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HPV as a marker for molecular characterization in head and neck oncology: Looking for a standardization of clinical use and of detection method(s) in clinical practice

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Abstract

Background: A consensus about the most appropriate diagnostic method(s) for head and neck human papillomavirus (HPV)-induced carcinogenesis is still lacking because most of the commercially available assays have been designed for the cervix.

Methods: This article summarizes current data and trends concerning HPV diagnostic strategies in oropharyngeal squamous cell carcinoma (OPSCC). Six main approaches are described.

Results: The diagnostic gold standard for HPV-related OPSCC, focusing on E6/E7 mRNA detection, requires fresh samples. Because most frequently available samples are formalin-fixed paraffin-embedded (FFPE), the pros and cons of the different approaches were analyzed.

Conclusions: In the FFPE samples, the immunohistochemistry of p16, which is considered appropriate to assess HPV-driven carcinogenesis in OPSCC according to the 8th American Joint Committee on Cancer TNM classification, may not be specific enough to become the diagnostic standard in the perspective of treatment deintensification. p16 may play a safer role in combination with another highly sensible assay. Other promising approaches are based on DNA detection through real-time polymerase chain reaction and RNAscope.

KEYWORDS

FFPE, HPV, molecular characterization, oropharynx, treatment deintensification

1 | HPV AS A MARKER FOR MOLECULAR CHARACTERIZATION IN THE HEAD AND NECK

The first works specifically evaluating molecular markers in head and neck squamous cell carcinomas (HNSCCs) in a translational research setting date back at least to the 1970s.[1] Translational research was expected to widely impact clinical practice in the context of prevention as "molecular epidemiology," diagnosis as "molecular diagnostics," prognosis assessment and treatment selection as "molecular characterization,"[2] and the synthesis of new drugs as "molecular targeting." However, after 40 years, the impact of translational research on the daily clinical practice in head and neck oncology is de facto limited to a single molecular-targeted drug approved by the Food and Drug Association (C225).

Currently, the National Comprehensive Cancer Network (NCCN) guidelines for head and neck cancer do not recommend the use of any molecular marker in routine decision making.[3]

The potential reasons for such failure of translational research in head and neck oncology are many; among them

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are the extreme clinical and molecular heterogeneity of the diseases, which due to the anatomical proximity and the common histology (squamous cell carcinoma, SCC) are often arbitrarily considered as a whole.[4–7] One of the main heterogeneity factors is the primary site and subsite of the SCC, as lesions arising just a few millimeters apart are very different under a clinical and molecular point of view (ie, glottis vs supraglottis, retromolar trigone vs tonsil). Therefore, HNSCCs are a group of different cancers, each one relatively rare, and this makes it difficult to reach a critical mass for clinical validation of any molecular marker, given that the specific mechanisms underlying neoplastic transformation at individual subsites are largely unknown.

Nevertheless, the scientific evidence that accumulated in the last 20 years[8–12] on the prognostic significance of high-risk human papillomavirus (hr-HPV) infection in SCCs from a single site, which is the oropharynx, resulted in this parameter being the only molecular characterization marker included in the NCCN guidelines. We know that approximately 50% of oropharyngeal SCC (OPSCC) in Western countries (with higher rates in the United States and Northern Europe and lower rates in Southern Europe) are hr-HPV+ and that HPV+ cases are characterized by a better prognosis, probably because HPV infection is associated with a lower (half) mutational rate and harbors a wt-p53, the main actor of radio-induced apoptosis.[13] Thus, HPV infection is considered a valid characterization marker for OPSCCs, as it is not always present in malignant cells but is associated with precise biological features and a clinical behavior as well as is easily detectable on small bioptic or cytological samples.[2] The American Joint Committee on Cancer (AJCC) recently included HPV-driven carcinogenesis as a decisive prognostic determinant, diversifying the TNM classification between HPV-related and HPV-unrelated OPSCC.[14]

Still, despite a notable volume of literature hypothesizing treatment modulation according to HPV status in OPSCC,[15] the same NCCN justifies, at present, such an approach only in clinical trials.[3]

The main reason for this is that, with a proper, careful attitude, the NCCN panel waits for the demonstration in randomized trials that deintensification of treatment in HPV-positive OPSCC, which is for sure beneficial as far as functional results are concerned, is also oncologically safe.[15]

At any rate, we do believe that there is also another fundamental concern hampering the safe introduction of most promising molecular markers, especially for HPV in the head and neck clinical practice, which is the consensus about the best diagnostic method(s).

It is important to state that although HPV positivity is sometimes reported in HNSCCs arising outside the oropharynx, a clear relationship between HPV infection at non-oropharyngeal sites and distinct clinical features, including responsiveness to treatments, is not yet established. Therefore, efforts to standardize protocols for the diagnosis of HPV infection should, at present, be limited to OPSCC, for which it has a clear clinical value.[16]

2 | DETECTION METHODS FOR HPV IN HNSCC

The detection of HPV in a tumor sample does not mean that the virus is transcriptionally active or that the cancer is virus related. However, what is required for a diagnostic method to be utilized in clinical practice is not to be as sensitive as to detect a small number of copies of the HPV genome (possibly coming from a transient/not relevant infection or from a contamination) but to demonstrate a clinically relevant number of copies of translationally active viral oncogenes, which are supposed to have impacted the carcinogenetic process, to currently contribute to the transformed phenotype and to be associated with the typical clinical features of HPV-induced cancers (HPV-driven carcinogenesis).

A plethora of methods are commercially available to diagnose hr-HPV infection in biological samples, and much more have been described in the scientific literature. Nevertheless, all the current options for detecting HPV infection in HNSCC in clinical practice follow one of the following strategies, or a combination of them:

- Detection of viral mRNA
- Detection of viral DNA with polymerase chain reaction (PCR)
- Detection of viral DNA without PCR
- Detection of viral DNA with in situ hybridization (ISH)
- Detection of indirect markers of HPV-induced carcinogenesis (eg, p16 protein, pRb, p53, cyclin D1)
- Detection of antibodies against HPV antigens in the serum

HPV+ HNSCC development in humans appears to be driven only by E6 and E7 oncogenes, because the expression of the other oncogene, E5, is not detected in cancer cells. If we stick to the current evidence, most of the HPV-related HNSCCs have the E6 and E7 genes integrated in the genome of the tumor cells. However, there is still discussion about the real frequency of carcinogenesis driven only by episomal viral DNA, as some authors observe that, unlike cervical cancer, a significant proportion of HPV-related OPSCC may contain extrachromosomal virus,[17] and using tagging enrichment and next-generation sequencing of HPV16[18] virus integration is reported to be considerably less frequent in OPSCC with HPV E6 mRNA expression than in cervical cancer (51% vs 79%).

Less evidence supports a “hit and run” role for HPV infection in head and neck carcinogenesis, as in the current carcinogenetic model, in which E6 and E7 proteins are fundamental for the maintenance of the transformed phenotype.[19–27] Thus, for the diagnosis of HPV-related oropharyngeal SCC, the

perfect tool should evaluate the expression of the E6 and E7 proteins, but in the absence of fully reliable immunohistochemical probes for E6 and E7 proteins, methods detecting E6 and E7 mRNA in cancer cells are currently the gold standard for diagnosing an HPV-related HNSCC. Unfortunately, this approach carries several limitations related to the complexity of the assays and the limited availability of adequate samples.[16,28–30]

Nonquantitative methods detecting viral DNA via PCR are usually considered quite sensitive but poorly specific[16,31–33] due to the risk of contamination (as HPVs are quite common) and the amplification of biologically not relevant HPV DNA fragments or incidental infections. These rational concerns about PCR have often involved other HPV DNA detecting methods, which instead have a better specificity,[16] and among these are methods that rely on viral load quantification with real time-PCR[13,29,30,34] or in vitro DNA hybridization with signal amplification (hybrid capture).[11,28,35] The latter approach is reported to perfectly correlate with mRNA detection in fresh samples[28] and is partly validated in cytological samples,[35] even if it may present sensitivity issues when a small amount of poor quality DNA is extracted from formalin-fixed paraffin-embedded (FFPE) samples.[11] Nevertheless, despite several limitations of DNA-based methods, the original work by Gillison demonstrated the clinical relevance of hr-HPV infection[8] in oropharyngeal carcinogenesis and its prognostic significance used a method detecting DNA, and so did a recent paper by Stransky et al.,[13] giving precious and definite insights about the mutational pattern of HPV-related OPSCC. Notably, none of these authoritative papers, nor studies using E6/E7 mRNA detection,[8,13,28] found a prevalence of hr-HPV infection higher than 5%-10% in head and neck sites outside the oropharynx.

ISH-based assays have been and still are very popular in the United States, as they are acknowledged by most authors to be highly specific.[13,16,30,36] However, they share, with p16 and other immunohistochemistry (IHC) methods, the requirement of an experienced histopathologist to correctly interpret the results, and in many papers, their diagnostic (particularly concerning sensitivity)[29–31] and prognostic[10,36] reliability is demonstrated to be limited or, in any case, lower than immunohistochemistry for $p16^{INK4A}$ protein (p16 IHC) itself. The cost and complexity of the procedure are also potential concerns.

p16 IHC has been used at least since 2003[37] and rapidly became the most used method for the diagnosis of HPV infection in HNSCCs. Despite its undeniable advantages, such as its simplicity, low cost, and feasibility,[38] p16 IHC, which is proven to be a valid diagnostic method in uterine cervix, is associated with many issues and pitfalls in the head and neck, even for an experienced histopathologist.[38] Despite these limitations, in particular its low specificity, the expression of p16 is the criterion used for patient enrollment in the original prospective trials of treatment deintensification[39,40] and is acknowledged as valid in assessing HPV-related carcinogenesis in OPSCC by the same AJCC classification.[14]

HPV-driven OPSCCs elicit a humoral response to early virus proteins, especially E6, with HPV-16 E6 antibodies being associated with a 132-fold increase in oropharyngeal cancer risk.[41] Conversely, HPV16 E6 seropositivity is present only in 0.7% of the healthy controls.[42] Interestingly, antibodies to HPV16 E6 and other early proteins develop more than 10 years before OPSCC diagnosis, indicating that yet unrevealed HPV-specific precursor lesions may exist many years prior to cancer.[43] E6 seropositivity and/or seropositivity to more than two other early viral proteins is shown to have a high diagnostic accuracy (98%) in detecting HPV-driven OPSCC, as defined by CxCa-like viral RNA pattern-positive status, and strongly predicts a better survival.[44] These data, even if still probably insufficient for supporting a routine clinical use of the HPV serology, look promising for a future role in diagnosing HPV infection also with a screening perspective.

3 | RATIONALE AND PITFALLS OF P16 IHC FOR THE DIAGNOSIS OF HR-HPV INFECTION IN HNSCC

3.1 | Cellular basis for p16IHC as a cellular marker of transforming HPV infection in cancer

After the isolation of the first hr-HPV genotypes, that is, HPV16 and HPV18, from cancer biopsies of the cervix and cloning of their genome,[45,46] the detection of E6 and E7 transcripts in cancer cell lines and cancer biopsies further confirmed the etiological role of these viruses in cervical carcinogenesis.[47]

Many of the molecular mechanisms of cervical HPV-driven carcinogenesis are fully elucidated.[48] Immediately after infection, the expression of the early genes results in the deregulation of pathways involved in crucial cellular events, such as cell cycle, apoptosis, senescence, and immune response. All these alterations of cellular pathways create the ideal situation for the efficient completion of the viral life cycle. As a side effect, persistent hr-HPV infections induce the accumulation of DNA damage that cooperates with viral oncoproteins in the malignant transformation of the infected cells. Three proteins encoded by the early viral genes E5, E6, and E7, whose primary role is probably to maintain such functions in differentiating epithelial cells, possess proliferation-stimulating activity. The most significant role for malignant transformation is assigned to the E6 and E7 genes and their respective proteins. They are consistently expressed in malignant tissues, and inhibiting their expression blocks the malignant phenotype of cervical cancer cells. Several functions are described for E6 and E7.[21–27]

Some of the prominent functions of the hr-E6 protein originate from its interaction with, followed by the degradation of, p53, whereas a relevant carcinogenic function of hr-HPV E7 is the binding and degradation of the product of the retinoblastoma tumor suppressor gene (pRB) and related proteins p107 and p130.[49] One of the major functions of pRB is to negatively regulate, via a direct association, the activity of the transcriptional factors, E2F1-3, maintaining the cell in a quiescent state during the G0/G1 phase of the cell cycle. pRb inactivation by hr-HPV E7 leads to the constitutive activation of E2F-regulated transcription and unscheduled cellular proliferation. The loss of the control of cellular proliferation promotes the accumulation of the cell cycle inhibitor p16, which is used as a surrogate marker for hr-HPV infection and to define the severity of cervical premalignant lesions. The p16 accumulation can be explained as a possible attempt of the hr-HPV-infected cells to counteract the impact of the viral oncoproteins in the regulation of the cell cycle. However, recent studies also provided lines of evidence that p16 accumulation in hr-HPV-infected cells is necessary for cell viability, highlighting a pro-proliferation function of p16 in a specific context.[50,51]

3.2 | Clinical framework for the use of P16 IHC in cancers of the uterine cervix and of the oropharynx

P16, and recently Ki67, overexpression detection by IHC was used and validated as a reliable method to diagnose or predict HPV-driven progression and transformation in squamous intraepithelial lesions of the uterine cervix.[52] Because there are no proven substantial differences in the carcinogenic mechanisms in the head and neck, such an approach to the diagnosis of HPV-induced carcinogenesis has spread rapidly also in head and neck oncology, in which it has been the most used diagnostic tool for hr-HPV infection in the last decade. However, the clinical framework of HPV-induced carcinogenesis in the two tumor sites is totally different (Table 1).

- Prevalence of HPV-related carcinogenesis: Substantially, all the SCCs of the uterine cervix are induced by HPV,[53] whereas in head and neck HPV-induced carcinomas, it is probably not more than 20% of the SCCs, and they are located almost exclusively in the oropharynx (in which they account for about half of the cases)[8,13,28,54–56]
- Role of HPV detection in prevention/early diagnosis: In the uterine cervix, hr-HPV-infected squamous intraepithelial lesions are clearly demonstrated and are often detected before the development of invasive cancer. Therefore, in the cervix, HPV detection is a precious epidemiologic marker for the management of identifiable precancerous lesions and the prevention of invasive carcinoma, whereas p16 overexpression is a valid diagnostic marker for the progression toward malignancy.[52] Conversely, in the head and neck, HPV-related precancerous lesions have not yet been demonstrated, and the use of hr-HPV infection as an epidemiologic marker is not justified by current evidence nor does it provide any indication that a target lesion would need to be treated with the intention of secondary prevention of invasive cancer or be clinically and pathologically monitored. Because of such different roles, an incorrect perception by patients, resulting from misleading information from the Internet or the media, by general practitioners as well as by some specialists, often leads subjects affected by HPV-positive precancerous or frankly neoplastic cervical or anal lesions, and their partners, to the otolaryngologist, who can only suggest a periodical examination (with palpation) of the oropharynx and a biopsy of possible suspicious lesions. Random biopsies with p16 IHC and/or the search for hr-HPV in cytologic or histologic samples or in saliva seem to be promising tools[57,58] but still need to be validated on the clinical setting, with particular attention to the site chosen for sampling.
- Clinical use of p16 IHC: Given that virtually all cervical carcinomas are HPV positive, p16 overexpression in the cervix is used as a marker of carcinogenetic progression more than that of HPV infection.[52] In the head and neck, the infection rate is much lower, and p16 overexpression is used as a marker of HPV infection itself, even if theoretically it can clearly derive from many other carcinogenic processes apart from HPV-induced ones.[38] In fact, outside the oropharynx, where the overall HPV infection rate is probably lower than 5%, p16 IHC is demonstrated to show very low or no correlation with HPV infection itself. Therefore, its prognostic role, if any,[59] has nothing to do with HPV-driven carcinogenesis, and it is not acceptable anymore as a diagnostic test for HPV infection outside the oropharynx.[15,28,56,60] In past decades, this inappropriate use of p16 staining as an HPV diagnostic tool probably led many studies to overestimate the real clinical relevance of HPV infection in head and neck cancers, especially outside the oropharynx.[28,56] In the oropharynx, p16 staining has a definite strong statistical correlation with HPV infection and has some diagnostic reliability,[10,29] but it is still associated with a number of

TABLE 1 The clinical framework of HPV-induced carcinogenesis in uterine cervix and in the head and neck

	HPV in cervical SCC	HPV in HNSCC
hr-HPV+ prevalence	95%-100%	~50% in oropharynx (~15% overall)
Clinical role of HPV	Epidemiologic marker	Characterization marker
Clinical utility of p16 IHC	Diagnosis of HPV infection and transformation	Prognosis

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus; p16 IHC, immunohistochemistry for $p16^{INK4A}$ protein; SCC, squamous cell carcinoma.

false-positive and false-negative cases.[61] As previously outlined,[38] other aspects currently limiting its use as a tool to diagnose HPV-associated carcinogenesis are the subjective nature of the IHC evaluation, the variable mechanisms of p16 expression in HNSCC, and the persistent lack of standardized scoring and interpretive criteria, such as the proposed H-score,[29] which still awaits extensive clinical validation.

It is of course clinically relevant to note what appears to be an independent prognostic value of p16 IHC for OPSCC, even in HPV-negative OPSCC. Similarly, it seems that some samples that are HPV DNA+ but p16 negative have a poor prognosis in comparison to HPV+ P16+ OPSCC. Such an intricate relationship between p16 and HPV does not seem to correlate with what we know for cervical cancers and could be indeed related to the interference of tobacco-related damage among OPSCC smokers. It may become a critical issue under a clinical point of view and still deserves to be thoroughly discussed in a separate work, as it appears beyond the scope of the present article, which focuses on the diagnosis of HPV infection.

4 | TOWARD THE STANDARDIZATION OF THE DIAGNOSIS OF HR-HPV INFECTION IN HNSCC

It should be stressed that we already have a gold standard for the detection in the clinical practice of HPV infection in OPSCC, which are the methods based on E6/E7 mRNA detection.[28–30] Many commercial kits, extensively validated in gynecology and approved for the clinical use, are available, their cost is decreasing, and they should be considered acceptable, because they are appropriately used for the molecular characterization of human malignancies. Nevertheless, for these tests to be reliable, a tissue sample stored in RNA later and possibly at −20°C is needed to warrant an adequate preservation of mRNA. mRNA detection assays on FFPE samples from HNSCC are described,[62,63] and, recently, an RNAscope ISH test, which is an ISH assay that detects hr-HPV oncogene mRNA on FFPE material, was described,[64] but extensive validation is still lacking. Therefore, in order to obtain accurate data about HPV-driven carcinogenesis in OPSCC, the clinician should ideally have a tube with RNA later available when performing the biopsy for the histological diagnosis of an OPSCC, in which part of the sample would be immediately stored. This would require a change in the clinicians' attitude in order to make it the only standard for HPV detection in OPSCCS. Furthermore, mRNA extraction and amplification is currently not widely feasible in clinical facilities in which OPSCC is routinely diagnosed. Therefore, the samples available for HPV testing, also in referral centers, in which it is not often considered, justify performing another biopsy to obtain a fresh sample, which are most often FFPE specimens. In this situation, a standard for HPV detection is still lacking.

4.1 | Diagnosing HPV infection in FFPE samples of OPSCC

p16 IHC, all the assays described above (with the exception of RNAscope), and those currently used for diagnosing HPV-driven carcinogenesis among OPSCC were first designed and validated for a totally different clinical setting, which is dysplasia and carcinoma of the uterine cervix. This is probably the reason why none of these assays are fully satisfying in head and neck oncology, leading some authors to conclude that none of these used singly is specific enough for routine clinical use on FFPE samples of OPSCCs.[15,30]

According to what was described and stated above, even if it is the most used method, accepted also by AJCC, for diagnosing HPV-related carcinogenesis in OPSCCs, p16 IHC, at least alone, presents some relevant issues when it comes to guiding treatment deintensification:

- False positive cases are present in every series[10,11,30,38] and do not presumably imply a better prognosis, and thus if the treatment is deintensified, survival is impaired. The false positive cases can be estimated to be between 5% and 15% of HPV-negative cases[10,28] and therefore to be higher in areas in which HPV prevalence in OPSCC is lower. When compared with ISH, p16 IHC alone may warrant a better prognostic prediction,[10,36] but recent studies show that it is definitely lower than what is obtained with DNA detection alone.[11,65] In any case, when the aim is to diagnose HPV infection in order to be allowed to deintensify the treatment, the specificity is obviously more desirable than the sensibility, or in other words, an overtreatment of an HPV-induced cancer is always more desirable than an undertreatment and a failure of an HPV-unrelated one (diagnosed as HPV positive by an inadequate, specific tool).
- The known advantages of the simplicity, low cost, and feasibility of p16 IHC have lost much of their attractiveness, as other methods based on nucleic acid detection, in particular DNA, have been developed for the management of cervical precancerous conditions, which are quite routine and low cost as well without being biased by the subjective reading of the histopathologist.
- Because p16 IHC has no value as a diagnostic assay outside the oropharynx and, in locally advanced lesions, the definition of the primary site may not be straightforward, the risk of incorrectly classifying as HPV-related hypopharyngeal and especially oral primaries by p16 IHC needs to be taken into account.

Nonquantitative diagnostic methods, including a DNA amplification phase, share with p16 IHC the concerns about specificity.

Therefore, in the search for a consensus, which should be reached by an inclusive expert panel, possibly also through the implementation of further clinical studies, three main strategies, based on the currently available options, appear at present to have the greatest strength as a potential standard for guiding treatment deintensification in HPV-driven carcinogenesis in OPSCC FFPE samples:

- A sequential strategy, including two highly sensitive methods as those already partially validated by Dutch[31,32,66] and English[30] groups. These authors propose an upfront P16 immunostaining, and when positive staining is observed, a PCR with virus-specific primers on DNA extracted from FFPE samples is used for confirmation. The Dutch authors report that the sensitivity and specificity of such approach can be as high as 100%. Still, this two-step procedure is definitely time consuming, may determine delays in the completion of the diagnostic workup in the daily clinical practice, and is more expensive than a single-step tool.
- The original approach, which definitely unveiled the HPV-driven carcinogenesis in the oropharynx, is based on DNA detection.[8] Lately, also because of the rational concerns about specificity, the quantification of HPV-DNA through real-time PCR has gained momentum[13,29,34] as well as in vitro DNA hybridization.[11,28,35] Such DNA-based techniques may be able to predict prognosis and relapse better than p16 IHC alone,[11,36] to correctly stratify cancers under a molecular point of view[13] and to warrant an adequate specificity. Real-time PCR assays have the great potential of adjusting the sensitivity and specificity by modifying the threshold for positivity, adapting it to different clinical settings and samples, but still need extensive validation in the OPSCC setting and standardization.

The picture described so far may change with the conclusive validation of new tests such as the RNAscope ISH test as a stand-alone assay. In comparison with the previously discussed assays, RNAscope is the first test that has not been transferred from cervical oncology to OPSCC. In fact, since its initial development, it has appeared as a promising tool to overcome several limitations of routine HPV testing methods in head and neck oncology, whereas its application in cervical cancer is very limited. Recent studies show that the performance of RNAscope is comparable with that of the validated sequential strategies[64,67] and highlight its significant advantages, including direct visualization on FFPE tissue samples, the minimal risk of contamination, and the fact that clinically relevant correlations are obtained with a single test. From a pathologist's point of view, the main issues still limiting the widespread application of the RNAscope test are the cost, the high technical requirements of the manual procedure associated with the risk of false-negative and false-positive results, and the limited automation.

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REFERENCES

1. Silverman NA, Alexander JC Jr, Chretien PB. CEA levels in head and neck cancer. *Cancer*. 1976;37(5):2204-2211.
2. Almadori G, Bussu F, Cadoni G, Galli J, Paludetti G, Maurizi M. Molecular markers in laryngeal squamous cell carcinoma: towards an integrated clinicobiological approach. *Eur J Cancer*. 2005;41(5):683-693.
3. National Comprehensive Cancer Network. *Head and Neck Cancers (Version 2. 2017-May 8, 20117)*. https://www.nccn.org/professionals/physician_gls/pdf/head-and-neck.pdf. Accessed on January 23, 2018.
4. Huang Q, Yu GP, McCormick SA, et al. Genetic differences detected by comparative genomic hybridization in head and neck squamous cell carcinomas from different tumor sites: construction of oncogenetic trees for tumor progression. *Genes Chromosomes Cancer*. 2002;34(2):224-233.
5. Bosch FX, Ritter D, Enders C, et al. Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. *Int J Cancer*. 2004;111(4):530-538.
6. Shah JP, Patel KJ. *Head and Neck Surgery and Oncology*. 3rd ed. Maryland Heights, Missouri: Mosby Ltd.; 2003.
7. De Vita V, Lawrence T, Rosenberg S, Depinho R, Weinberg R. *DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology*. 8th ed. Philadelphia: Lippincott Williams & Wilkins; 2008.
8. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst*. 2000;92(9):709-720.
9. Gillison ML. HPV and prognosis for patients with oropharynx cancer. *Eur J Cancer*. 2009;45(Suppl 1):383-385.
10. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363(1):24-35.
11. Bussu F, Sali M, Gallus R, et al. Human papillomavirus (HPV) infection in squamous cell carcinomas arising from the oropharynx: detection of HPV DNA and p16 immunohistochemistry as diagnostic and prognostic indicators--a pilot study. *Int J Radiat Oncol Biol Phys*. 2014;89(5):1115-1120.
12. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. *Int J Cancer*. 2007;121(8):1813-1820.
13. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science*. 2011;333(6046):1157-1160.
14. Amin MB. *AJCC Cáncer Staging Manual Eighth Edition*. New York City: Springer; 2017.
15. Bhatia A, Burtness B. Human papillomavirus-associated oropharyngeal cancer: defining risk groups and clinical trials. *J Clin Oncol*. 2015;33(29):3243-3250.
16. Westra WH. Detection of human papillomavirus (HPV) in clinical samples: evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas. *Oral Oncol*. 2014;50(9):771-779.
17. Speel EJ. HPV integration in head and neck squamous cell carcinomas: cause and consequence. *Recent Results Cancer Res*. 2017;206:57-72.
18. Xu B, Chotewutmontri S, Wolf S, et al. Multiplex identification of human papillomavirus 16 DNA integration sites in cervical carcinomas. *PLoS One*. 2013;8(6):e66693.
19. Begum S, Cao D, Gillison M, Zahurak M, Westra WH. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clin Cancer Res*. 2005;11(16):5694-5699.
20. Mehrad M, Zhao H, Gao G, Wang X, Lewis JS Jr. Transcriptionally-active human papillomavirus is consistently retained in the distant metastases of primary oropharyngeal carcinomas. *Head Neck Pathol*. 2014;8(2):157-163.
21. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and

sufficient for transformation of primary human keratinocytes. *J Virol*. 1989; 63(10):4417-4421.

22. McDougall JK. Immortalization and transformation of human cells by human papillomavirus. *Curr Top Microbiol Immunol*. 1994;186: 101-119.

23. Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev*. 1997; 11(16):2101-2111.

24. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene*. 1996;13(11):2323-2330.

25. Duensing S, Duensing A, Crum CP, Munger K. Human papillomavirus type 16 E7 oncoprotein-induced abnormal centrosome synthesis is an early event in the evolving malignant phenotype. *Cancer Res*. 2001;61(6):2356-2360.

26. Band V, Zajchowski D, Kulesa V, Sager R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc Natl Acad Sci U S A*. 1990;87(1):463-467.

27. Halbert CL, Demers GW, Galloway DA. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol*. 1991;65(1):473-478.

28. Bussu F, Sali M, Gallus R, et al. HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker? *Br J Cancer*. 2013; 108(5):1157-1162.

29. Jordan RC, Lingen MW, Perez-Ordonez B, et al. Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. *Am J Surg Pathol*. 2012;36(7):945-954.

30. Schache AG, Liloglou T, Risk JM, et al. Evaluation of human papilloma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity, and prognostic discrimination. *Clin Cancer Res*. 2011; 17(19):6262-6271.

31. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer*. 2007;121(11):2465-2472.

32. Braakhuis BJ, Brakenhoff RH, Meijer CJ, Snijders PJ, Leemans CR. Human papilloma virus in head and neck cancer: the need for a standardised assay to assess the full clinical importance. *Eur J Cancer*. 2009;45(17):2935-2939.

33. Weinberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus--associated oropharyngeal cancers with favorable prognosis. *J Clin Oncol*. 2006;24(5):736-747.

34. Cohen MA, Basha SR, Reichenbach DK, Robertson E, Sewell DA. Increased viral load correlates with improved survival in HPV-16-associated tonsil carcinoma patients. *Acta Otolaryngol*. 2008;128(5):583-589.

35. Bishop JA, Maleki Z, Valsamakis A, et al. Application of the hybrid capture 2 assay to squamous cell carcinomas of the head and neck: a convenient liquid-phase approach for the reliable determination of human papillomavirus status. *Cancer Cytopathol*. 2012;120(1):18-25.

36. Shi W, Kato H, Perez-Ordonez B, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *J Clin Oncol*. 2009;27(36):6213-6221.

37. Klussmann JP, Gultekin E, Weissenborn SJ, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol*. 2003;162(3):747-753.

38. El-Naggar AK, Westra WH. p16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma: a guide for interpretative relevance and consistency. *Head Neck*. 2012;34(4):459-461.

39. Mehra R, Ang KK, Burtness B. Management of human papillomavirus-positive and human papillomavirus-negative head and neck cancer. *Semin Radiat Oncol*. 2012;22(3):194-197.

40. Kimple RJ, Harari PM. Is radiation dose reduction the right answer for HPV-positive head and neck cancer? *Oral Oncol*. 2014;50(6):560-564.

41. Anantharaman D, Gheit T, Waterboer T, et al. Human papillomavirus infections and upper aero-digestive tract cancers: the ARCAGE study. *J Natl Cancer Inst*. 2013;105(8):536-545.

42. Lang Kuhs KA, Anantharaman D, Waterboer T, et al. Human papillomavirus 16 E6 antibodies in individuals without diagnosed cancer: a pooled analysis. *Cancer Epidemiol Biomarkers Prev*. 2015;24(4):683-689.

43. Kreimer AR, Johansson M, Waterboer T, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *J Clin Oncol*. 2013;31(21):2708-2715.

44. Liang C, Marsit CJ, McClean MD, et al. Biomarkers of HPV in head and neck squamous cell carcinoma. *Cancer Res*. 2012;72(19):5004-5013.

45. Durst M, Gissmann L, Ikenberg H, Zur HH. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A*. 1983;80(12):3812-3815.

46. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, Zur HH. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J*. 1984;3(5):1151-1157.

47. Schwarz E, Freese UK, Gissmann L, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature*. 1985; 314(6006):111-114.

48. Tommasino M. The human papillomavirus family and its role in carcinogenesis. *Semin Cancer Biol*. 2014;26:13-21.

49. Cobrinik D. Pocket proteins and cell cycle control. *Oncogene*. 2005;24(17): 2796-2809.

50. McLaughlin-Drubin ME, Park D, Munger K. Tumor suppressor p16INK4A is necessary for survival of cervical carcinoma cell lines. *Proc Natl Acad Sci U S A*. 2013;110(40):16175-16180.

51. Pauck A, Lener B, Hoell M, et al. Depletion of the cdk inhibitor p16INK4a differentially affects proliferation of established cervical carcinoma cells. *J Virol*. 2014;88(10):5256-5262.

52. Bergeron C, Ronco G, Reuschenbach M, et al. The clinical impact of using p16(INK4a) immunochemistry in cervical histopathology and cytology: an update of recent developments. *Int J Cancer*. 2015;136(12):2741-2751.

53. Bosch FX, Robles C, Diaz M, et al. HPV-FASTER: broadening the scope for prevention of HPV-related cancer. *Nat Rev Clin Oncol*. 2016 Feb;13(2): 119-132.

54. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011;29(32):4294-4301.

55. Gheit T, Abedi-Ardekani B, Carreira C, Missad CG, Tommasino M, Torrente MC. Comprehensive analysis of HPV expression in laryngeal squamous cell carcinoma. *J Med Virol*. 2014;86(4):642-646.

56. Lingen MW, Xiao W, Schmitt A, et al. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncol*. 2013;49(1):1-8.

57. D'Souza G, Gross ND, Pai SI, et al. Oral human papillomavirus (HPV) infection in HPV-positive patients with oropharyngeal cancer and their partners. *J Clin Oncol*. 2014;32(23):2408-2415.

58. Rettig EM, Wentz A, Posner MR, et al. Prognostic implication of persistent human papillomavirus type 16 DNA detection in oral rinses for human papillomavirus-related Oropharyngeal carcinoma. *JAMA Oncol*. 2015;1(7): 907-915.

59. Chung CH, Zhang Q, Kong CS, et al. p16 protein expression and human papillomavirus status as prognostic biomarkers of nonoropharyngeal head and neck squamous cell carcinoma. *J Clin Oncol*. 2014;32(35): 3930-3938.

60. Ha PK, Pai SI, Westra WH, et al. Real-time quantitative PCR demonstrates low prevalence of human papillomavirus type 16 in premalignant and malignant lesions of the oral cavity. *Clin Cancer Res*. 2002;8(5): 1203-1209.

61. Castellsague X, Alemany L, Quer M, et al. HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients. *J Natl Cancer Inst*. 2016;108(6):djv403.

62. Halec G, Holzinger D, Schmitt M, et al. Biological evidence for a causal role of HPV16 in a small fraction of laryngeal squamous cell carcinoma. *Br J Cancer*. 2013;109(1):172-183.

63. Schache AG, Liloglou T, Risk JM, et al. Validation of a novel diagnostic standard in HPV-positive oropharyngeal squamous cell carcinoma. *Br J Cancer*. 2013;108(6):1332-1339.

64. Mirghani H, Casiraghi O, Amen F, et al. Diagnosis of HPV-driven head and neck cancer with a single test in routine clinical practice. *Mod Pathol*. 2015; 28(12):1518-1527.

65. Lohaus F, Linge A, Tinhofer I, et al. HPV16 DNA status is a strong prognosticator of loco-regional control after postoperative radiochemotherapy of

locally advanced oropharyngeal carcinoma: results from a multicentre explorative study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). *Radiother Oncol*. 2014;113(3):317-323.

66. Rietbergen MM, Leemans CR, Bloemena E, et al. Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by a validated test algorithm. *Int J Cancer*. 2013; 132(7):1565-1571.

67. Mirghani H, Casiraghi O, Guerlain J, et al. Diagnosis of HPV driven oropharyngeal cancers: comparing p16 based algorithms with the RNAscope HPV-test. *Oral Oncol*. 2016;62:101-108.