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Current Perspective

Human papilloma virus in head and neck cancer: The need for a standardised assay to assess the full clinical importance

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ABSTRACT

Recent studies have revealed an important and intriguing role for human papillomavirus (HPV) in head and neck squamous cell carcinoma (HNSCC). There are indications that the prevalence of HPV-positive HNSCC has recently increased, and genetic analyses point to a completely distinct class of HNSCCs. Most studies suggest that patients with this type of tumour have a better prognosis and some argue that an adjusted therapeutic approach is needed. One crucial point in the research of HNSCC–HPV involvement has often been neglected, which is the lack of a standardised assay to detect HPV. This has resulted in a considerable variation in the frequency of HPV-positive tumours between studies reported thus far. Especially for PCR-based tests, the risk exists that the assay is too sensitive and detects virus without implying a causal involvement in HNSCC. A reliable algorithm to detect a clinically relevant HPV infection in formalin-fixed paraffin embedded tissue has recently become available. Here, we address important biological and analytical aspects of HPV involved in the development of HNSCC and it is emphasised that a standardised HPV assay is a prerequisite for assessing the clinical importance of a HPV infection in HNSCC.

1. HPV in HNSCC

Oral and oropharyngeal squamous cell carcinomas constitute together with hypopharyngeal and laryngeal squamous cell carcinomas and several other entities the group of head and neck squamous cell carcinomas (HNSCCs). HNSCCs comprise about 5% of all newly diagnosed cancer cases in North-Western Europe and the United States, and they are the fifth most common cancer worldwide.[1] Tobacco smoking and excessive alcohol consumption are well established risk factors for HNSCCs. Recent studies have revealed human papillomavirus (HPV) infection, particularly HPV type 16 (HPV-16), as an aetiological factor for HNSCCs.[2] Data from numerous (nested) case-control studies, in part based on serology, point to HPV infection being a risk factor for most notably oropharyngeal cancer.[3] Molecular differences were found between HPV-positive and HPV-negative HNSCCs, at the DNA,[4–6] the mRNA,[7–10] and the protein level,[11] supporting the idea of two separate

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carcinogenic pathways to HNSCC: one determined by lifestyle carcinogens and the other by HPV.[5,7,12] Patients with HPV-positive HNSCC are generally younger, have a history of less tobacco usage, have had a higher exposure to marijuana, and have had more oral sex partners as compared to those with HPV-negative carcinomas.[13,14] Moreover, most studies show a better prognosis for HPV-positive HNSCCs, although there is some controversy.[15,16] The demonstration of HPV in a HNSCC may have essential consequences.[3]

A serious problem in research in this field, which may undermine the true importance of HPV for HNSCC patients, is that no consensus consists on how to identify HNSCCs that are attributable to HPV. There are many assay variables that differ between studies making it intricate to compare various studies and generalise results. For instance, the variation in prevalence between studies is surprisingly large; some studies report 14% HPV-positive oropharyngeal cancers,[17] whereas others observe 72%.[14] Nevertheless, when analysing sixty different studies, Kreimer et al. found several distinct trends.[18] Overall, HPV-16 prevalence was significantly higher in oropharyngeal SCCs (mean value of 35%) than in oral (23%) or laryngeal SCCs (24%); in particular SCC of the tonsil is often caused by SCC. Prevalence of HPV-16 is higher in the United States and Asia than in Europe (mean values of 47% versus 28%).[18]

This perspective addresses important biological and analytical aspects of HPV involved in the development of HNSCC and it is concluded that a standardised HPV assay is a prerequisite for assessing the full clinical importance of a HPV infection in HNSCC.

2. Aetiology and biology of HPV in HNSCCs

Human papillomaviruses are epitheliotropic DNA viruses with a genome of approximately 8 kb. Over 100 genotypes of HPV have been recognised, of which about 15 are considered to be oncogenic (high risk, HR) in squamous epithelium.[19] HR-HPV is aetiologically involved in almost all cervical cancers. HNSCC belongs together with vulvar and penile cancers to the cancer types in which a proportion can be attributed to HR-HPV. The virus produces two oncoproteins encoded by the *E6* and *E7* genes, which are responsible for the induction of the transformed phenotype of the epithelial cells. Both *E6* and *E7* bind to multiple cellular targets, leading to abrogation of important cellular processes like cell cycle control and apoptosis.[20] The interactions that are proposed to be most relevant are the binding of *E6* to the tumour suppressor gene product p53,[21] and the binding of *E7* to the retinoblastoma tumour suppressor gene product pRb.[22,23] Abnormal expression of *E6* and *E7* in proliferating cells of squamous epithelium is believed to initiate transformation mediated by this virus[24,25] and this phenomenon is associated with an accumulation of $p16^{INK4a}$, a product of CDKN2A regulating pRb activity, in the proliferating cell layers of the epithelium.[25–27] *In vitro* studies have shown that HR-HPVs are able to immortalise primary human keratinocytes, including those of the oral cavity, and upon prolonged culturing it was found that fully tumourigenic clones can emerge.[28] This process of *in vitro* progression to a tumourigenic phenotype coincides with an accumulation of specific (epi-)genetic alterations of cancer genes, mediated by genetic instability induced by continuous expression of *E6* and *E7*.[29] Interference with *E6* and *E7* expression in cancer cell lines results in the loss of the transformed phenotype, indicating that HR-HPV is essential not only for the initiation, but also for the maintenance of the malignant phenotype.[30] It must be noted that the development of HR-HPV-induced cancers may last for decades, and that multiple additive crucial (epi-)genetic alterations are considered to be essential throughout the carcinogenic process.[25]

3. Detection of HPV

The frequency of HR-HPV positive tumours in HNSCC shows a considerable variation between the studies reported thus far. Tumour site and many technical aspects influence the outcome of these analyses. In addition, different results may be obtained when using frozen or paraffin material.[31] Another problem is associated with the type of assay and the criterion of positivity. With the presently available assays with extremely high analytical sensitivities, the term 'positive' must be regarded with caution. There is the risk that the test is too sensitive and detects virus without underlying biological or clinical consequences. In this respect, the analytical sensitivity and specificity should be discriminated from the clinical ones, the latter pointing to virus-induced disease instead of to coincidental virus infections.[32] There is sufficient evidence that increased analytical sensitivities of assays result in decreased clinical specificities (i.e. more positive test results in the absence of virus-induced disease).[32,33] Thus, a positive HR-HPV-test does not automatically mean that the virus has been involved in the initiation or maintenance of carcinogenesis.

Many assays for HPV detection are available at present, each with its own analytical sensitivity.[34] There are the PCR-based assay systems, often linked to a specific genotyping system. Very sensitive systems are Amplicor[35] and SPF10[36] as well as various type-specific PCR methods, whereas GP5+/GP6+-PCR[37] and PGMY[38] are somewhat less sensitive in detecting HPV. In addition, methods are available that are based on DNA hybridisation with specific labelled RNA probes, such as HC2 (from Digene).[39] ISH (*in situ*-hybridisation) can also be applied to detect HPV in slides (scrapes or tissue sections)[40] and examples are Inform® HPV test from Ventana and the Genpoint™ system from Dako. There is strong evidence that various methods differ in their performance of detecting HPV-related disease,[32] and for cervical screening purposes guidelines have been formulated for HPV test requirements to ensure high specificity and at the same time high sensitivity for clinically relevant disease.[41] Notably, detection of very low viral copy numbers in cervical specimens does not reflect (risk of) high grade disease.[42] This likely also holds true for head and neck cancer, and is supported by recent findings of Cohen and colleagues, indicating that amongst tonsillar cancers with detectable HPV, only the presence of higher HPV copy numbers was associated with a different clinical course compared to that of HPV-negative carcinomas.[43]

4. Standardised detection of clinically relevant HPV in HNSCC

What is the best way to detect aetiological involvement of HR-HPV, meaning that the virus was implicated in the initiation and maintenance of the cancer phenotype? Data from recent studies suggest that detectable expression of *E6* and *E7* mRNA in carcinomas is a reason to assume that the virus has been aetiologically involved.[44] Convincing arguments in favour are provided by genetic studies: the mRNA expression of *E6* and *E7* runs parallel with a characteristic geno- and phenotype that can be detected by loss of heterozygosity (LOH), array comparative hybridisation and array expression analysis.[4,5,7] Measuring this, however, has limited applicability, as high quality freshly frozen material is needed with intact mRNA. A recent multi-centre study compared various HPV detection techniques that used paraffin-embedded tissue and had frozen tissue as the reference.[45] LOH analysis had been used as evidence for viral involvement. HPV-*E6* could be measured with an RT-PCR-based method with primers especially designed for the use in paraffin material, but its application was restricted to HPV-16. More importantly, an algorithm was developed for HPV detection in paraffin material that combines satisfactory performance with the option for high-throughput analysis. This is based on the combination of two tests: a $p16^{INK4a}$ immunostaining upfront, followed by HPV-DNA PCR (e.g. GP5+/6+) on the $p16^{INK4a}$-positive cases. In this published series a 100% sensitivity and 100% specificity could be reached, taking an aberrant LOH profile and the mRNA expression of *E6* on fresh-frozen material as reference.[45] Other methods were compared, but were less sensitive (FISH) or less specific (antibody detection in serum). This study was based on a small series and only a part of all available HPV detection methods were addressed. It cannot be excluded that other technique(s) or combination(s) of assays are just as good or even better. Choice of the best assay method is facilitated by the fact that HPV-positive HNSCCs possess a unique genetic profile.[4–6] The ultimate aim is to establish a standardised HPV–HNSCC assay. In addition, to be able to compare different studies, we would like to advocate that the authors provide details in their publications on the protocols that have been used for identification of HPV-positive HNSCCs.

5. Clinical importance of HR-HPV in HNSCCs

Thus far, the definitive picture has not yet emerged regarding the relation between survival and a HR-HPV infection, and some important issues remain unsolved. The majority of the studies done before 2007 indicated a trend towards a better survival and a lower risk of recurrence for patients with a HR-HPV-positive HNSCC, and the average risk of death collected in large number of studies was reduced by 18% in patients with HR-HPV positive carcinomas in comparison with those with HPV-negative carcinomas.[15] This positive HR-HPV-effect has also been observed in other more recent reports studying oropharyngeal carcinomas.[26,46,47] Still, there are issues to be resolved: some studies report a worse survival in case of HR-HPV positive carcinomas, because of a higher risk of local recurrence.[15,48] In addition, the positive effect of HR-HPV on overall and disease-specific survival was only observed for oropharyngeal carcinomas,[15] suggesting that HPV-positive oropharyngeal carcinomas are aetiologically different from tumours from non-oropharyngeal sites. Also, it cannot be excluded that HR-HPV has been scored falsely positive in the latter group. As stated above, part of the controversy may be attributed to variations related to the type of tissue investigated and assay employed.

Improved survival observed for patients with a HPV-positive carcinoma may be related to type of treatment. Preliminary data from small studies indicate that HPV-positive tumours are more likely to respond to induction chemotherapy[47] and to radiation.[49] However, in another study a possible better effect of radiation in HPV-positive tumours was not observed.[50] The biological basis of the improved survival among the HPV-positive patients is unclear at present.

To make headway in HPV–HNSCC research three pertinent issues should urgently be clarified: (1) what is prevalence of HNSCC with aetiologically involved HPV, (2) do these patients have improved survival and (3) is there an effect of therapy? There are basically two approaches to solve these questions and these could run in parallel. The first option is to perform a retrospective study in a cohort of treated HNSCC patients (preferably from multiple centres). This population should contain a number of patients that would allow for comparing various treatment arms and the difference by HPV-status. Paraffin blocks of the tumours will be needed and the HPV status should be determined with a reliable detection assay. Considering treatment arms it would be interesting to compare protocols with and without radiotherapy. The second option is to perform a prospective study with HPV status as the study variable. This would be an observational study in which all patients will receive the same treatment. A more practical approach along the same line is a randomised trial comparing two treatment arms, with stratification for HPV status. A secondary aim of the study would be the analysis of the interaction between HPV and type of treatment.

Conflicts of interest statement

None declared.

REFERENCES

1. Pisani P, Bray F, Parkin DM. Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. *Int J Cancer* 2002;**97**:72–81.
2. Mork J, Lie AK, Glattre E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *New Engl J Med* 2001;**34**:1125–31.
3. Psyrri A, Gouveris P, Vermorken JB. Human papillomavirus-related head and neck tumors: clinical and research implication. *Curr Opin Oncol* 2009;**21**:201–5.
4. Braakhuis BJM, Snijders PJF, Keune WJH, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. *J Natl Cancer Inst* 2004;**96**:998–1006.

5. Smeets SJ, Braakhuis BJM, Abbas S, et al. Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. *Oncogene* 2006;**25**:2558–64.
6. Klussmann JP, Mooren JJ, Lehnen M, et al. Genetic signatures of HPV-related and unrelated oropharyngeal carcinoma and their prognostic implications. *Clin Cancer Res* 2009;**15**:1779–86.
7. Slebos RJC, Yi YJ, Ely K, et al. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. *Clin Cancer Res* 2006;**12**:701–9.
8. Lohavanichbutr P, Houck J, Fan W, et al. Genomewide gene expression profiles of HPV-positive and HPV-negative oropharyngeal cancer: potential implications for treatment choices. *Arch Otolaryngol Head Neck Surg* 2009;**135**:180–8.
9. Schlecht NF, Burk RD, Adrien L, et al. Gene expression profiles in hpv-infected head and neck cancer. *J Pathol* 2007;**213**:283–93.
10. Martinez I, Wang J, Hobson KF, Ferris RL, Khan SA. Identification of differentially expressed genes in HPV-positive and HPV-negative oropharyngeal squamous cell carcinomas. *Eur J Cancer* 2007;**43**:415–32.
11. Melle C, Ernst G, Winkler R, et al. Proteomic analysis of human papillomavirus-related oral squamous cell carcinoma: identification of thioredoxin and epidermal-fatty acid binding protein as upregulated protein markers in microdissected tumor tissue. *Proteomics* 2009;**9**:2193–201.
12. 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**:709–20.
13. Gillison ML, D'souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst* 2008;**100**:407–20.
14. D'Souza G, Kreimer AR, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *New Engl J Med* 2007;**356**:1944–56.
15. Ragin CCR, 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**:1813–20.
16. Rosenquist K, Wennerberg J, Annertz K, et al. Recurrence in patients with oral and oropharyngeal squamous cell carcinoma: human papillomavirus and other risk factors. *Acta Otolaryngol* 2007;**127**:980–7.
17. Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *J Natl Cancer Inst* 2003;**95**:1772–83.
18. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomark Prev* 2005;**14**:467–75.
19. Zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst* 2000;**92**:690–8.
20. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. *Virus Res* 2002;**89**:213–28.
21. Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* 1993;**13**:775–84.
22. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;**243**:934–7.
23. Munger K, Basile JR, Duensing S, et al. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* 2001;**20**:7888–98.
24. Cheng S, Schmidt-Grimminger DC, Murant T, Broker TR, Chow LT. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev* 1995;**9**:2335–49.
25. Snijders PJF, Steenbergen RDM, Heideman DAM. Meijer Cjlm. Hpv-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* 2006;**208**:152–64.
26. Hafkamp HC, Manni JJ, Haesevoets A, et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. *Int J Cancer* 2008;**122**:2656–64.
27. Wittekindt C, Gultekin E, Weissenborn SJ, et al. Expression of p16 protein is associated with human papillomavirus status in tonsillar carcinomas and has implications on survival. *Adv Otorhinolaryngol* 2005;**62**:72–80.
28. Chen TM, Pecoraro G, Defendi V. Genetic analysis of in vitro progression of human papillomavirus-transfected human cervical cells. *Cancer Res* 1993;**53**:1167–71.
29. Steenbergen RD, Walboomers JM, Meijer CJ, et al. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene* 1996;**13**:1249–57.
30. Rampias T, Sasaki C, Weinberger P, Psyrri A. E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells. *J Natl Cancer Inst* 2009;**101**:412–23.
31. Brink AA, Snijders PJ, Meijer CJ. HPV detection methods. *Dis Markers* 2007;**23**:273–81.
32. Snijders PJ, van den Brule AJ, Meijer CJ. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. *J Pathol* 2003;**201**:1–6.
33. Hesselink AT, van den Brule AJ, Brink AA, et al. Comparison of hybrid capture 2 with in situ hybridization for the detection of high-risk human papillomavirus in liquid-based cervical samples. *Cancer* 2004;**102**:11–8.
34. Iftner T, Villa LL. Chapter 12: human papillomavirus technologies. *J Natl Cancer Inst Monogr* 2003;**31**:80–8.
35. van Ham MA, Bakkers JM, Harbers GK, et al. Comparison of two commercial assays for detection of human papillomavirus (HPV) in cervical scrape specimens: validation of the Roche AMPLICOR HPV test as a means to screen for HPV genotypes associated with a higher risk of cervical disorders. *J Clin Microbiol* 2005;**43**:2662–7.
36. Kleter B, van Doorn LJ, ter Schegget J, et al. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* 1998;**153**:1731–9.
37. Jacobs MV, Snijders PJ, van den Brule AJ, et al. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997;**35**:791–5.
38. Gravitt PE, Peyton CL, Alessi TQ, et al. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000;**38**:357–61.
39. Clavel C, Masure M, Putaud I, et al. Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. *J Clin Pathol* 1998;**51**:737–40.
40. Hopman AH, Kamps MA, Smedts F, et al. HPV in situ hybridization: impact of different protocols on the detection of integrated HPV. *Int J Cancer* 2005;**115**:419–28.
41. Meijer CJ, Berkhof J, Castle PE, et al. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. *Int J Cancer* 2009;**124**:516–20.

42. Hesselink AT, van Ham MA, Heideman DA, et al. Comparison of GP5+/6+-PCR and SPF10-line blot assays for detection of high-risk human papillomavirus in samples from women with normal cytology results who develop grade 3 cervical intraepithelial neoplasia. *J Clin Microbiol* 2008;**46**:3215–21.
43. 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**:583–9.
44. Van Houten VMM, Snijders PJF, Van Den Brekel MWM, et al. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. *Int J Cancer* 2001;**93**:232–5.
45. Smeets SJ, Hesselink AT, Speel EJM, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer* 2007;**121**:2465–72.
46. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst* 2008;**100**:261–9.
47. Worden FP, Kumar B, Lee JS, et al. Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. *J Clin Oncol* 2008;**26**:3138–46.
48. Rosenquist K, Wennerberg J, Annertz K, et al. Recurrence in patients with oral and oropharyngeal squamous cell carcinoma: human papillomavirus and other risk factors. *Acta Otolaryngol* 2007;**127**:980–7.
49. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. *Cancer* 2001;**92**:805–13.
50. Licitra L, Perrone F, Bossi P, et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2006;**24**:5630–6.