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Review Article

P16^{INK4A} as a surrogate biomarker for human papillomavirus-associated oropharyngeal carcinoma: Consideration of some aspects

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Human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinomas (OPSCCs) frequently show different clinical and pathological features, which tend to be younger age, better performance status, less tobacco and alcohol consumption, more poorly differentiated histopathology, but usually with better treatment response and prognosis compared with HPV-negative OPSCCs. In tumor tissue, HPV infection is closely correlated with p16^{INK4A} expression, which has been suggested to be a surrogate biomarker of HPV infection. However, there is diversity of sensitivity and specificity about p16^{INK4A} in surrogate detection of HPV status. Herein, we summarize the current knowledge and note some aspects for consideration concerning p16^{INK4A} as a surrogate biomarker for HPV-associated OPSCC. (*Cancer Sci* 2013; 104: 1553–1559)

As one of the cyclin-dependent kinase inhibitors that inhibit cyclin-dependent kinases 4 and 6, p16^{INK4A} is encoded by the tumor suppressor gene *CDKN2A*. In non-human papillomavirus (HPV) infected carcinomas, p16^{INK4A} frequently showed in low levels or loss for epigenetic alteration and gene mutation.[1] However, in HPV-related cervical lesions and head and neck squamous cell carcinomas (HNSCCs), oncoprotein E7 could combine with Rb, and cause the dysfunction of Rb. The functional inactivation of Rb by E7 therefore results in the release of the transcriptional factor E2F from the Rb–E2F protein complex and the promotion of cell cycle progression, and also leads to release of the *p16^{INK4A}* gene from its transcriptional inhibition, causing p16^{INK4A} to be expressed at a high level.[2–4] Because of this molecular event, the fact that p16^{INK4A} turned out to be substantially overexpressed in virtually all HPV-transformed cells in cervical lesions, p16^{INK4A} expression has been used for distinguishing high-risk from low-risk HPV infection,[5] for ancillary confirmation and grading of histological diagnosis of cervical intraepithelial neoplasia,[6] and for predicting progression or regression of low-grade cervical intraepithelial neoplasia.[7,8] Considering the etiological link with HPV in HNSCCs, p16^{INK4A} expression has been detected in many HPV-related HNSCCs.[9–14] Those investigations suggest that overexpression of p16^{INK4A} is caused by HPV infection, induced by dysfunction of the *Rb* tumor suppressor gene; also, theoretically, tumors with a high level of p16^{INK4A} are the result of HPV infection.[15] However, it is arbitrary to consider p16^{INK4A} as a surrogate biomarker of HPV infection or HPV-related carcinomas solely according to the theory mentioned above. First, the definition of "surrogate" should be identified. Does the high expression of p16^{INK4A} indicate HPV infection, or the initiation of carcinogenesis induced by HPV, or tumors originating from HPV? Second, if p16^{INK4A} serves as a surrogate biomarker, it should represent the biological and clinical features of HPV-related carcinomas. If p16^{INK4A} expression can represent or partially represent the features of HPV-related carcinomas, it would be valuable as a surrogate biomarker.

Expression of p16^{INK4A} and HPV Infection in Oropharyngeal Carcinomas

Human papillomavirus infection was found in 25.9% of head and neck cancers and 35.6% of oropharyngeal squamous cell carcinomas (OPSCCs).[16] Patients with HPV-associated OPSCC are frequently of a younger age at the time of diagnosis, with better performance status, less tobacco and alcohol exposure,[17] with oral–genital sexual behavior,[18,19] poorly differentiated disease, and better prognosis than OPSCC patients without HPV infection.[13,20] We collected published reports on OPSCCs with p16^{INK4A} expression and simultaneous HPV DNA detection (PCR and/or *in situ* hybridization [ISH]) in the past 5 years in the PubMed medical literature database,[9,21–29] and found that the sensitivity of p16^{INK4A} for HPV DNA detection varied from 46% to 98%. Although it was recognized that overexpression of p16^{INK4A} was closely correlated with HPV infection in OPSCCs, 3–51% of HPV DNA-negative OPSCCs were found to express p16^{INK4A}, and 2–54% of HPV DNA-positive OPSCCs were found to display negative expression of p16^{INK4A} (Table 1). In fact, regarding HPV DNA ISH, p16^{INK4A} expression immunohistochemistry (IHC) detection shows excellent sensitivity, but there were too many p16^{INK4A}(+)/HPV(−) cases (18–51%).[9,22,27,29] Similarly, regarding HPV DNA PCR, there were many p16^{INK4A}(−)/HPV(+) cases (25–54%).[26,28,30] We can ascribe the discrepancy between the p16^{INK4A} IHC and HPV DNA tests to the excessive sensitivity of the PCR test and the specificity of the ISH test. Additionally, the pathology grade of tumors and the cut-off point of p16^{INK4A} interpretation must be considered. Data from Thavaraj *et al.*[24] (Table 1) showed excellent concordance between the p16^{INK4A} IHC and HPV DNA tests, partially due to the majority of poorly differentiated samples and suitably stringent criteria for p16^{INK4A} staining (scored as positive if there was strong and diffuse staining present in >70% of the malignant cells). A published guide for interpretation of p16^{INK4A} expression[31] provides much

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Table 1. Expression of p16^{INK4A} in oropharyngeal carcinomas with different human papillomavirus (HPV) DNA status, as reported in research published 2009–2013

Study	Total cases	P16+/HPV+ (sensitivity, %)	P16+ /HPV− (false positivity, %)	P16− /HPV+, false (negativity, %)	P16− /HPV−, (specificity, %)	Pathology grade	HPV DNA detection	P16 IHC expression pattern†
Shi *et al.*[9]	111	61/62 (98)	8/44 (18)	2	82	No data	ISH	Neu and cyto[a]
Gao *et al.*[29]	150	54/55 (98)	22/43 (51)	2	49	No data	ISH	Neu and cyto[b]
Thavanaj *et al.*[24]	142	88/90 (98)	2/52 (4)	2	96	Grade I, 0; II, 71; III, 71	ISH or PCR‡	Neu and cyto[c]
Lewis *et al.*[22]	239	158/163 (97)	29/76 (38)	3	62	K, 54; H*, 50; NK, 126	ISH or PCR‡	Neu and/or cyto[d]
Jordan *et al.*[27]	235	138/143 (97)	27/89 (30)	3	70	No data	ISH	No data[e]
Evans *et al.*[25]	30	20/22 (91)	2/4 (50)	9	50	No data	PCR	No data[f]
Nasman *et al.*[21]	175	119/136 (88)	4/35 (11)	12	89	High, 5; medium, 54; low, 91	PCR	No data[g]
Hong *et al.*[23]	198	62/83 (75)	3/115 (3)	25	97	Grade I & II, 118; III, 77	PCR	Neu and cyto[h]
Junor *et al.*[26]	254	77/133 (58)	4/51 (8)	42	92	HPV+ and p16+ are likely to be grade III	PCR	Neu and/or cyto[i]
Holzinger *et al.*[28]	199	42/92 (46)	12/85 (14)	54	86	No differential impact of p16 overexpression	PCR	Neu and cyto[j]

†Criteria for p16INK4A expression: [a]A tumor was considered positive when strong signals were detected in both the tumor nuclei (Neu) and the cytoplasm (cyto). [b]Classified in a binary manner as positive when >50% of the cells showed nuclear and cytoplasmic staining. [c]Scored as positive if there was strong and diffuse staining present in >70% of the malignant cells, other staining patterns were scored as negative. [d]Graded in a quartile manner for its extent: 0, negative; 1+, 1–25% of cells positive; 2+, 26–50%; 3+, 51–75%; 4+, 76–100%. Cases were also divided into positive (1–4 +) and negative (0) groups. [e]Significant differences in AUC were observed for both intensity score and percentage staining. A p16INK4A intensity score cut-off point of 2 on a scale of 0–3 was most sensitive, and a percentage staining cut-off point of 35% on a scale of 0–100% was most specific. *A hybrid (H) score cut-off point of 60 on a scale of 0–300 yielded an average sensitivity of 91.6% and specificity of 90.4%. [f]p16INK4A was positive in diffuse staining and patchy staining. [g]No detailed data were found. [h]Weak focal staining was recorded as negative. [i]Staining was scored as negative, focal positive, and positive based on both nuclear and cytoplasmic staining. Diffuse and continuous cytoplasmic and nuclear staining was considered a positive reaction. [j]Scoring p16INK4A-high required strong nuclear and cytoplasmic staining in the proliferating tumor cells. Patchy and negative staining was recorded as p16INK4A-low. ‡Positive result, when either PCR or *in situ* hybridization (ISH) test was positive; negative result, when both PCR and ISH were negative. K, keratinizing; NK, non-keratinizing.

instruction for p16^{INK4A} expression as a surrogate biomarker for HPV-associated OPSCCs, in which the pathology grade and stringent criteria were also emphasized.

Apart from the detection techniques that can disturb the accuracy of p16^{INK4A} staining for HPV status detection, we cannot deny the theoretical possibility that there are the p16^{INK4A}(+)/HPV(−) and p16^{INK4A}(−)/HPV(+) cases (Fig. 1). Results regarding aberrant expression of p16^{INK4A} had been reported in breast cancer[32] and small-cell lung cancer.[33] Klingenberg *et al.*[34] previously found p16^{INK4A} overexpression was frequently detected in tumor-free tonsil tissue without association with HPV infection (detected by PCR and FISH analysis). Therefore, mechanisms other than HPV infection are implicated in p16^{INK4A} upregulation. Little is known concerning the topic, and further investigation is warranted. Of course, we can improve the criteria of p16^{INK4A} interpretation to decrease the incidence of p16^{INK4A}(+)/HPV(−) cases, however, which would take risk of increasing p16^{INK4A}(−)/HPV(+) cases. Junor *et al.*[26] excessively restricted the criteria of p16^{INK4A} staining (Table 1), resulting in a p16^{INK4A}(−)/HPV(+) set whose survival was much closer to that of p16^{INK4A}(+)/HPV(+) patients than p16^{INK4A}(−)/HPV(−) patients. Hoffmann *et al.*[35] found that p16^{INK4A} expression was closely correlated to HPV E6/E7 mRNA expression. In the HPV-associated OPSCCs, HPV DNA-negative sets and HPV DNA(+)/mRNA(−) sets showed similar survival curves.[36] Human papillomavirus DNA detection can reflect the status of existing HPV infection but may be insufficient to indicate whether HPV is transformally active or not. No impact on survival was reported when the presence of HPV DNA was focused as a single factor, but HPV E6/E7 mRNA expression, p16^{INK4A} overexpression, or the HPV DNA/p16^{INK4A} combined test clearly showed statistical significance for better overall survival.[35] Overexpression of p16^{INK4A} or HPV E6/E7 mRNA expression were thought to be the parameter that described an activity of viral oncogenes, a finding that exactly explained that the p16^{INK4A}(−)/HPV DNA(+) events were the results of HPV inactive infection. Consequently, HPV status was decided by HPV infection and transactivation (Fig. 1). The HPV DNA PCR test can detect HPV infection but cannot detect its activation. Although p16^{INK4A} expression and HPV DNA infection are correlated

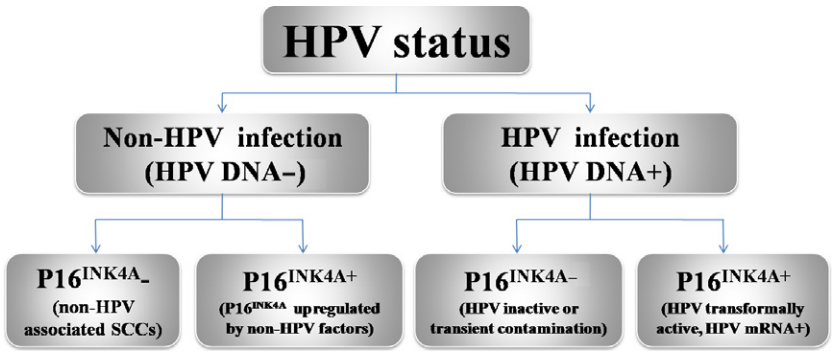

Fig. 1. Human papillomavirus (HPV) status impacted by HPV infection and p16^{INK4A} expression. SCC, squamous cell carcinoma.

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with HPV-associated OPSCCs, neither of the tests alone is the optimal method for HPV status detection.

Detection of HPV-Associated OPSCC

Gold standard for HPV status detection. Although $p16^{INK4A}$ staining or the HPV DNA test can well reflect the HPV status of OPSCCs, inevitable discrepancy was found between $p16^{INK4A}$ staining and the HPV DNA test in a few cases. The use of HPV E6/E7 mRNA RT-PCR detection can directly provide the present level of HPV oncoproteins in existing clinical samples (e.g., formalin-fixed paraffin-embedded tumors),[37] and it can remove the situation of HPV-inactive status and transient HPV infection or contamination. Survival analysis showed that the HPV mRNA RT-PCR test can well stratify survival[9,29] and was superior to the HPV DNA test.[36,38] Detection HPV E6/E7 mRNA by RT-PCR has been considered the gold standard for meaningful HPV infection. Another HPV RNA detection method is RNA ISH, which can also be used for detecting transcriptionally active HPV infection in formalin-fixed paraffin-embedded samples. Currently, the application of HPV RNA ISH for clinical detection is rare; Gao *et al.*[29] has reported a perfect correlation (100% sensitivity and specificity) with HPV mRNA real-time quantitative PCR. In contrast to the gold standard HPV mRNA RT-PCR, RNA ISH is not quantitative and requires positive and negative controls. However, because HPV RNA ISH is slide based, it is convenient for clinical use. In addition, HPV RNA ISH requires less tissue and allows for visualization of viral transcripts directly in tumor cells. The HPV RNA ISH technique has shown perfect concordance with HPV mRNA RT-PCR,[29] HPV DNA ISH, $p16^{INK4A}$ staining, and survival.[39,40] Thus, HPV RNA ISH is thought to be an ideal platform for HPV detection, but further supportive data is needed.

Widely used methods for HPV status detection. Other widely used methods for HPV status detection include HPV DNA consensus PCR detection and type, HPV DNA ISH/FISH, HPV DNA load real-time PCR, and indirect detections such as the $p16^{INK4A}$ expression IHC test and serum HPV L1/E6/E7 antibody tests. Table 2 shows the different sensitivities and specificities of each method according to the HPV E6 and/or E7 mRNA test (RT-PCR or RNA ISH) as the gold standard.

In OPSCCs, the HPV DNA PCR assay was used early and widely in HPV status detection with a high sensitivity range from 97% to 100% and a relative low specificity of 66–91%.[11,27,36,38,41] Human papillomavirus DNA was usually amplified with the L1 consensus HPV MY09/MY11 or $GP5^{+}/6^{+}$ primer set, or HPV E6/E7 specific primers. HPV DNA PCR assay usually overestimates the results for inactive HPV infection. In addition, the possibility of a false-negative HPV L1 consensus PCR assay exists because of the presence of integrated virus with loss or disruption of the L1 ORF.[42,43] Additionally, HPV DNA PCR assay does not distinguish the integrated form from the episomal form of the virus, a finding that argues against the use of PCR alone for classification of HPV status.

Another commonly used method is the type-specific HPV DNA detection by ISH or FISH assays. Usually punctuated nuclear (F)ISH signals indicate HPV DNA integrated into the host genome, and areas with diffuse nuclear (F)ISH staining indicate episomal HPV DNA. These assays allow visual confirmation of HPV DNA within individual tumor cell nuclei,[37] making excellent specificity for HPV status detection, at approximately 88–100%.[9,27,29,38,43] However, the interpretation of staining is subjective, leading to ambiguous interpretation for non-specific staining *in situ*.[43]

The assay with HPV DNA copy number has not yet formed a standardized protocol for HPV transcriptional activity, and it is very labor-intensive as the neoplastic cells need to be enriched by microdissection.[44] Jung *et al.*[36] showed that all the HPV16 transcriptionally active tumors had elevated viral load values, with a cut-off point at 1 copy per diploid genome equivalent. Considering some normal tissue contamination in the microdissected tumor samples, Smeets *et al.*[11] scored tumors with >0.5 copies per cell as positive and showed a high sensitivity and specificity of 92% and 97%, respectively, for HPV status detection.

Detection of the antibody to HPV E6 and E7 in serum was investigated in several large clinical studies concerning cervical cancer patients.[45,46] Seropositivity validated by HPV 16 L1 virus-like particles was associated with a significantly increased risk of oropharyngeal cancer.[47] Smeets *et al.* detected antibodies against the proteins HPV 16 L1, E6, and E7, indirectly reflecting HPV 16 infection, and the highest sensitivity was reached when positive serology was defined with any of the three antibodies (91%), but the specificity was then limited (74%).[11] Better results from Rotnaglova *et al.*[41] showed a high correlation between HPV DNA/RNA status and seropositivity of E6/E7 oncoproteins, and indicated that antibodies against HPV 16 E6 and E7 oncoproteins reached high sensitivity (96%) and specificity (89%) for detection of HPV-associated tonsillar cancer. Serological testing is simple, convenient, and cheap, but the value of clinical application of HPV L1/E6/E7 and other antigens requires further investigations.

Immunohistochemical staining of $p16^{INK4A}$ for HPV status detection in OPSCCs. Apart from HPV status mentioned above, $p16^{INK4A}$ expression can also be regulated through epigenetic control and multiple transcription factors, such as PRC1, PRC2, YY1, Id1, CTCF, Sp1, Ets, and HBP-1.[48] Frequently, HPV-positive OPSCCs are less likely to carry genetic alterations compared with HPV-negative ones, including chromosomal aberration,[49] gene mutation,[50] and transcriptional expression.[36] The difference of gene profiling would indirectly lead to diametrically opposed expression of $p16^{INK4A}$, for example, 11q is frequently found lost in HPV-positive but gained in HPV-negative OPSCCs, on which Ets (a protein that can raise the level of $p16^{INK4A}$) is located. The different genetic landscapes associated with transcriptionally active HPV are consistent with epidemiologic and clinical features (e.g., age, tobacco and alcohol exposure, tumor stage, grade, and response to treatment). Selection of the patients with different features can in turn reflect the initiation and genotype of tumors, which could impact $p16^{INK4A}$ expression in OPSCCs with different HPV status. Additionally, there were different interpretations for $p16^{INK4A}$ IHC staining between different investigators. The results of IHC staining usually depended on comprehensive elements, including the expression pattern (nuclear and/or cytoplasmic), intensity of staining, and percentage of stained tumor cells. The selection of the experimental reagents and interpretation of the staining may lead to different results. Considering the reasons mentioned above, controversy appeared regarding the capacity of $p16^{INK4A}$ to indicate HPV status.

The interpretation of $p16^{INK4A}$ IHC staining likely contributes to most of the discrepancy regarding $p16^{INK4A}$ detection and is the only element we can easily control. Cut-off points for the intensity and percentage of tumor cell staining are equally important. A single limitation of the cut-off point for either intensity[9,27] or percentage[38] of the staining will certainly result in increased sensitivity and decreased specificity (Table 2). Suitably restricting both the cut-off points can eliminate the false-positivity of $p16^{INK4A}$ expression induced by low-risk HPV infection and non-HPV factors, and even cause the specificity of $p16^{INK4A}$ detection to surpass its sensitivity.[43] Easy to carry out, $p16^{INK4A}$ IHC detection is also low cost and has a high sensitivity. However, there is a

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tendency toward false-positive results in $p16^{INK4A}$ staining, and there is a lack of a direct and exclusive mechanistic link between HPV DNA integration and $p16^{INK4A}$ expression. Cautious interpretation and stringent criteria for $p16^{INK4A}$ IHC staining with attached information of various histologic, anatomic, clinical, and technical considerations were advocated.[31] And further HPV testing was suggested when $p16^{INK4A}$ staining was absent/weak or when keratinizing squamous cell carcinoma staining was present.

Further HPV testing, for instance, $p16^{INK4A}$ IHC in combination with HPV DNA PCR assays or ISH assays, was frequently used in studies and in clinics. Table 3 shows the comparison of the detection of $p16^{INK4A}$ combined with HPV DNA PCR or ISH assays based on HPV mRNA detection as the gold standard.[11,27,29,38,41] In the combined detection using the $p16^{INK4A}$ IHC and HPV DNA ISH assays, if results set both positive as a positive test and all others as a negative test, we can obtain a perfect specificity but with decreased sensitivity; if results set either positive as a positive test and both negative as a negative test, we can scarcely obtain any enhancement in sensitivity or specificity (Table 3). Unlike the combination with the ISH assay, HPV IHC combined with PCR can conspicuously improve the specificity on the premise of continuously high sensitivity (Table 3). Using HPV DNA PCR combined with $p16^{INK4A}$ IHC for HPV status detection, Hong *et al.* and Heath *et al.*[30,51] both showed that the HPV DNA(+)/$p16^{INK4A}$(+) groups had better survival than the HPV DNA(+)/$p16^{INK4A}$(−) and HPV DNA(−)/$p16^{INK4A}$(−) groups. Clearly, the combined detection of $p16^{INK4A}$ IHC and HPV DNA PCR can not only eliminate inactive infection and transient contamination but can also omit the contingently elevated $p16^{INK4A}$ expression by non-viral related alterations. The $p16^{INK4A}$ IHC/HPV DNA PCR combination test offers a valuable alternative to RNA analysis, with excellent sensitivity /specificity and prognostic value.[38,41] Additionally, $p16^{INK4A}$ combined with other cellular proteins has been reported as a feasible biomarker to identify OPSCCs with active HPV, for example, combined with pRb (sensitivity 78%, specificity 93%), with p53 (sensitivity 67%, specificity 95%), or with cyclin D1 (sensitivity 78%, specificity 90%).[52]

In the combined detection of $p16^{INK4A}$/p53 IHC staining, active HPV infection is inversely associated with *p53* mutation. The HPV-associated OPSCCs with wild-type *p53* gene always show a low level ("negative" in standard IHC and "normal low" in TSA-IHC staining) of p53 protein due to ubiquitination and degradation through viral E6 protein. Intriguingly, HPV-negative OPSCCs (inclined to *p53* mutation) always show "absent" or "high" p53 protein level (detected with standard IHC and TSA-IHC)[52] because absent or high p53 staining was correlated to nonsense or missense *p53* mutations,[53] respectively, and mutant p53 protein was more stable and had heavier staining than wild-type p53 protein. Mannweiler *et al.*[54] reported completely consistent p53 staining with $p16^{INK4A}$/HPV DNA combined detection in penile lesions and suggested p53 expression along with $p16^{INK4A}$ negativity to identify HPV-negative cancers. However, the method of p53 detection was questionable and normal p53 and nonsense mutations were not taken into consideration.

Significance of $p16^{INK4A}$ for Prognosis and Treatment in HPV-Associated OPSCCs

Despite the controversy concerning the significance of HPV status at other sites of HNSCCs, its particular meaning for OPSCCs has been extensively recognized.[55] The HPV-associated HNSCCs, particularly OPSCCs, have been defined as a distinct entity with different epidemiology, etiology, pathogenesis, pathology and molecular pathology, clinical manifestations, treatment response, and prognosis. Most interestingly, despite the poor differentiation, and early cervical metastasis, HPV-associated OPSCCs usually demonstrate a better treatment response and prognosis than HPV-negative OPSCCs.

Immunohistochemical staining for $p16^{INK4A}$ can not only represent HPV status but can also indicate the prognosis of HPV-associated OPSCCs.[56,57] $P16^{INK4A}$ and HPV status is a strong and consistent determinant of superior survival, regardless of treatment strategy, such as surgery,[12] radiotherapy,[58] chemoradiotherapy,[56] or induction chemotherapy plus chemoradiotherapy.[13] Of course, most of the existing clinical trials reflecting advantageous prognosis were related to radio-

Table 2. Sensitivities and specificities of detection methods for human papillomavirus (HPV) status in oropharyngeal squamous cell carcinomas with HPV E6 or E7 mRNA (RT-PCR or RNA *in situ* hybridization [ISH]) as the gold standard

Study	Total cases	Tumor site	HPV DNA PCR, %		ISH/FISH, %		Viral load, %†		$P16^{INK4A}$ IHC, %‡		P16 IHC interpretation	
			Sens.	Spec.	Sens.	Spec.	Sens.	Spec.	Sens.	Spec.	Intensity	%
Smeets *et al.*[11]	48	Oropharynx, 18 Oral, 30	100	89	83	100	92	97[a]	100	79[d]	≥1+ Scale, 0–3	>10
Jordon *et al.*[27]	235	Oropharynx	100	66	88	94	91	96[b]	97	84[e]	≥2+ Scale, 0–3	>35
Rotnaglova *et al.*[41]	109	Tonsillar	100	89	–	–	–	–	96	94[f]	None	>50
Gao *et al.*[29]	150	Oropharynx	–	–	69	95	–	–	95	90[g]	None	>50
Schache *et al.*[38]	108	Oropharynx	97	87	88	88	–	–	94	82[h]	None	>70
Schlecht *et al.*[43]	110	Oropharynx, 30	–	–	Ventana 67 Dako 38	30 100	–	–	90	100[i]	≥2+ Scale, 0–3	>75
Shi *et al.*[9]	111	Oropharynx	–	–	84	92	–	–	89	81[j]	Strong	None
Jung *et al.*[36]	231	Oropharynx	100	91	–	–	100	100[c]	–	–	–	–

†Criteria for viral load as follows. [a]Tumors with >0.5 copies per cell were scored as positive. [b]No data found. [c]Low viral loads <1 copy/diploid genome equivalent, higher HPV16 loads >1 copy/diploid genome equivalent. ‡Criteria for p16INK4A immunohistochemical (IHC) staining as follows: [d]Staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed, positive defined as intensity >1 and percentage >10%. [e]Significant differences in AUC were observed for both intensity score and percentage staining. A p16INK4A intensity score cut-off point of 2 on a scale of 0–3 was most sensitive, and a percentage staining cut-off point of 35% on a scale of 0–100% was most specific. An H score cut-off point of 60 on a scale of 0–300 yielded an average sensitivity of 91.6% and specificity of 90.4%. [f]Positive for p16INK4A expression had to show more than 50% of positive cells and reveal nuclear and/or cytoplasmic staining. [g]Classified in a binary manner as positive when >50% of the cells showed nuclear and cytoplasmic staining. [h]P16INK4A IHC was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in >70% of the malignant cells. [i]P16INK4A positivity was defined by a mean intensity cut-off >2 and diffuse (>75%) staining distribution in either the nuclei or cytoplasm. [j]A tumor was considered positive when strong signals were detected in both the tumor nuclei as well as the cytoplasm. –, no data; Sens., sensitivity; Spec., specificity.

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Table 3. Human papillomavirus (HPV) status detection using $p16^{INK4A}$ immunohistochemical (IHC) staining in combination with HPV DNA PCR or *in situ* hybridization (ISH) assay

Study	Cases	Gold standard	Combined with PCR, %		Combined with ISH, %				Compared with single $p16^{INK4A}$ IHC staining, %†	
			Both positive		Both positive		Either positive			
			Sens.	Spec.	Sens.	Spec.	Sens.	Spec.	Sens.	Spec.
Smeets *et al.*[11]	48	HPV E6/E7 mRNA	100	100	–	–	–	–	100	79[a]
Rotnaglova *et al.*[41]	109	HPV E6*I mRNA	100	88	–	–	–	–	96	94[b]
Schache *et al.*[38]	108	HPV E6 mRNA	97	94	88	90	–	–	94	82[c]
Jordon *et al.*[27]	232	HPV E6/E7 mRNA	–	–	86.1	97.3	98.7	81.1	97	84[d]
Gao *et al.*[29]	150	HPV E6/E7 mRNA ISH	–	–	69	100	95	85	95	90[e]

†Criteria for p16INK4A immunohistochemical (IHC) staining as follows. [a]Staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed, positive defined as intensity >1 and percentage >10%. [b]Positive for p16INK4A expression had to show more than 50% of positive cells and reveal nuclear and/or cytoplasmic staining. [c]P16INK4A IHC was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in >70% of the malignant cells. [d]Significant differences in AUC were observed for both intensity score and percentage staining. A p16INK4A intensity score cut-off point of 2 on a scale of 0–3 was most sensitive, and a percentage staining cut-off point of 35% on a scale of 0–100% was most specific. An H score cut-off point of 60 on a scale of 0–300 yielded an average sensitivity of 91.6% and specificity of 90.4%. [e]Classified in a binary manner as positive when >50% of the cells showed nuclear and cytoplasmic staining. –, no data; Sens., sensitivity; Spec., specificity.

therapy and/or chemotherapy. Parts of the studies investigated the effect of radical surgery on HPV-associated OPSCCs; however, either most of the included patients received postoperative radiotherapy[12] or no statistically significant differences were found between HPV-positive and -negative groups.[59] Lassen reviewed the clinical data, addressing the impact of HPV on radiotherapy, including conventionally fractionated radiotherapy, accelerated fractionated radiotherapy, hypoxic modification in radiotherapy, and chemoradiotherapy.[60] Human papillomavirus-positive or $p16^{INK4A}$-positive tumors support a better prognosis in various schedules of radiotherapy and chemotherapy except the hypoxic modification in radiotherapy.[61] The HPV-associated OPSCCs have been considered to show an excellent sensitivity to radiotherapy and chemotherapy. Given that HPV-associated OPSCCs are distinct from HPV-negative carcinomas in treatment response and prognosis, numerous clinical trials that deintensify treatment for HPV-associated carcinomas are underway, with the aim of reducing treatment toxicity and improving the quality of life. A review authored by Mehra *et al.*[62] summarized the ongoing clinical trials.

Reduced-intensity therapy mostly focuses on reducing the radiation dose and replacing concurrent chemotherapy with cetuximab. However, little is known concerning the mechanism of enhanced sensitivity to radiotherapy and chemotherapy in HPV-associated OPSCCs. The HPV-associated OPSCCs possess fewer *p53* mutations and lower *EGFR* expression, which may play a role in better prognosis, simultaneously questioning the replacement of treatment with cetuximab. Human papillomavirus E7 can bind to the catalytic and structural subunits of protein phosphatase 2A and inhibit their interaction with Akt, thereby maintaining PKB/Akt signaling by inhibiting its dephosphorylation.[63] The activated PI3K/Akt pathway is known to be a potent inducer of radiation resistance in cervical carcinoma, whereas little is known regarding its role in head and neck carcinoma. In terms of radiation, hypoxic cells in tumors are resistant to treatment. It has been reported that hypoxic modification improved the outcome in HPV- or $p16^{INK4A}$-negative tumors but was of no significant benefit in HPV- or $p16^{INK4A}$-positive tumors.[61] The hypothesis supported that the extent of hypoxia may be more pronounced in $p16^{INK4A}$-negative tumors compared with $p16^{INK4A}$-positive tumors.[64] Additionally, elevated $p16^{INK4A}$ is induced by functional inactivation of the tumor suppressor gene *Rb*, which may also contribute to $p16^{INK4A}$-positive tumors' sensitivity to chemo- or radiotherapy. Conclusions regarding the mechanism to explain why $p16^{INK4A}$-positive /HPV-associated OPSCCs possess a superior prognosis are difficult to draw from inconsistent data. With the current findings of molecular biology and clinicopathology of HPV-associated OPSCCs, we hypothesize that increased sensitivity to radiotherapy and/or chemotherapy is the aggregate result of poor differentiation, continuous proliferation, abrogation of the inhibition of DNA synthesis induced by radiation,[65] slight hypoxia status, non-mutation but dysfunctional *p53*, and its own genomic instability.[66]

Oropharyngeal SCC is a distinct entity frequently associated with younger age, better performance status, less tobacco and alcohol consumption, improved adaptive immunity[67] and different patterns of gene-expression profiles.[68] Additionally, the mechanism of superior prognosis is unclear and needs further investigation. As described above, the excellent prognosis of the HPV-positive entity is the result of the comprehensive effect of multiple factors. Additionally, change to any single factor may cause alteration of the outcome, for instance, tobacco consumption.[56] Likewise, no single factor alone can reflect all the characteristics of an individual patient, particularly regarding treatment response and prognosis. Although a series of clinical trials on deintensification for reducing treatment toxicity are underway, we believe the deintensification should proceed with caution when considering the potential cost of treatment efficacy, particularly the formulation and mastery of the indications for deintensified treatment. By contrast, HPV-associated OPSCCs show an excellent radiotherapy or chemoradiotherapy response. Thus, the following questions arise: why should these effective adjuvant treatments be discarded when radical surgery is the primary treatment algorithm, and could HPV-positive and/or $p16^{INK4A}$ expression be a clinical indication for postoperative radiotherapy or chemoradiotherapy? There is a lack of research regarding this subject area. With different treatment intensifications, as well as different efficacies and toxicities, which is the key point is determined by the patient's choice.

Conclusions and Future Perspectives

The tumor suppressor $p16^{INK4A}$ plays an important role in cell cycle regulation. When $p16^{INK4A}$ is expressed in HNSCCs, particularly in OPSCCs, it is associated with a new implication: HPV status and superior prognosis. Immunohistochemical staining of $p16^{INK4A}$ does not exactly match the HPV DNA

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test, and two inconsistent patterns of $p16^{INK4A}(+)$/HPV DNA (−) and $p16^{INK4A}(-)$/HPV DNA(+) cases are evidence. Restricting the cut-off point of criteria can improve the specificity of $p16^{INK4A}$ IHC staining as a surrogate biomarker for HPV-associated OPSCC detection. The combination of $p16^{INK4A}$ staining with the HPV DNA PCR test can produce almost perfect sensitivity and specificity with HPV E6 or E7 mRNA (RT-PCR or RNA ISH) as a gold standard. Granted, $p16^{INK4A}$ can stratify prognosis in OPSCCs, but the mechanism for better survival remains unclear, warranting further investigation. Despite the advantageous treatment response and prognosis of HPV-associated carcinomas, decreasing the treatment intensification would lead to a potential risk of reducing treatment efficacy. Additionally, the clinical indication and implementation of deintensification should be done with caution. Finally, we would like to reiterate the features of HPV-associated OPSCCs: they are not only carcinomas associated with HPV infection but are OPSCCs associated with high-risk HPV infection and activation of malignant transformation in carcinogenesis, with distinct characteristics in epidemiology, etiology and pathogenesis, clinical manifestations, pathology and molecular phenotype, treatment response and survival.

Disclosure Statement

The authors have no conflict of interest.

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