Human Papillomavirus-Related Head and Neck Cancer

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Summary

Human papillomavirus(HPV)-related head and neck cancer is recognized as a distinct tumor entity with rising incidence reported for several countries. These tumors arise from squamous cells, typically in the oropharynx. In contrast to cancer associated with other risk factors, HPV-related cancer is driven by viral oncoprotein activity and has individual profiles regarding protein expression, and genetic and epigenetic alterations. Molecular characteristics are p16^{INK4A} overexpression, absence of p53 inactivating mutations, and PI3K/AKT and Wnt pathway modulation. Patients with HPV-related head and neck cancer have improved survival compared to those with HPV-negative tumors, and p16^{INK4A} staining has been introduced into tumor staging recently. However, no specific or toxicity-reduced treatment modalities have been established for this entity so far. Although the still incomplete and partially inconsistent data in this field needs further study, particular features of HPV-related cancers such as specific microRNA expression, immunology, or gene methylation patterns certainly have the potential to be implemented in future diagnostic and therapeutic concepts.

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Introduction

Human papillomavirus(HPV)-related carcinogenesis has been attributed to a subset of head and neck cancers [1, 2]. Those cancers comprise squamous cell carcinoma of the head and neck (HNSCC), and primarily arise in the oropharynx (OPSCC) and especially in the tonsils [3, 4]. HPV-related HNSCC are characterized by absent somatic mutations in TP53, deregulation of retinoblastoma protein(Rb)-mediated cell cycle control, and overexpression of the tumor suppressor protein p16^{INK4a}.

The epidemiology of HPV-related HNSCC is variable and highly dependent on tumor subsite and region. It is assumed that HPV-related HNSCC have different risk factors compared to HPV-negative HNSCC, e.g., sexual behavior instead of tobacco and alcohol consumption. A trend towards higher smoking prevalence from north to south is reported for Europe [5], while the opposite trend in HPV prevalence persists. Although smoking is decreasing, the overall prevalence of OPSCC is remaining constant or even increasing in certain countries over time. This trend of an increase in HPV-related and a decrease in HPV-negative OPSCC was published for the USA in 2011, with a total HPV prevalence in OPSCC of 70% in the last time period evaluated between 2000 and 2004 [6]. In a meta-analysis of data for 12,163 HNSCC patients from 44 countries, the pooled HPV DNA prevalence was 46% for oropharynx, 22% for larynx, and 24% for oral cavity. The overall HPV-attributable fraction was higher in North America (60%) compared to Europe (41%) [7]. A recent review confirmed an increase in OPSCC incidences in many countries and mainly in men [8]. The impact of tumor site and region was also confirmed by an international study with uniform HPV testing of 3,680 specimen from pharyngeal, oral cavity, and laryngeal cancers. Inconsistent with the above study, a higher HPV-attributable fraction was found in women for cancers of the oropharynx in Europe [9].

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Fig. 1. Natural life cycle and human papillomavirus (HPV)-induced carcinogenesis. Deregulation of HPV E2 protein leads to aberrant expression of E6 and E7 oncoproteins, the hallmark of HPV-related carcinogenesis. **a** As a consequence of E7 activity, the cellular tumor suppressor protein p16^{INK4a} is overexpressed, which can serve as a biomarker for HPV-driven cancers in immunohistochemically staining of tumor tissue. **b** Fluorescent staining of cultured HPV-positive squamous cell carcinoma of the head and neck (HNSCC) cell shows uniform cytoplasmic expression of p16^{INK4a} (red) in relation to membranous staining of β -catenin (green) and nuclear DNA staining (blue).

Natural HPV Life Cycle and Oncogene Regulation

Numerous papillomaviruses are known, with more than 150 mostly low-risk types in humans. Currently, 15 high-risk HPV types have been identified in precancerous and cancerous lesions, which are considered causative for cancer. HPV infects undifferentiated cells in the basal layer of epithelia through microwounds. HPV DNA replication is coupled with cellular DNA replication and maintained at a low copy number as long as infected cells remain at the basal membrane. Viral gene expression is linked to the differentiation process when infected cells are moved towards the epithelial surface, involving regulatory 'E' proteins (E1–E8) expressed early in the HPV life cycle. Two proteins expressed late in the life cycle (L1 and L2) build the capsid structure of the viral particles which are released together with cells of the uppermost epithelial layers. In HPV-related cancers, the viral life cycle is interrupted as cancer cells remain in an undifferentiated state. Thus, viral replication cannot be completed, and infectious particles are not produced and released.

Rising incidences of HPV-related OPSCC have been attributed to changes in sexual behavior [6, 10]. Nevertheless, although HPV is highly associated with tonsillar cancer [4, 11], less than 1% of tumor-free tonsillar tissue harbors HPV DNA [12]. Even in women with genital HPV infections, HPV DNA detection in the oropharynx is low (5.7%) and comparable to HPV DNA detection rates in females negative for genital HPV (5.1%), suggesting that sexual transmission of HPV from the cervix uteri to the oropharynx is rare [13]. However, in a recent study in patients undergoing tonsillectomy for benign indications, the HPV prevalence was 3.6% in tonsil brushings and 13.1% in gargles (with an HPV16 prevalence of 2.2 and 4.1%, respectively), indicating significant differences caused by sampling techniques [14] which may account for inconsistency in this field.

Viral Transformation

Unlike cervical cancer, HPV-induced precancerous lesions are absent in the upper aerodigestive tract. Tonsillar cancers may be surrounded by p16-positive mucosal fields, being an indicator for HPV16 in OPSCC and adjacent dysplasia [15]. However, transcriptionally active HPV could not be detected in the mucosa surrounding HPV-positive OPSCC, suggesting the absence of HPV-induced field cancerization [16].

Despite lacking knowledge about the exact ways of oral HPV infection and the very first steps in viral transformation, HPV-driven carcinogenesis is well understood at the molecular level (fig. 1). The same pathways altered in the natural life cycle are involved in HPV-related carcinogenesis and similar to HPV-negative cancers, but deregulation is largely based on protein-protein interactions rather than driven by genetic alteration.

Protein Expression Alterations

The hallmark of HPV-related carcinogenesis is viral protein-mediated deregulation of cell cycle control and interruption of p53-dependent apoptosis activation. Both depend on the activity of the HPV oncoproteins E6 and E7 (fig. 1). E7 targets Rb for ubiquitin-mediated proteasomal degradation. This releases and activates E2F transcription factors, driving S-phase gene expression and cell cycle progression. Unlike previously thought, p16^{INK4a} overexpression observed in HPV-related cancers (fig. 1a, b), is not a consequence of this Rb inactivation but triggered by a cellular senescence response via activation of the histone lysine demethylase KDM6B, which has been directly linked to E7 expression in cervical carcinoma cell lines [17].

E6 binds to p53 and recruits ubiquitin ligase E6AP. This leads to p53 ubiquitylation, its proteasomal degradation, and inhibition of p21. As a consequence, growth arrest is released, p53-induced ap-

Fig. 2. Viral DNA integration as a prerequisite for human papillomavirus(HPV)-driven cancer. **a** Linearization of the viral DNA (schematic drawing) within the E2 open reading frame and subsequent integration into the host's DNA abrogates the controlling function of E2 on E6 and E7 protein expression. **b** Fluorescence-labeled HPV DNA (red in **b**, green in **c**) can be detected within the nucleus (blue) of squamous cell carcinoma of the head and neck (HNSCC) tumor cells (**b**; tumor area marked by dashed lines) and HPV-positive HNSCC cell lines (**c**).

optosis is inhibited, and DNA damage repair and cellular senescence are deregulated. Further, induction of the hTERT promoter by E6 via interaction with E6AP (and other proteins) is known, and direct E6/hTERT interactions mediating telomerase activation have been shown recently [18]. Interestingly, p21 expression is described as another surrogate marker of HPV-related tonsillar cancer being strongly associated with favorable patient outcome [19], although reduced p21 expression could be expected with E6-mediated p53 inactivation. Results of other studies are somewhat inconsistent, but a recent study indicates that E7 bypasses the inhibitory effect of p21 on cell cycle progression [20].

Other proteins with altered expression are epidermal growth factor receptor (EGFR), Baculoviral IAP repeat-containing protein 5 (survivin), and metabolic proteins which are related but not essential for HPV-induced carcinogenesis. Survivin is negatively regulated by p53. It suppresses apoptosis and plays a role in cell division. Nuclear survivin expression was correlated with a poor disease-free survival rate and negative HPV status in OPSCC [21].

Thioredoxin (TRX) and epidermal fatty acid binding protein (E-FABP) were identified by proteomics to be upregulated in HPV-related tumors [22]. TRX is a redox mediator promoting cell survival, e.g., under hypoxic conditions and oxidative stress. It has been linked to treatment resistance. E-FABP was discovered as a lipid carrier in human epidermis, but data indicates that E-FABP plays a role in keratinocyte differentiation and other cellular signaling processes; its role in HNSCC is uncertain.

Expression of the EGFR has been in focus for its potential as a molecular marker and therapeutic target in many malignancies. EGFR is normally expressed in low quantities on the surface of most cells. Overexpression is reported for HNSCC and associated with treatment resistance, aggressive clinical behavior, and poor prognosis. A trend toward better prognosis has been shown for EGFR-negative OPSCC, and HPV-related OPSCC tended to have decreased EGFR expression in the same study [23]. Sequencing data support this finding, since amplification of EGFR and fibro-

blast growth factor receptor 1 (FGFR1) appears to be restricted to HPV-negative HNSCC [24–27]. Currently, replacement of classical chemotherapy by therapeutic EGFR antibodies is being investigated in clinical trials for HPV-related HNSCC (NCT01855451, NCT01302834, NCT01874171).

HPV DNA Integration

Linearization of the viral DNA within the E2 open reading frame abrogates the controlling function of E2 on E6 and E7 protein expression. Thus, viral DNA integration was considered a prerequisite for HPV-driven cancer (fig. 2). Although this classical model is reasonable, in more than 60% of HPV-associated OPSCC only episome-derived DNA was detected by polymerase chain reaction (PCR) [28]. Also, viral oncogene expression has been shown to be independent of viral copy number and DNA integration [29]. This was confirmed by RNA-Seq and whole genome sequencing data showing that 3 possible states of the HPV genome (episomal, integrated, and a hybrid of both forms) were evenly split in HPV16-positive HNSCC [30], indicating overlapping mechanisms in HPV oncoprotein regulation (e.g., by methylation of E2 binding sites (E2BS)).

Although the role of HPV DNA integration may be challenged, chromosomal instability seems to be associated with HPV DNA integration and cancer progression [31]. In an in-vitro study of HPV-transfected keratinocytes, integration sites were detected in various chromosomal locations but also in or near genes encoding growth control proteins [32]. This supports the idea of clonal selection by HPV integration towards more aggressive tumors. Interestingly, HPV DNA integration in the AKR1C3 gene (a hydroxysteroid dehydrogenase involved in the regulation of androgens and estrogens) has been attributed to malignant transformation in a patient with HPV type 6-related juvenile-onset recurrent respiratory papillomatosis [33]. However, in tonsillar cancer, HPV DNA integration was found to be associated with a favorable prognosis [31].

Table 1. Deregulated microRNA expression in human papillomavirus-positive and -negative squamous cell carcinoma of the head and neck

	Mirghani et al., 2016 [40]	Hui et al., 2013 [43]	Gao et al., 2013 [44]	Lajer et al., 2012 [41]	Lajer et al., 2011 [42]
miR-324-5p	XXX	XXX		XXX	
miR-155		XXX	XXX	XXX	
miR-107	XX	XX			
miR-9		XX	XX		
miR-145		XX			XX
miR-99b-3p	XX			XX	
miR-18a-5p	XX		XX		
miR-26b				XX	XX
miR-363				XX	XX
miR-381		X			X
miR-101		X			X

XXX = Common between 3 studies; XX = common between 2 studies; X = common between 2 studies but regulated in opposite direction.

Genetic Alterations: Copy Number Variations and Mutations

Certain genetic alterations are shared in HNSCC independent of HPV, while others are restricted to either HPV-related or HPV-negative HNSCC [34]. Chromosomal amplifications of 3q, 8q, and 20p have been frequently detected in HNSCC, irrespective of HPV status, by comparative genomic hybridization in earlier studies [35] and by sequencing later on [24, 26, 36]. Important genes like PIK3CA, TP63, and SOX2 are located in the 3q26–28 region. HPV-negative HNSCC show frequent loss of chromosome arms 3p and 9p, and amplification of 11q13 [35, 37]. CDKN2A and CCND1 are located on 9p and 11q13, encoding p16^{INK4A} and cyclin D1. Both are involved in Rb signaling and therefore, unsurprisingly, less frequently altered in HPV-related HNSCC. The same applies for TP53 mutations which are found in 60–80% of HPV-negative but not in HPV-related HNSCC. Also, amplifications of EGFR and FGFR1 appear to be restricted to HPV-related HNSCC [24–27]. In contrast, deletion (14%) or mutation (8%) of tumor necrosis factor receptor-associated factor 3 (TRAF3, encoded on 14q32.32), involved in the innate and acquired antiviral immune response, is overrepresented in HPV-related HNSCC [24]. Innate immunity appears to be significant for HPV-related HNSCC, since genes coding for HLA I components are frequently mutated and higher numbers of CD56-positive natural killer cells have been reported for HPV-related OPSCC recently [38].

Currently available data indicates that higher TpC mutation frequencies are found in HPV-positive HNSCC, and CpG transversions in HPV-negative HNSCC, while the overall mutation rate does not differ by HPV status [24]. However, regarding the still small number of genome-wide analyzed samples, general statements should be handled with care. For example, KRAS mutations were found in 5.8% (3/51) of HPV-positive tumors in a comparative genomic analysis of HPV-positive and -negative HNSCC [36], which could not be confirmed in another study [39].

Epigenetic Alteration and miRNAs

So far, microRNA (miRNA) expression analyses according to HPV status have been performed in 5 studies for HNSCC [40–44]. In the most recent study, 15 HPV-negative and 11 HPV-related

OPSCC were analyzed by microarrays targeting 1,719 sequences. 25 differentially expressed miRNAs were detected. Biological importance was in silico predicted for PI3K and Wnt signaling, focal adhesion, and cytoskeleton regulation [40]. However, only 11 differentially expressed miRNAs were reported in more than 1 study, with 2 of them (miR-381 and miR-101) regulated in opposite directions (table 1). It is important to note that different methods have been used and that the number of analyzed miRNA sequences ranges between 96 and 1,719 among the studies.

Even HPV-encoded miRNAs have been identified and experimentally validated. Their putative target genes are within the viral genome itself [45], but reciprocal interactions between viral and host genes and miRNAs can be assumed. Recently, a target site for human miRNAs (miR-875 and miR-3144) was found in the HPV E6 gene. Both miRNAs inhibit E6 oncogene expression, and in HPV16-positive cell lines growth was inhibited and apoptosis promoted by high-level expression of both miRNAs [46].

Modification, e.g., by acetylation, methylation, ubiquitylation, or phosphorylation, alters histone structure and binding to DNA. Therefore, chromosomal regions become more or less accessible for transcription. Interactions of HPV E7 with histone-modifying proteins are known. One consequence is p16^{INK4A} overexpression due to the activity of histone lysine demethylase KDM6B as described above [17]. Further, E7 recruits histone deacetylase HDAC1 and histone demethylase KDM5B to the Toll-like receptor 9 (TLR9) regulatory region and to promoters containing interferon regulatory factor 1 (IRF1) response elements, both involved in immune response, apoptosis regulation, DNA damage, and tumor suppression [47, 48].

In contrast to the classical HPV DNA integration-based idea of HPV E2 deregulation, and in particular in tumors with non-integrated HPV genomes, methylations at E2BS in the upstream regulatory region of E6 and E7 appear to regulate their expression in the presence of E2 [49, 50]. DNA methylation patterns have been published for viral-driven cancers including HPV-associated neoplasms [51, 52]. Based on viral DNA integration and the E2BS methylation status, distinct subgroups of HPV-associated SCC causally linked with viral oncogene expression have been identified

[28, 49, 50]. Interestingly, the clinical outcome of HNSCC patients, including HPV-related OPSCC, could be predicted depending on differentially methylated promoters of 5 host genes (ALDH1A2, OSR2, IRX4, GRIA4, and GATA4) [51, 53].

HPV Diagnostics, Patient Survival, and Treatment

HPV diagnosis relies on HPV DNA detection and proof of its oncogenic activity. PCR-based methods are advantageous compared to in-situ hybridization with regard to time, cost, and sensitivity. HPV-typing can be performed using amplified DNA for hybridization to type specific probes or for type determination by sequencing. Additional p16^{INK4A} immunostaining or HPV mRNA detection can compensate the lower specificity of HPV DNA PCR. Although HPV mRNA detection is considered the ‘gold standard’ of HPV diagnosis, mRNA expression is not necessarily equal to protein expression or activity. In contrast, cellular p16^{INK4A} overexpression has been directly linked to viral oncogenic activity, and p16^{INK4A} overexpression is rare in other cancers. Although p16^{INK4A} expression can also be detected in tumor-free tonsils [12], p16 immunostaining has become a reliable surrogate for HPV-driven cancers, especially for high-risk HPV-induced HNSCC and OPSCC [11, 15, 54, 55] and has recently been introduced into TNM staging [56]. HPV serology as an alternate biomarker also seems to be relevant to the etiology and natural history of HPV16-related OPSCC. HPV16 E6 antibodies were found to be strongly associated with HPV16-related OPSCC in a large case control study [57], but further studies are needed in this field.

It is accepted that HPV is a risk factor and indicator of improved survival in HNSCC patients. Nevertheless, in patients with advanced non-resectable tumors, HPV status had a positive impact on progression-free survival but not on overall survival [58]. This indicates that in subsets of HPV-related HNSCC, additional factors may overcome the influence of HPV, and stratification strategies have to be refined.

Treatment is either with a focus on radiotherapy or upfront surgery, depending largely on regional/national preference rather than on evidence-based criteria, with no clear advantage of one over the other. Currently, de-escalation is being evaluated in several clinical trials [59]. For example, in ECOG3311, an intermediate risk group of OPSCC patients is randomized to receive a reduced or standard radiation dose (50 vs. 60 Gy) after transoral tumor resection (NCT01898494). In the ADEPT trial, patients with complete tumor resection but with extracapsular spread are randomized to receive platinum-based radiochemotherapy versus radiation only (NCT01687413). Both studies are ongoing.

Recent molecular findings suggest novel treatment options, especially in HPV-related HNSCC, using demethylating agents or PI3K and immune checkpoint pathway modulation. However, the identification of suitable patient subgroups for targeted treatment is still under investigation.

Outlook and Conclusion

In contrast to cancers associated with other risk factors, HPV-related HNSCC are driven by viral oncoprotein activity and have individual profiles regarding protein expression, and genetic and epigenetic alterations. Patients with HPV-related HNSCC have improved survival compared to those with HPV-negative tumors, but no specific or toxicity-reduced treatment modalities have been established for this tumor entity so far. Although the still incomplete and partially inconsistent data in this field needs further study, certain features of HPV-related HNSCC have the strong potential to be implemented in future diagnostic and therapeutic concepts.

Disclosure Statement

The authors declare no conflict of interest.

References

- Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuutinen J: Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. *Int J Oral Surg* 1983;12:418–424.
- Andl T, Kahn T, Pfuhl A, Nicola T, Erber R, Conradt C, Klein W, Helbig M, Dietz A, Weidauer H, Bosch FX: Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. *Cancer Res* 1998;58:5–13.
- Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D: Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92:709–720.
- Klussmann JP, Weissenborn SJ, Wieland U, Dries V, Kolligs J, Jungehuelsing M, Eckel HE, Dienes HP, Pfister HJ, Fuchs PG: Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. *Cancer* 2001;92:2875–2884.
- World Health Organization: WHO Report on the Global Tobacco Epidemic, 2015. www.who.int/tobacco/global_report/2015/en/.
- Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML: Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29:4294–4301.
- Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsague X, Laporte L, Bosch FX, de Sanjose S, Trottier H: HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. *Lancet Oncol* 2014;15:1319–1331.
- Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C: Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. *J Clin Oncol* 2015;33:3235–3242.
- Castellsague X, Alemany L, Quer M, Halc G, Quiros B, Tous S, Clavero O, Alos L, Biegner T, Szafarowski T, Alejo M, Holzinger D, Cadena E, Claros E, Hall G, Laco J, Poljak M, Benevolo M, Kasamatsu E, Mehanna H, Ndiaye C, Guimera N, Lloveras B, Leon X, Ruiz-Cabezas JC, Alvarado-Cabrero I, Kang CS, Oh JK, Garcia-Rojo M, Iljazovic E, Ajayi OF, Duarte F, Nessa A, Tinoco L, Duran-Padilla MA, Pirog EC, Viarheichyk H, Morales H, Costes V, Felix A, Germar MJ, Mena M, Ruacan A, Jain A, Mehrotra R, Goodman MT, Lombardi LE, Ferrera A, Malami S, Albanesi EI, Dabed P, Molina C, Lopez-Revilla R, Mandys V, Gonzalez ME, Velasco J, Bravo IG, Quint W, Pawlita M, Munoz N, de Sanjose S, Xavier Bosch F; ICO International HPV in Head and Neck Cancer Study Group: HPV Involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients. *J Natl Cancer Inst* 2016;108:djv403.
- Rettig E, Kiess AP, Fakhry C: The role of sexual behavior in head and neck cancer: implications for prevention and therapy. *Expert Rev Anticancer Ther* 2015;15:35–49.

- 11 Klussmann JP, Gultekin E, Weissenborn SJ, Wieland U, Dries V, Dienes HP, Eckel HE, Pfister HJ, Fuchs PG: Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol* 2003;162:747–753.
- 12 Klingenberg B, Hafkamp HC, Haesevoets A, Manni JJ, Slootweg PJ, Weissenborn SJ, Klussmann JP, Speel EJ: p16 INK4A overexpression is frequently detected in tumour-free tonsil tissue without association with HPV. *Histopathology* 2010;56:957–967.
- 13 Meyer MF, Huebbers CU, Siefer OG, Vent J, Engbert I, Eslick GD, Valter M, Klussmann JP, Preuss SF: Prevalence and risk factors for oral human papillomavirus infection in 129 women screened for cervical HPV infection. *Oral Oncol* 2014;50:27–31.
- 14 Combes JD, Dalstein V, Gheit T, Clifford GM, Tommasino M, Clavel C, Lacau St Guily J, Franceschi S; SPLIT study group: Prevalence of human papillomavirus in tonsil brushings and gargles in cancer-free patients: the SPLIT study. *Oral Oncol* 2017;66:52–57.
- 15 Mooren JJ, Gultekin SE, Straetmans JM, Haesevoets A, Peutz-Kootstra CJ, Huebbers CU, Dienes HP, Wieland U, Ramaekers FC, Kremer B, Speel EJ, Klussmann JP: P16(INK4A) immunostaining is a strong indicator for high-risk-HPV-associated oropharyngeal carcinomas and dysplasias, but is unreliable to predict low-risk-HPV-infection in head and neck papillomas and laryngeal dysplasias. *Int J Cancer* 2014;134:2108–2117.
- 16 Rietbergen MM, Braakhuis BJ, Moukhtari N, Bloemen E, Brink A, Sie D, Ylstra B, Baatenburg de Jong RJ, Snijders PJ, Brakenhoff RH, Leemans CR: No evidence for active human papillomavirus (HPV) in fields surrounding HPV-positive oropharyngeal tumors. *J Oral Pathol Med* 2014;43:137–142.
- 17 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;110:16175–16180.
- 18 Liu X, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R: HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proc Natl Acad Sci U S A* 2009;106:18780–18785.
- 19 Hafkamp HC, Mooren JJ, Claessen SM, Klingenberg B, Voogd AC, Bot FJ, Klussmann JP, Hopman AH, Manni JJ, Kremer B, Ramaekers FC, Speel EJ: P21 Cip1/WAF1 expression is strongly associated with HPV-positive tonsillar carcinoma and a favorable prognosis. *Mod Pathol* 2009;22:686–698.
- 20 Fan X, Chen JJ: Role of Cdk1 in DNA damage-induced G1 checkpoint abrogation by the human papillomavirus E7 oncogene. *Cell Cycle* 2014;13:3249–3259.
- 21 Preuss SF, Weinell A, Molitor M, Stenner M, Semrau R, Dreber U, Weissenborn SJ, Speel EJ, Wittekindt C, Guntinas-Lichius O, Hoffmann TK, Eslick GD, Klussmann JP: Nuclear survivin expression is associated with HPV-independent carcinogenesis and is an indicator of poor prognosis in oropharyngeal cancer. *Br J Cancer* 2008;98:627–632.
- 22 Melle C, Ernst G, Winkler R, Schimmel B, Klussmann JP, Wittekindt C, Guntinas-Lichius O, von Eggeling F: Proteomic analysis of human papillomavirus-related oral squamous cell carcinoma: identification of thioredoxin and epidermal-fatty acid binding protein as up-regulated protein markers in microdissected tumor tissue. *Proteomics* 2009;9:2193–2201.
- 23 Reimers N, Kasper HU, Weissenborn SJ, Stutzer H, Preuss SF, Hoffmann TK, Speel EJ, Dienes HP, Pfister HJ, Guntinas-Lichius O, Klussmann JP: Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. *Int J Cancer* 2007;120:1731–1738.
- 24 Cancer Genome Atlas Network: Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* 2015;517:576–582.
- 25 Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR: The mutational landscape of head and neck squamous cell carcinoma. *Science* 2011;333:1157–1160.
- 26 Lechner M, Frampton GM, Fenton T, Feber A, Palmer G, Jay A, Pillay N, Forster M, Cronin MT, Lipson D, Miller VA, Brennan TA, Henderson S, Vaz F, O'Flynn P, Kalavrezos N, Yelensky R, Beck S, Stephens PJ, Bosshoff C: Targeted next-generation sequencing of head and neck squamous cell carcinoma identifies novel genetic alterations in HPV+ and HPV- tumors. *Genome Med* 2013;5:49.
- 27 Agrawal N, Frederick MJ, Pickering CR, Bettgeowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs R, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN: Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* 2011;333:1154–1157.
- 28 Olthof NC, Speel EJ, Kolligs J, Haesevoets A, Henfling M, Ramaekers FC, Preuss SF, Dreber U, Wieland U, Silling S, Lam WL, Vucic EA, Kremer B, Klussmann JP, Huebbers CU: Comprehensive analysis of HPV16 integration in OSCC reveals no significant impact of physical status on viral oncogene and virally disrupted human gene expression. *PLoS One* 2014;9:e88718.
- 29 Olthof NC, Huebbers CU, Kolligs J, Henfling M, Ramaekers FC, Cornet I, van Lent-Albrechts JA, Stegmann AP, Silling S, Wieland U, Carey TE, Walline HM, Gollin SM, Hoffmann TK, de Winter J, Kremer B, Klussmann JP, Speel EJ: Viral load, gene expression and mapping of viral integration sites in HPV16-associated HNSCC cell lines. *Int J Cancer* 2015;136:E207–218.
- 30 Nulton TJ, Olex AL, Dozmorov M, Morgan IM, Windle B: Analysis of the cancer genome atlas sequencing data reveals novel properties of the human papillomavirus 16 genome in head and neck squamous cell carcinoma. *Oncotarget* 2017;8:17684–17699.
- 31 Mooren JJ, Kremer B, Claessen SM, Voogd AC, Bot FJ, Peter Klussmann J, Huebbers CU, Hopman AH, Ramaekers FC, Speel EJ: Chromosome stability in tonsillar squamous cell carcinoma is associated with HPV16 integration and indicates a favorable prognosis. *Int J Cancer* 2013;132:1781–1789.
- 32 Lace MJ, Anson JR, Klussmann JP, Wang DH, Smith EM, Haugen TH, Turek LP: Human papillomavirus type 16 (HPV-16) genomes integrated in head and neck cancers and in HPV-16-immortalized human keratinocyte clones express chimeric virus-cell mRNAs similar to those found in cervical cancers. *J Virol* 2011;85:1645–1654.
- 33 Huebbers CU, Preuss SF, Kolligs J, Vent J, Stenner M, Wieland U, Silling S, Dreber U, Speel EJ, Klussmann JP: Integration of HPV6 and downregulation of AKR1C3 expression mark malignant transformation in a patient with juvenile-onset laryngeal papillomatosis. *PLoS One* 2013;8:e57207.
- 34 Wagner S, Wurdemann N, Hubbers C, Reuschenbach M, Prigge ES, Wichmann G, Hess J, Dietz A, Durst M, Tinhofer I, von Knebel-Doberitz M, Wittekindt C, Klussmann JP: (HPV-associated head and neck cancer: mutational signature and genomic aberrations). *HNO* 2015;63:758–767.
- 35 Klussmann JP, Mooren JJ, Lehnen M, Claessen SM, Stenner M, Huebbers CU, Weissenborn SJ, Wedemeyer I, Preuss SF, Straetmans JM, Manni JJ, Hopman AH, Speel EJ: Genetic signatures of HPV-related and unrelated oropharyngeal carcinoma and their prognostic implications. *Clin Cancer Res* 2009;15:1779–1786.
- 36 Seiwert TY, Zuo Z, Keck MK, Khattri A, Pedamallu CS, Stricker T, Brown C, Pugh TJ, Stojanov P, Cho J, Lawrence MS, Getz G, Bragelmann J, DeBoer R, Weichselbaum RR, Langerman A, Portugal L, Blair E, Stenson K, Lingen MW, Cohen EE, Vokes EE, White KP, Hammerman PS: Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. *Clin Cancer Res* 2015;21:632–641.
- 37 Smeets SJ, Braakhuis BJ, Abbas S, Snijders PJ, Ylstra B, van de Wiel MA, Meijer GA, Leemans CR, Brakenhoff RH: Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. *Oncogene* 2006;25:2558–2564.
- 38 Wagner S, Wittekindt C, Reuschenbach M, Hennig B, Thevarajah M, Wurdemann N, Prigge ES, von Knebel Doeberitz M, Dreyer T, Gattenlohner S, Klussmann JP: CD56-positive lymphocyte infiltration in relation to human papillomavirus association and prognostic significance in oropharyngeal squamous cell carcinoma. *Int J Cancer* 2016;138:2263–2273.
- 39 Prigge ES, Urban K, Stiegler S, Muller M, Kloor M, Mai S, Ottstadt M, Lohr F, Wenz F, Wagner S, Wittekindt C, Klussmann JP, Hampl M, von Knebel Doeberitz M, Reuschenbach M: No evidence of oncogenic KRAS mutations in squamous cell carcinomas of the anogenital tract and head and neck region independent of human papillomavirus and p16(INK4a) status. *Hum Pathol* 2014;45:2347–2354.
- 40 Mirghani H, Ugolin N, Ory C, Goisard M, Lefevre M, Baulande S, Hofman P, Guily JL, Chevillard S, Lacave R: Comparative analysis of micro-RNAs in human papillomavirus-positive versus -negative oropharyngeal cancers. *Head Neck* 2016;38:1634–1642.
- 41 Lajer CB, Garnaes E, Friis-Hansen L, Norrild B, Therkildsen MH, Glud M, Rossing M, Lajer H, Svane D, Skotte L, Specht L, Buchwald C, Nielsen FC: The role of miRNAs in human papilloma virus (HPV)-associated cancers: bridging between HPV-related head and neck cancer and cervical cancer. *Br J Cancer* 2012;106:1526–1534.
- 42 Lajer CB, Nielsen FC, Friis-Hansen L, Norrild B, Borup R, Garnaes E, Rossing M, Specht L, Therkildsen MH, Nauntofte B, Dabelsteen S, von Buchwald C: Different miRNA signatures of oral and pharyngeal squamous cell carcinomas: a prospective translational study. *Br J Cancer* 2011;104:830–840.
- 43 Hui AB, Lin A, Xu W, Waldron L, Perez-Ordóñez B, Weinreb I, Shi W, Bruce J, Huang SH, O'Sullivan B, Waldron J, Gullane P, Irish JC, Chan K, Liu FF: Potentially prognostic miRNAs in HPV-associated oropharyngeal carcinoma. *Clin Cancer Res* 2013;19:2154–2162.
- 44 Gao G, Gay HA, Chernock RD, Zhang TR, Luo J, Thorstad WL, Lewis JS Jr, Wang X: A microRNA expression signature for the prognosis of oropharyngeal squamous cell carcinoma. *Cancer* 2013;119:72–80.
- 45 Qian K, Pietila T, Ronty M, Michon F, Frilander MJ, Ritari J, Tarkkanen J, Paulin L, Auvinen P, Auvinen E: Identification and validation of human papillomavirus encoded microRNAs. *PLoS One* 2013;8:e70202.
- 46 Lin L, Cai Q, Zhang X, Zhang H, Zhong Y, Xu C, Li Y: Two less common human microRNAs miR-875 and miR-3144 target a conserved site of E6 oncogene in most high-risk human papillomavirus subtypes. *Protein Cell* 2015;6:575–588.

- 47 Hasan UA, Zannetti C, Parroche P, Goutagny N, Malfoy M, Roblot G, Carreira C, Hussain I, Muller M, Taylor-Papadimitriou J, Picard D, Sylla BS, Trinchieri G, Medzhitov R, Tommasino M: The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the Toll-like receptor 9 promoter. *J Exp Med* 2013;210:1369–1387.
- 48 Park JS, Kim EJ, Kwon HJ, Hwang ES, Namkoong SE, Um SJ: Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. *J Biol Chem* 2000;275:6764–6769.
- 49 Chaiwongkot A, Vinokurova S, Pientong C, Ekalaksananan T, Kongyingyoes B, Kleeckaow P, Chumworathayi B, Patarapadungkit N, Reuschenbach M, von Knebel Doeberitz M: Differential methylation of E2 binding sites in episomal and integrated HPV 16 genomes in preinvasive and invasive cervical lesions. *Int J Cancer* 2013;132:2087–2094.
- 50 Reuschenbach M, Huebbers CU, Prigge ES, Bermejo JL, Kalteis MS, Preuss SF, Seuthe IM, Kolligs J, Speel EJ, Olthof N, Kremer B, Wagner S, Klussmann JP, Vinokurova S, von Knebel Doeberitz M: Methylation status of HPV16 E2-binding sites classifies subtypes of HPV-associated oropharyngeal cancers. *Cancer* 2015;121:1966–1976.
- 51 Kostareli E, Holzinger D, Bogatyrova O, Hielscher T, Wichmann G, Keck M, Lahrmann B, Grabe N, Flechtenmacher C, Schmidt CR, Seiwert T, Dyckhoff G, Dietz A, Hofer D, Pawlita M, Benner A, Bosch FX, Plinkert P, Plass C, Weichenhan D, Hess J: HPV-related methylation signature predicts survival in oropharyngeal squamous cell carcinomas. *J Clin Invest* 2013;123:2488–2501.
- 52 Minarovits J, Demcsak A, Banati F, Niller HH: Epigenetic dysregulation in virus-associated neoplasms. *Adv Exp Med Biol* 2016;879:71–90.
- 53 Kostareli E, Hielscher T, Zucknick M, Baboci L, Wichmann G, Holzinger D, Mucke O, Pawlita M, Del Misstro A, Boscolo-Rizzo P, Da Mosto MC, Tirelli G, Plinkert P, Dietz A, Plass C, Weichenhan D, Hess J: Gene promoter methylation signature predicts survival of head and neck squamous cell carcinoma patients. *Epigenetics* 2016;11:61–73.
- 54 Prigge ES, Arbyn M, von Knebel Doeberitz M, Reuschenbach M: Diagnostic accuracy of p16INK4a immunohistochemistry in oropharyngeal squamous cell carcinomas: a systematic review and meta-analysis. *Int J Cancer* 2017;140:1186–1198.
- 55 Gultekin SE, Senguen B, Klussmann JP, Dienes HP: P16(INK 4a) and Ki-67 expression in human papilloma virus-related head and neck mucosal lesions. *Invest Clin* 2015;56:47–59.
- 56 Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, Jessup JM, Brierley JD, Gaspar LE, Schilsky RL, Balch CM, Winchester DP, Asare EA, Madera M, Gress DM, Meyer LRE: *AJCC Cancer Staging Manual*. New York, NY, Springer, 2017.
- 57 Anantharaman D, Gheit T, Waterboer T, Abedi-Ardekani B, Carreira C, McKay-Chopin S, Gaborieau V, Marron M, Lagiou P, Ahrens W, Holcatova I, Merletti F, Kjaerheim K, Talamini R, Simonato L, Castellsague X, Macfarlane TV, Biggs AM, Thakker N, Znaor A, Thomson P, Canova C, Conway DI, Healy CM, Tommasino M, Pawlita M, Brennan P: Human papillomavirus infections and upper aero-digestive tract cancers: the ARCAGE study. *J Natl Cancer Inst* 2013;105:536–545.
- 58 Semrau R, Duerbaum H, Temming S, Huebbers C, Stenner M, Drebber U, Klussmann JP, Muller RP, Preuss SF: Prognostic impact of human papillomavirus status, survival, and epidermal growth factor receptor expression on survival in patients treated with radiochemotherapy for very advanced nonresectable oropharyngeal cancer. *Head Neck* 2013;35:1339–1344.
- 59 Mirghani H, Amen F, Blanchard P, Moreau F, Guigay J, Hartl DM, Lacau St Guily J: Treatment de-escalation in HPV-positive oropharyngeal carcinoma: ongoing trials, critical issues and perspectives. *Int J Cancer* 2015;136:1494–1503.