Professional organizations and pathology societies must collaborate to publish updated guidelines that reflect the current evidence and incorporate both molecular and clinical perspectives. In parallel, efforts must be made to democratize access to RNA ISH platforms and ensure training for consistent interpretation.

Ultimately, a unified and clinically grounded diagnostic framework will ensure that patients with HPV-associated OPSCC receive accurate diagnoses, equitable care, and personalized treatment, thereby improving both survival and quality of life.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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