Chapter 12
Prognostic Role of p16/HPV in Non-oropharyngeal Head and Neck Squamous Cell Cancer (HNSCC)

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Introduction

HPV infection has been established as an etiologic and prognostic factor for a subset of oropharyngeal squamous cell carcinoma (OPC) with distinct clinical and pathologic characteristics [1]. Also established is that the expression of p16/INK4A gene correlates with HPV infection in the oropharynx and, consequently, the product of this gene, detected with immunohistochemistry (IHC), can be used as a surrogate biomarker for HPV-related OPC [2]. This information is vital for properly staging HPV(+) OPC, for determining the prognosis of the disease, and for implementing de-intensification strategies in the context of clinical trials. On the contrary, conflicting evidence exists regarding the role of p16/HPV as a biomarker in non-OPC head and neck cancer, more specifically for oral, for laryngeal and for hypopharyngeal primaries. Most of the relevant data are inconsistent and derive from retrospective and heterogeneous series of patients. Herein, a brief review of the existing information regarding the utility of p16 as a surrogate marker for HPV in non-OPC HNSCC is presented, and the potential prognostic role of p16/HPV in non-OPC primaries is analyzed.

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HPV Life Cycle

HPV is a small, non-enveloped, double-stranded DNA virus that encodes a total of 8–9 proteins in approximately 8000 base pairs and has the ability to infect cutaneous or mucosal tissues. The viral genome consists of three distinct regions that have specific functional properties. The early (E) region encodes proteins regulating viral transcription (E2), viral DNA replication (E1, E2), cell proliferation (E5, E6, E7), and viral particle release (E4). E6 and E7 viral oncogenes also encode proteins associated with malignant lesions that are capable of immortalizing primary human keratinocytes. The continuous expression of E6 and E7 is crucial in maintaining the cancer phenotype in infected cells, as repression of their expression reverses the malignant phenotype of HPV(+) cancer cells. The late (L) region encodes for two structural viral capsid proteins (L1 and L2). Finally, the long control (LCR) or non-coding region (NCR) regulates viral gene expression and replication [3].

HPV has a complex gene expression that requires a synchronization of transcription, mRNA stability, splicing, and polyadenylation with keratinocyte differentiation and distinct phases of the viral life cycle. The life cycle of HPV is directly related to the cellular differentiation program of the host cell. In the initial phase of the HPV life cycle, keratinocytes of the basal cell layer are infected by the virus that has passed through the above epidermal barrier through erosions and microwounds. For efficient establishment of infection from the high-risk subtypes it is vital that they infect actively dividing basal, or stem epithelial cells. Initial infection is followed by a phase of viral genome amplification; subsequently, the viral genome is maintained as an extrachromosomal circular element, known as episome, at a low copy number. Alternatively, it can be integrated into the host-cell genome. This integration usually occurs downstream of the early genes E6 and E7, often in the E1 or E2 region. HPV uses the host cell replication machinery to initiate viral DNA replication. In the case of high risk HPVs, proteins E6 and E7 promote cell-cycle progression and viral DNA replication in differentiated keratinocytes as they move towards the surface epithelium. As a result, HPV DNA replicates in a high copy number in differentiated cells located near the epithelial surface. Both E7 and E6 have a vital role in manipulating the cellular replication mechanisms in order to create the optimal environment for viral genome replication. Viral protein E6 is required for episomal genome maintenance, while E7 forces the infected cell to re-enter S-phase. The integration of HPV DNA into the host genome disrupts the expression of the main viral transcription/replication factor E2, which acts as transcriptional repressor of E6 and E7 viral oncogenes. Furthermore, the E6 protein causes degradation of the p53 tumor suppressor protein via a ubiquitin-mediated process, while the HPV-E7 protein binds cullin 2 ubiquitin ligase complex and ubiquitinates the retinoblastoma (Rb) tumor suppressor protein. Consequently, the p53 and pRb tumor suppressor pathways are dormant but active in cancer cells due to the continuous expression of E6 and E7 oncogenes. Degradation of Rb induces expression of p16INK4A, which is the hallmark of HPV(+) OPC. pRb downregulates p16 protein at the transcriptional level and low pRb levels, inversely, lead to p16

upregulation. This is the reason why HPV-associated cancers contain high p16 protein levels. The final stage of the life cycle of HPVinvolves the exit from the cell and the expression of late viral proteins L1 and L2 to enable packing of the viral genome [4].

Risk Factors for HPV(+) Non-OPC

HPV(+) OPC has distinct risk factors that are different from the common risk factors of HPV(−) OPC, e.g. excessive exposure to tobacco and alcohol. It is correlated with younger age, male gender, multiple sexual partners and higher socio-economic status, to name a few [5]. It is, however, unclear, whether these same demographic variables apply for HPV(+) non-OPC as well.

To our knowledge, there is only one study that has tried to answer that query. In a multicenter, case-control study of SCCs called the Papillomavirus Role in Oral Cancer Viral Etiology study (PROVE), Windon et al. have discovered that HPV(−) non-OPC patients were more likely to be ever smokers than HPV(+) OPC (n = 185, OR 3.28, 95%CI 1.10–10.2). Also, compared with their HPV(+) OPC counterparts, HPV(+) non-OPC were less likely to have had over 3 oral sexual partners (OR 0.29, 95%CI 0.06–0.9), more likely to have multimorbidity (OR 3.30, 95%CI 1.04–10.5), and less likely to have antibodies to HPV16 E6 (90% vs 28%, OR 0.05, 95%CI 0.02–0.2). Although this was a small study, it provided potential evidence that HPV is not an adequate factor to promote carcinogenesis in non-OPC sites, in contrast to OPC, but rather a second hit of chemical-induced carcinogenesis is required for cancer progression in these cases, as it also happens in HPV(−) HNSCC [6].

p16 as a Surrogate Biomarker for a Transcriptionally Active HPV Infection in Non-OPC

The gold standard for determining that HPV is actively contributing to the oncogenic process in OPC is the detection of the E6/E7 viral oncogene expression through quantitative reverse transcriptase–PCR (qRT-PCR) [3, 4]. Detection of HPV mRNA, however, in formalin-fixed paraffin-embedded (FFPE) tissue has variable sensitivity depending on the quality of the clinical sample. Moreover, many HPV(+) patients identified in the next-generation sequencing study by Parfenov et al. had low levels of E6/E7 expression and could be misclassified by E6/E7mRNA detection [7]. A method that is commonly used is HPV DNA detection by either in situ hybridization (ISH) or polymerase chain reaction (PCR) [2]. DNA ISH can differentiate between integrated and episomal forms of HPV in tumors but lacks sensitivity. HPV DNA PCR is a sensitive method for determination of HPV status but it lacks specificity. HPV DNA presence in tumors per se cannot prove causality, as

HPV is ubiquitously present in humans. Also, as has already been mentioned, the detection of p16 protein expression by IHC staining is used as a surrogate marker of oncogenic HPV infection. A negative autoregulatory loop between p16 and pRb has been described and degradation of pRb by HPVE7 oncoprotein leads to p16 upregulation in HPV(+) cancers [8]. p16 IHC followed by PCR for HPV DNA detection in p16(+) cases has been proposed as a reliable algorithm for determination of HPV status in paraffin-embedded OPC specimens. p16 protein expression, however, is not a reliable surrogate biomarker for HPV infection outside the oropharynx.

Using these methods, it has been found that in OPC, HPV positivity ranges between 57% and 72%, with the variation attributed to differences in assay selection and study populations [9–11]. In addition, a high concordance rate of approximately 90% is noted between HPV ISH and p16 IHC in OPC, and this is partly a result of the high rates of active and persistent HPV infection in this site [12].

In non-OPC sites, however, the rates of active HPV infection are substantially lower, as only 1.3% to 7% of non-OPCs, including cancers of the oral cavity, hypopharynx, and larynx, are HPV positive [13–15]. Similarly, the concordance rates between these two methods of HPV detection also seem to be lower. For example, in oral cavity squamous cell cancer, sensitivity of p16 IHC compared with high-risk HPV E6/E7 mRNA expression is 79%, specificity is 93%, positive predictive value (PPV) is 41%, and negative predictive value is 99% [14].

Harris et al. examined biomolecular profiles in a cohort of 25 young adults with squamous cell cancers (SCCs) of the oral tongue diagnosed between 1989 and 2007. Median age at diagnosis was 30 years (range: 20–39 years). Patients with non-squamous histology, prior history of malignancy and distant metastatic disease at presentation were excluded. Further demonstrating the discrepancy between p16 expression and HPV DNA positivity in non-OPC, p16 overexpression was observed in 11 of 25 patients, whereas HPV-16 DNA positivity was observed in none of the tumor samples by ISH and 2 of the tumor samples by PCR. Interestingly, neither of these HPV DNA(+) patients were found to be p16(+) as well. In this study, p16INK4a positivity was correlated with improved relapse-free survival (HR = 0.23, p = 0.01) and overall survival (OS) (HR = 0.28, P = 0.05). In this trial p16 positivity was correlated with favorable prognosis, while p16INK4a overexpression was not a reliable predictor of HPV positivity. The authors concluded that a mechanism alternative to HPV infection that is leading to p16 positivity may exist in this particular subset of tumors, and that p16INK4a status is the truly important prognosticmarker in HNSCC, independent of HPV infection. However, the small cohort size and the selected patient populationare serious limitations for generalization of these results [16].

Furthermore, in a study by Chung et al. p16 expression and high-risk HPV status in non-OPCs from RTOG 0129, 0234, and 0522 studies were determined by IHC for p16 and HPV DNA ISH for high-risk HPV DNA. A total of 683 eligible patients with non-OPSCC tumors, including primary sites in the oral cavity, hypopharynx, and larynx, were identified among the 1921 patients enrolled onto the above-mentioned trials. Tumors from 356 (52.1%) of 683 patients with non-OPSCC were tested for p16 expression, which could be determined in 90.4% (322 of 356) of the

tumors. HPV status could be determined in 95.5% (297 of 311). Overall, 19.3% (62 of 322) of non-OPC were p16 positive and 9.4% (28 of 297) were HPV ISH positive. p16 expression was positive in 14.1% (12 of 85), 24.2% (23 of 95), and 19.0% (27 of 142) and HPV ISH was positive in 6.5% (six of 93), 14.6% (15 of 103), and 6.9% (seven of 101) of non-OPCsfrom RTOG 0129, 0234, and 0522 studies, respectively. Cancer of the oral cavity had the highest rate of p16 positivity (21 [26.3%] of 80), followed by the larynx (31 [17.1%] of 181) and hypopharynx (10 [16.4%] of 61). Also, cancer of the oral cavity had the highest rate of HPV ISH positivity (13[14.6%] of 89), followed by the larynx (12 [7.9%] of 151) and hypopharynx (three [5.3%] of 57). HR for p16 expression were 0.63 (95% CI, 0.42 to 0.95; P = 0.03) and 0.56 (95% CI, 0.35 to 0.89, P = 0.01) for progression-free (PFS) and OS, respectively. Poor concordance was observed between p16 and HPV ISH among the subsites of the oral cavity, hypopharynx, and larynx, where oral cavity tumors had the worst concordance. Although this trial also showed that patients with p16-negative non-OPC have worse outcomes than patients with p16-positive non-OPC, HPV status was not found to be prognostic, so once again it was demonstrated that p16 was not a good surrogate biomarker for HPV positivity [17].

Finally, in a retrospective study of 409 cases of oral cavity SCC treated at 4 North American Hospitals, fifteen high-risk HPV types were detected in tumors by consensus PCR followed by type-specific HR-HPV E6/7 oncogene expression by quantitative reverse-transcriptase PCR. P16 expression was evaluated by IHC. Twenty-four (5.9%) were high-risk HPV E6/7 expression positive; 3.7% (95%CI 1.8–5.5) for HPV16 and 2.2% (95%CI 0.8–3.6) for other high-risk HPV types. HPV(+) tumors originated from throughout the oral cavity (floor of mouth [n = 9], anterior tongue [6], alveolar process [4], hard palate [3], gingiva [1] and lip [1]) and were significantly correlated with male gender, small tumor stage, poor tumor differentiation, and basaloid histopathology. In this trial, p16 IHC had very good-to-excellent sensitivity (79.2%, 95%CI 57.9–92.9), specificity (93.0%, 95%CI 90.0–95.3), and negative-predictive value (98.6%,95%CI 96.8–99.6), but poor positive-predictive value (41.3%, 95%CI 27.0–56.8) for HR-HPV E6/7 expression in oral cavity SCC [14].

Conclusively, the data at our disposal suggest that p16 is a poor surrogate marker for transcriptionally active HPV infection in non-OPC sites.

p16/HPV as a Prognostic Factor in Non-OPC

In addition to the above-mentioned studies, several other trials have attempted to elucidate the prognostic role of p16 and/or HPV status in non-OPC disease sites. Young et al. evaluated a cohort of 324 laryngeal SCC patients for the expression of p16 by IHC and for high-risk HPV E6 and E7 mRNA transcripts by RNA ISH. The median age of patients at diagnosis was 66 years (range 36–88 years). Males comprised 94% of the patients, with 95% being current or former smokers. p16 expression and HPV status were correlated with clinicopathological features and outcomes.

In this trial, 6.5% of the patients were p16(+) and only 7 cases were HPV RNA(+), all of which were also p16 IHC positive. There was no difference in OS between p16-positive and p16-negative patients with 2-year survival of 79% in each group (HR = 0.83, 95% CI 0.36–1.89, P = 0.65). Also, no statistically significant difference in OS was found between patients with HPV RNA ISH-positive tumors compared with ISH-negative tumors with 2-year survival of 86% and 71%, respectively (HR = 0.76, 95% CI 0.23–2.5, P = 0.65). The most significant strength of this study is the large cohort consisting of a single site of head and neck cancer only, namely the larynx, while its major limitation is its retrospective nature. The researchers concluded that p16 overexpression in laryngeal cancer is infrequent as are the proportion of cases with high-risk HPV transcripts, and there are no statistically significant correlations with survival outcomes [18].

Furthermore, in a retrospective, multi-institution study by Fakhry et al. 239 patients with OPC and 621 patients with non-OPC of the oral cavity, larynx, and nasopharynx, diagnosed from 1995 to 2012, were centrally tested for p16 and HPV by HPV16 DNA and high-risk HPV E6/E7mRNA ISH. The prevalence of HPV(+) tumors among cancers of the oropharynx, oral cavity, larynx, and nasopharynx was 56%, 2%, 5%, and 10%, respectively. The tumor HPV status and p16 were not of prognostic significance in HNSCCs of the oral cavity (n = 253; P = 0.22), larynx (n = 243; P = 0.72), or nasopharynx (n = 125; P = 0.23). Also, the study did not find any correlation of p16 with OS for non-OPC (P = 0.26) [19].

Also, D′ Souza et al. analyzed data from 1362 HNSCC cases diagnosed between 2002–2011 and registered in epidemiologic studies in Brazil (GENCAPO study, n = 388), U.S. (CHANCE study, n = 472), and Europe (ARCAGE study, n = 502). Tumors were centrally tested for p16 and HPV16 DNA by PCR. In total, 517 OPC and 845 non-OPC cases (397 laryngeal, 382 oral cavity, and 66 hypopharyngeal SCC) were identified. Although HPV-related OPC had similar survival benefits across these three regions, among non-OPC, neither p16 (aHR = 0.83, 95%CI = 0.60–1.14), HPV16 DNA (aHR = 1.20, 95%CI = 0.89–1.63), or p16(+)/HPV16(+) (aHR = 0.59, 95%CI = 0.32–1.08) were statistically significant predictors of mortality. The researchers concluded that the prognostic utility of HPV among non-OPC patients is limited and, although cases with dual p16 and HPV positivity appeared to have better outcome, tumor HPV/p16 testing should not be routinely done in non-OPC [20].

In addition, Lassen et al. analyzed retrospectively p16 expression via IHC in a cohort of 1294 patients enrolled in previously conducted DAHANCA-trials between 1992 and 2012. The study included patients with stage III–IV pharynx and larynx cancer treated with primary CRT. Thirty-eight percent (490/1294) of the tumors were p16-positive with a significantly higher frequency in OPC (425/815) than in non-OPC (65/479) (p < 0.0001). As expected, in OPC p16-positivity correlated with significantly improved locoregional control (LRC), event-free survival (EFS) and OS. However, in non-OPC no prognostic impact of p16-status was found for either endpoint: LRC (HR = 1.13 [0.75–1.70]), EFS (HR = 1.06 [0.76–1.47]), and OS

(HR = 0.82 [0.59–1.16]). This trial further suggests that, in non-OPC sites, p16 positivity is rare and does not carry any prognostic significance [21].

On the contrary, results from a retrospective analysis of 19,993 non-OPC patients registered in the National Cancer Data Base (NCDB), of whom 5070 were positive for HPV via PCR, revealed that OS was significantly higher for patients with HPV(+) versus HPV(−) non-OPC, and that the robust survival advantage of HPV was maintained in all subsites. Improved outcomes were more pronounced in patients with locally advanced compared to early stage disease. The main limitation of this trial is that, since routine HPV testing in non-OPC is not standard of care, selection bias must exist in the data set. Therefore, factors driving the decision to test for HPV status may be contributing to the improved outcomes of the HPV(+) non-OPC cohort [22].

Moreover, high-risk HPV positivity was associated with OS in certain non-OPC primaries in a large analysis of 24,470 patients diagnosed with HNSCC between 2010 and 2013 who had been registered in the NCDB. Of these patients, 9907 patients had been diagnosed with non-OPC SCCs: 1085 with SCC of the hypopharynx, 4804 with SCC of the larynx, and 4018 with SCC of the oral cavity. The rate of high-risk HPV positivity for those patients varied by primary tumor site: 17.7% of patients with SCCs of the hypopharynx were high-risk HPV(+), as were 11% and 10.6%, respectively, of those with SCCs of the larynx and oral cavity. HPV status was found to be prognostic in multiple unadjusted and propensity-adjusted non-OPC populations. HPV positivity was associated with superior OS in patients with hypopharyngeal SCC with a HR of 0.61 (P < 0.001), in patients with AJCC stage III to IVB laryngeal SCC (HR = 0.79; P = 0.019), and in patients with AJCC stage III to IVB SCC of the oral cavity (HR = 0.78; P = 0.03). However, as the researchers themselves have pointed out, there are certain serious limitations in this trial. First of all, the results of this trial derive from retrospective, administratively collected data. Then, important information such as patterns of response/failure to treatment, salvage therapies, cause of death and smoking status are not captured by NCDB. Finally, the method of testing is not prespecifiedby the NCDB, so HPV testing wasperformed as part of clinical care and was, therefore, heterogeneous. The results of this study, therefore, should be interpreted with caution [23].

Conclusions

The studies evaluating the prognostic impact of HPV infection in non-oropharyngeal head and neck cancers have shown conflicting results (Table 12.1). Variations in sample sizes, geography, the method of HPV detection and other factors may have contributed to this fact. It seems that p16 is a poor surrogate biomarker for oncogenic HPV infection for non-OPC disease sites. The majority of studies so far suggest that the prognostic impact of HPV positivity is reserved for the oropharynx, so routine HPV testing is not recommended for other sites.

Table 12.1 Clinical Trials investigating the prognostic role of p16/HPV in non-OPC

Trial	Trial Design	Evaluation of HPV positivity	Disease Site	N	HPV (+) cases	Survival Outcomes
Young et al (ref 18)	Retrospective, single cohort study	Expression of p16 by IHC and for high-risk HPV E6 and E7 mRNA transcripts by RNA ISH	Larynx	324	6.5%: p16(+) 7 pts.: HPV RNA (+)	**HPV (+) vs HPV (−): 2-year survival: 86% VS 71%** (HR = 0.76, 95% CI 0.23–2.5, P = 0.65)
Fakhry et al (ref 19)	Retrospective, multi-institution study	HPV16 DNA and high-risk HPV E6/E7 mRNA ISH	Larynx, oral cavity, nasopharynx	**OPC:** 239 **Non-OPC:** 621	Oropharynx:56% Oral cavity: 2% Larynx: 5% Nasopharynx:10%	**Tumor HPV status-p16 not prognostic** Oral cavity: (n = 253; P = 0.22) Larynx: (n = 243; P = 0.72) Nasopharynx: (n = 125; P = 0.23)
D'Souza et al (ref 20)	Retrospective data analysis from the studies GENCAPO, CHANCE, ARCAGE	Central analysis for p16 and HPV16 DNA by PCR	Larynx, oral cavity, hypopharynx	**OPC:** 517 **Non-OPC:** 845 (397 laryngeal, 382 oral cavity, 66 hypopharyngeal SCC)	**p16-neg /HPV16-pos: 61/125 p16-pos /HPV16-neg: 52/122 p16-pos /HPV16-pos: 12/39**	**No survival differences noted: p16:** (aHR = 0.83, 95%CI = 0.60–1.14) **HPV16 DNA:** (aHR = 1.20, 95%CI = 0.89–1.63) **p16(+)/HPV16(+):** (aHR = 0.59, 95%CI = 0.32–1.08)

Trial	Trial Design	Evaluation of HPV positivity	Disease Site	N	HPV (+) cases	Survival Outcomes
Lassen et al (ref 21)	Retrospective analysis of pts. enrolled in a DAHANCA cohort	p16 expression via IHC	Pharynx, larynx	1294	**OPC: 425/815 Non-OPC: 65/479**	**No prognostic impact of p16-status: LRC:** (HR = 1.13 [0.75–1.70]) **EFS:** (HR = 1.06 [0.76–1.47]) **OS:** (HR = 0.82 [0.59–1.16])
Ko et al (ref 22)	Retrospective analysis of non-OPC patients registered in the NCDB	HPV DNA via PCR	Oral cavity, larynx, hypopharynx	19,993	**HPV(+)/HPV(−): 5070/14,923**	**OS associated with HPV status: Early stage:** (HR = 0.68; 95% CI 0.51–0.92) **Late stage:** (HR = 0.46; 95% CI 0.39–0.53)
Tian et al (ref 23)	Retrospective analysis of non-OPC patients registered in the NCDB	HPV DNA via PCR	Oral cavity, hypopharynx, larynx	9907	**HPV (+) cases:** Oral cavity: 10.6% Hypopharynx: 17.7% Larynx: 11%	**OS associated with HPV status: Hypopharynx:** HR: 0.61 (P < 0.001) **Larynx (stage III to IVB:** HR = 0.79 (P = 0.019) **Oral cavity (stage III to IVB:** HR = 0.78 (P = 0.03)

IHC immunohistochemistry; *ISH* in situ hybridization; *HR* Hazard Ratio; *aHR* adjusted Hazard Ratio; *CI* Confidence Interval; *OS* overall survival; *OPC* oropharyngeal cancer; *NCDB* National Cancer Data Base

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