ADVANCES IN THE INVOLVEMENT OF HUMAN PAPILLOMA VIRUS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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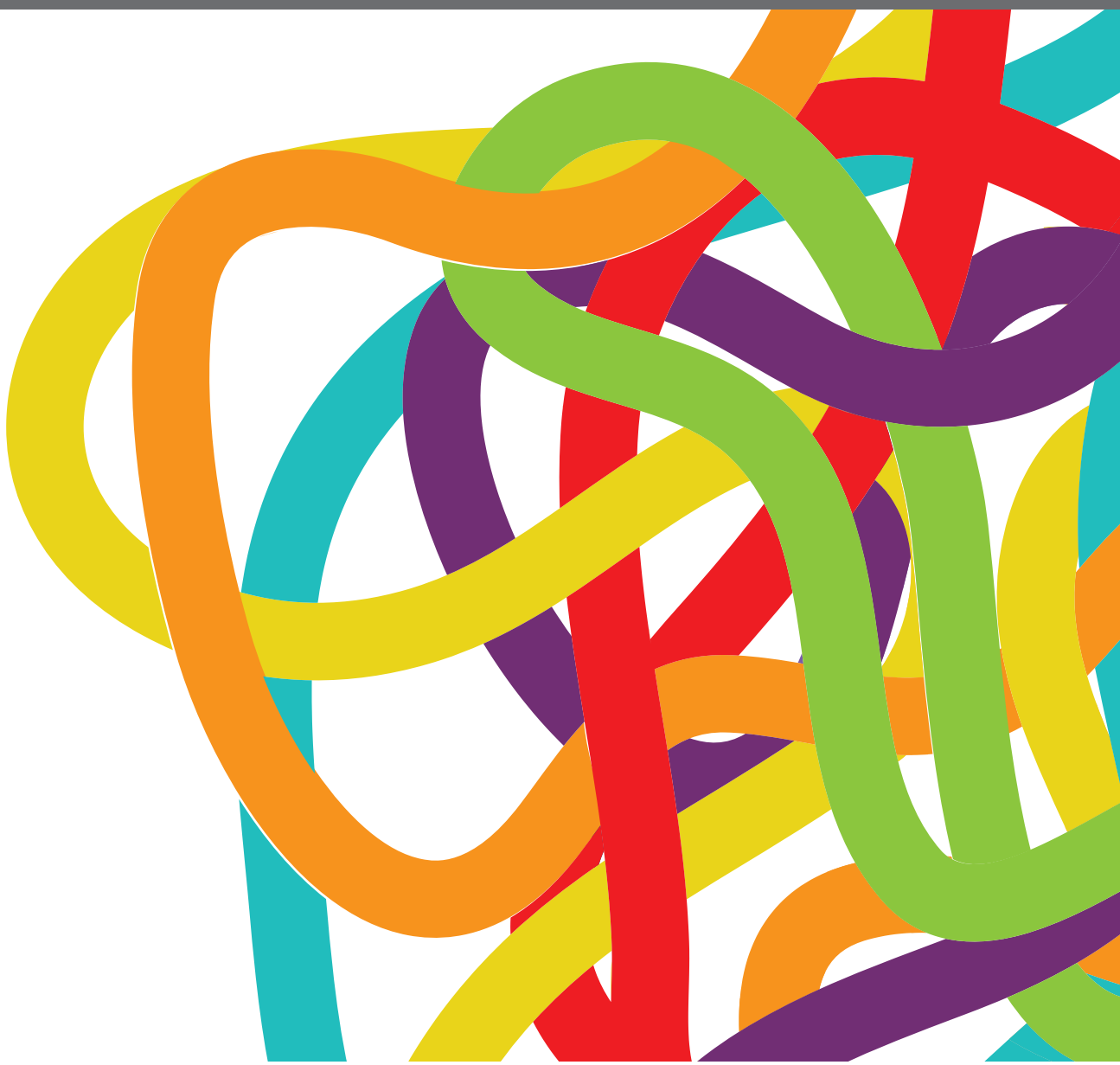

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ADVANCES IN THE INVOLVEMENT OF HUMAN PAPILLOMA VIRUS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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EDITORIAL
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Editorial: Advances in the Involvement of Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma

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Keywords: cancer, head neck, HPV, human papillomavirus, carcinoma, otolaryngology, head neck, surgery

Editorial on the Research Topic

Advances in the Involvement of Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma

BACKGROUND

Head and neck squamous cell carcinomas (HNSCC) are the sixth most common cancers in males and the eighth most common in females worldwide, accounting for over 890,000 new cases in 2017 (1, 2). HNSCCs represent 5.3% of all cancers. From a mortality standpoint, they involve more than 500,000 deaths worldwide, representing 5.3% of all cancer deaths (2). Irrespective to the world region, the most prevalent HNSCCs in 2017 were lip and oral cavity (390,000 new cases), laryngeal (211,000 new cases), pharyngeal (179,000 new cases) and nasopharyngeal (110,000 new cases) carcinomas (2). The main risk factors for HNSCCs are alcohol and tobacco consumption and persistent infection with human papillomavirus (HPV) that is associated with the rising incidence of oropharyngeal squamous cell carcinoma (OSCC) in the United States and Europe (3, 4). The incidence of HPV-induced HNSCC depends on the world region and the method used to assess the presence of HPV (5). The incidence of HPV-induced HNSCC has increased since the 80s (5).

HPV infection is predominantly attributable to subtypes HPV-16 and HPV-18 even if there is geographical heterogeneity between continents (3, 6, 7). Young, nondrinking and nonsmoking individuals with HPV-induced OSCC often have advanced carcinoma, but they have a better prognosis compared with patients with tobacco- and alcohol-induced OSCC (6, 7). The mechanisms underlying the better prognosis of patients with HPV-induced HNSCC remain poorly understood and may involve interactions between HPV antigens and the host-immune system, leading to an improved responses of immune cells against tumor (8). In that way, an increasing number of studies reported significant differences in the composition of tumor-infiltrating immune cells in HPV-positive and HPV-negative HNSCC (8). Most authors recognized that the overexpression of some immune cells may be considered as a significant prognostic factor for HNSCC patients (8). In this Research Topic, several research groups published clinical and experimental studies about the involvement of HPV in the development of OSCC and non-OSCC.2

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IMMUNOLOGICAL FEATURES OF HPV-INDUCED CARCINOMA

The better survival outcomes of patients with HPV-positive OSCC may be explained by immunological differences between HPV-positive and HPV-negative HNSCCs. The HPV infection is associated with an increased CD8+ T tumor cell infiltration and PD-1 expression by CD8+ T cells in both OSCC and non-OSCC, which is associated with better overall survival (OS). The interaction between the tumor environment and CD8+ T cells is mediated through many cytokines, including IFN-g and IL-2, -4, -8, -12 and -17 (9, 10). Recent studies supported interactions between CD8+ and CD4+ T cells in HPV-positive HNSCC (8). Precisely, in tumor environment, CD4+ T cells may be converted into Th17 cells, which could potentiate the cytotoxic effects of CD8+ T cells against HPV-induced tumor cells (8). M1 and M2 macrophages are additional immune cells that may have an important role in HPV-induced carcinogenesis (11). M2 macrophages are involved in the enhancement of immunosuppression through the stimulation of regulatory T cells (Tregs) that induces a favorable environment for tumor growth through the secretion of TGF-b, TNF-a, and IL-10 (8). In HPV-negative HNSCC, an increased proportion of M2 cells is an important factor underlying the improvement of OS (8). The involvement of Foxp3 Tregs in HPV-positive and HPV-negative HNSCC remains uncertain. Indeed, several researches showed that an increase of Tregs in HPV-positive HNSCC environment was associated with improved OS (8, 12), while others reported similar findings in HNSCC but no influence of the HPV status (8, 13). Foxp3 Tregs may inhibit the protumoral effects of inflammatory immune cells, which is a favorable prognostic marker at some tumor sites, whereas in other tumor sites, Treg infiltration may be associated with poor survival outcomes regarding their conventional regulatory function (13). The activation of Tregs may be associated with myeloid derived suppressor cells infiltration, these cells being associated with tumor progression and PD-L1 expression (14).

LIMITATIONS AND HETEROGENEITY STUDIES

The findings of studies investigating the immune cell features of HPV-induced HNSCCs have to be considered with cautious because many limitations. The method used to determine the HPV status may significantly vary from one to another study. In many centers, the detection of HPV infection is made with p16 immunostaining. Some authors used p16 immunostaining to compare data from HPV-positive and HPV-negative HNSCCs, while others used polymerase chain reaction (PCR) or other direct methods of DNA identification (15). In fact, some tumors can be HPV-positive/p16- or HPV-negative/p16+, which can be related to the lack of specificity of p16 in identifying HPV infection. As demonstrated in two studies (15, 16), the use of p16 immunostaining *versus* DNA detection may lead to substantial differences in the study results, which may bias the comparison between studies.

From an epidemiological standpoint, the analysis of study results (e.g. OS, disease-free survival) has to consider inclusion criteria. For example, the survival analysis according to the HPV stratus has to consider the ethnicity, tumor site, tobacco and alcohol histories, and the types of treatment (e.g. surgery, chemoradiation, radiotherapy or immunotherapy) (15); all of these points being known to influence the immune cell infiltrate.

PERSPECTIVES AND CONCLUSION

Epidemiological, clinical, and immunological outcomes of HPV-induced HNSCCs are all points investigated in studies of this Research Topic. Precisely, the following outcomes were studied in the present Research Topic: prevalence data of HPV-induced HNSCC in Syria (16), importance of extranodal extension HPV-positive OSCC (Gupta et al.), tumor microenvironment and immunotherapy in OSCC (Beltz et al.), survival outcomes according to lymph node invasion in oral SCC (Welters et al.), nonsmoking and nondrinking outcomes in HNSCC (Li et al.; Yang et al.), the impact of tonsillectomy in the management of unknown primary HNSCC regarding p16 status (Mulder et al.), and recurrent respiratory papillomatosis risk factors (Podeur et al.). Many grey areas have to be explored in the next few years to better understand the prognosis differences between HPV-positive and HPV-negative HNSCC patients, as well as the influence of some conditions on both immunological and survival features. A promising topic is the study of the influence of gut, laryngopharyngeal and oral microbiota on the immune regulation in tumor environment and its interaction with HPV infection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Global Burden of Disease Cancer Collaboration. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol* (2019) 5:1749–68. doi: 10.1001/jamaoncol.2019.2996
2. Aupérin A. Epidemiology of Head and Neck Cancers: An Update. *Curr Opin Oncol* (2020) 32(3):178–86. doi: 10.1097/CCO.0000000000000629
3. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, 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):709–20. doi: 10.1093/jnci/92.9.709
4. Filleul O, Preillon J, Crompot E, Lechien J, Saussez S. Incidence of Head and Neck Cancers in Belgium: Comparison With World Wide and

French Data. *Bull Cancer* (2011) 98(10):1173–83. doi: 10.1684/bdc.2011.1456
5. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma. *J Clin Oncol* (2015) 33:3235–42. doi: 10.1200/JCO.2015.61.6995
6. O'Rorke MA, Ellison MV, Murray LJ, Moran M, James J, Anderson LA. Human Papillomavirus Related Head and Neck Cancer Survival: A Systematic Review and Meta-Analysis. *Oral Oncol* (2012) 48(12):1191–201. doi: 10.1016/j.oraloncology.2012.06.019
7. Descamps G, Karaca Y, Lechien JR, Kindt N, Decaestecker C, Remmelink M, et al. Classical Risk Factors, But Not HPV Status, Predict Survival After Chemoradiotherapy in Advanced Head and Neck Cancer Patients. *J Cancer Res Clin Oncol* (2016) 142(10):2185–96. doi: 10.1007/s00432-016-2203-7
8. Lechien JR, Seminerio I, Descamps G, Mat Q, Mouawad F, Hans S, et al. Impact of HPV Infection on the Immune System in Oropharyngeal and Non-Oropharyngeal Squamous Cell Carcinoma: A Systematic Review. *Cells* (2019) 8(9):1061. doi: 10.3390/cells8091061
9. Russell S, Angell T, Lechner M, Liebertz DJ, Correa AJ, Sinha UK, et al. Immune Cell Infiltration Patterns and Survival in Head and Neck Squamous Cell Carcinoma. *Head Neck Oncol* (2013) 5(3):24.
10. Partlová SM, Bouček JE, Kloudová KG, Lukešová E, Zábrodský M, Grega M, et al. Distinct Patterns of Intratumoral Immune Cell Infiltrates in Patients With HPV-Associated Compared to Non-Virally Induced Head and Neck Squamous Cell Carcinoma. *Oncoimmunology* (2015) 4(1):e965570. doi: 10.4161/21624011.2014.965570
11. Ou D, Adam J, Garberis I, Blanchard P, Nguyen F, Levy A, et al. Influence of Tumor-Associated Macrophages and HLA Class I Expression According to HPV Status in Head and Neck Cancer Patients Receiving Chemo/Bioradiotherapy. *Radiother Oncol* (2019) 130:89–96. doi: 10.1016/j.radonc.2018.08.013
12. Seminerio I, Descamps G, Dupont S, de Marrez L, Laigle JA, Lechien JR, et al. Infiltration of FoxP3+ Regulatory T Cells is a Strong and Independent Prognostic Factor in Head and Neck Squamous Cell Carcinoma. *Cancers (Basel)* (2019) 11 (2):227. doi: 10.3390/cancers11020227
13. De Meulenaere A, Vermassen T, Aspeslagh S, Zwaenepoel K, Deron P, Duprez F, et al. Prognostic Markers in Oropharyngeal Squamous Cell Carcinoma: Focus on CD70 and Tumour Infiltrating Lymphocytes. *Pathology* (2017) 49(4):397–404. doi: 10.1016/j.pathol.2017.02.002
14. Yu GT, Bu LL, Huang CF, Zhang WF, Chen WJ, Gutkind JS, et al. PD-1 Blockade Attenuates Immunosuppressive Myeloid Cells Due to Inhibition of CD47/Sirpα Axis in HPV Negative Head and Neck Squamous Cell Carcinoma. *Oncotarget* (2015) 6(39):42067–80. doi: 10.18632/oncotarget.5955
15. Nordfors C, Grün N, Tertipis N, Ährlund-Richter A, Haeggblom L, Sivars L, et al. CD8+ and CD4+ Tumour Infiltrating Lymphocytes in Relation to Human Papillomavirus Status and Clinical Outcome in Tonsillar and Base of Tongue Squamous Cell Carcinoma. *Eur J Cancer* (2013) 49(11):2522–30. doi: 10.1016/j.ejca.2013.03.019
16. Punt S, Dronkers EA, Welters MJ, Goedemans R, Koljenović S, Bloemena E, et al. A Beneficial Tumor Microenvironment in Oropharyngeal Squamous Cell Carcinoma Is Characterized by a High T Cell and Low IL-17(+) Cell Frequency. *Cancer Immunol Immunother* (2016) 65(4):393–403. doi: 10.1007/s00262–016–1805-x

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CASE REPORT
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An Occult HPV-Driven Oropharyngeal Squamous Cell Carcinoma Discovered Through a Saliva Test

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Oropharyngeal cancer (OPC) caused by human papillomavirus (HPV) is a rising global concern. Early lesions are small and are often located in difficult to access areas (such as the crypts of the tonsils or base of tongue). Unlike cervical cancer, there is no standard or routine screening program for HPV-driven OPC. HPV DNA from OPC tumors may shed directly into saliva, and this can be used as a biomarker for early diagnosis. In this study, we report the first-ever clinically occult OPC in an asymptomatic patient discovered through a saliva test. This case relied upon serial measurements of HPV-16 DNA in saliva, which fell to undetectable levels following low morbidity, curative treatment.

Keywords: human papillomavirus, oropharyngeal cancer, saliva, screening tools, biomarker

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INTRODUCTION

The incidence of high-risk human papillomavirus (HR-HPV−16,-18,-33) driven oropharyngeal cancer (OPC) is rapidly increasing in developed countries (1–3). HPV-driven OPCs have surpassed cervical cancer as the most common HPV-driven cancer in the USA. The prevalence of HR-HPV has been reported as 3.7% of the USA population, with a bimodal age distribution of incidence (4). It remains unclear why some individuals go on to develop OPC, while others clear the initial HPV infection (5). The strong association between HR-HPV infection and cervical cancer has led to screening programmes in primary healthcare settings, resulting in earlier diagnosis and a reduction in cancer deaths (6). Unlike cervical cancer, no screening test is available for OPC and current HPV vaccines have yet to demonstrate any reduction in future OPC development (7). Here, we report the first ever case of occult OPC detected as a direct result of a theoretical screening test—in this case HPV-16 DNA analysis in salivary oral rinse samples. Our clinical and pathological findings increase our understanding of both the natural history of the disease and the potential for wider screening to identify early stage OPC, facilitating less morbid treatments.

CASES PRESENTATION

An ongoing HPV-16 DNA prevalence study was approved by institutional ethics committees from the University of Queensland; Queensland University of Technology and the Royal Brisbane and Women's Hospital. A total of 665 cancer-free healthy individuals from Queensland Region,

Australia between May 2016 and October 2017 were recruited. All participants gave written informed consent prior to sample collection.

Six hundred and fifty cancer-free healthy individuals with sufficient amount of DNA were tested for oral HPV-16 DNA. Of these 3 have been identified to have persistent oral HPV-16 DNA infection. Following discussion with our ethics team we have approached these three participants and offered them consultation with an Ear, Nose, and Throat (ENT) surgeon. A 63-year-old caucasian male was assessed as part of this consultation process. He had consistently been HPV-16 DNA positive for a period of 36 months, with a steadily rising HPV-16 viral load in his salivary oral rinse samples (**Figure 1A**). He was invited to attend the ENT clinic for assessment and discussion.

He is an ex-smoker, having quit 15 years ago, with a 45 pack year history of smoking. He drinks two standard drinks (2.5 units of alcohol) per day. He is heterosexual, and his social history includes multiple oral sex partners in the past (>5), followed by a long term monogamous relationship. Initial clinical examination of the oropharynx including palpation and white light revealed no significant abnormalities. Both tonsils were irregular due to mucous retention cysts and there was slight tonsillar asymmetry (Left < Right) but no evidence of any malignant lesions. Narrow band imaging (NBI) showed some mild vascular changes at the left glosso-tonsillar sulcus. There were no palpable lymph nodes in the neck. An MRI examination of the oropharynx and neck demonstrated no occult lesions of the tonsils or the base of tongue and no cervical lymphadenopathy.

He was offered continued surveillance, or a biopsy of the area of NBI change with bilateral tonsillectomy. The patient elected for bilateral tonsillectomy and biopsy of the base of tongue with NBI guidance under general anesthetic and informed consent was obtained. The surgical specimens were sent for histology and tissue HPV-16 DNA testing. The patient was discharged from hospital the same day. He had a routine postoperative course with a sore throat for 1 week and recovered fully. An ultrasound scan of his neck was performed 2 months post-surgery which showed no cervical lymphadenopathy. He is currently under routine oncological surveillance. The patient has a very high likelihood of cure with minimal morbidity from single modality treatment.

CLINICAL SPECIMENS' COLLECTION AND PROCESSING

Salivary oral rinse samples of this individual were collected at baseline, 6, 12, 36 month, and 2 weeks after his bilateral tonsillectomy using previously published method (8–10). Briefly, participants were asked to swish and gargle for 1–2 min with 2 × 10 mL volumes of 0.9% saline, prior to expectorating the rinse sample into a 50 mL falcon tube. Tissue biopsies from the tonsil and base of tongue were obtained after surgical resection. All samples were immediately frozen on dry ice upon collection and transported back to the laboratory for subsequent processing.

HPV-16 DNA QPCR ANALYSIS

Total DNA was extracted from salivary oral rinse and tonsillar tissue samples using the QIAmp DNA Mini Kit (Qiagen, Germantown, MD, USA) as per manufacturer's protocol. For detection of HPV-16 genotyping, the qPCR assay targeting the opening reading frame (ORF) region of HPV16 E6/7 was carried out with the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (11, 12). For quantification of HPV-16 DNA viral copies in salivary oral rinse and tissue samples, a standard calibration curve was generated using qPCR by plotting threshold cycle (Ct values) against the logarithm of the copy number of 8-fold serially diluted (1×10^1-1×10^8 copies) of pHPV-16 plasmid DNA [American Type Culture Collection (ATCC)® 45113™].

IMMUNOHISTOCHEMISTRY

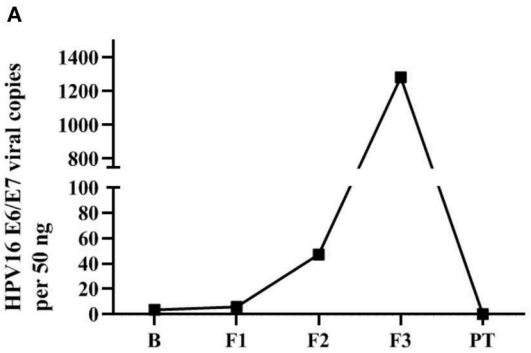
H&E (Haemotoxylin and Eosin stains) staining on formalin-fixed paraffin-embedded (FFPE) slide was performed to investigate the cellular and tissue structure/morphology. HPV status was evaluated using CINtec® p16INK4a Histology Kit (E6H4 clone) (Roche MTM Laboratories, Heidelberg, Germany) according to manufacturer's instructions. p16INK4a was considered positive by two independent pathologists when there was a strong, diffuse nuclear and cytoplasmic staining pattern in the majority (>70%) of tumor cells.

SALIVARY HPV-16 DNA AS A BIOMARKER-BASED TOOL FOR HPV-DRIVEN OPC SCREENING

Salivary oral rinse samples from this individual had been collected at baseline, 6, 12, and 36 month follow-up as well as 2 weeks after his bilateral tonsillectomy. HPV-16 DNA genotyping and viral loads in all samples were analyzed using an in-house developed qPCR assay. HPV-16 DNA viral load in saliva increased exponentially across the 36 month follow-up period (from 3.43 to 1,281.69 copies/50 ng) and subsequently declined to undetectable levels post-tonsillectomy (**Figure 1A**).

HISTOLOGICALLY CONFIRMED DIAGNOSIS OF AN OCCULT P16INK4A POSITIVE OPC

This individual was diagnosed as having a 2 mm squamous cell carcinoma (T1N0M0) in the left tonsil (**Figure 1B**) using Haemotoxylin and Eosin (H&E) staining. He had only foci of stromal invasion with a depth of <1 mm. The remainder of the left tonsil showed follicular lymphoid hyperplasia. Further, HPV-16 DNA was only positive in left tonsillar tissue (**Figure 1C**). Immunohistochemistry (IHC) staining for p16INK4a demonstrated diffuse and strong staining in more than 70% of tumor cells (**Figure 1D**). However, the non-affected remainder of the left tonsil as well as the right tonsil were negative for p16INK4a with usual mosaic pattern of staining. The excision



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Tissue Location	HPV-16 viral copies per 50 ng
Left Tonsil	3277.3
Right Tonsil	Negative
Base of Tongue	Negative

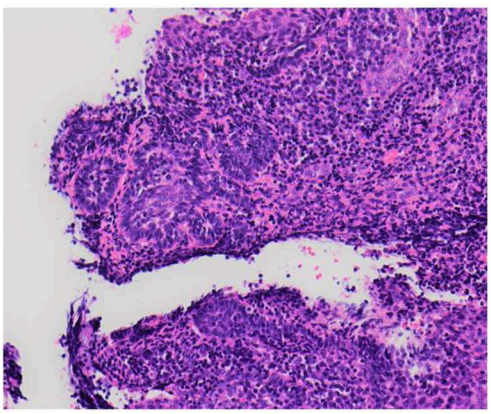

FIGURE 1 | Occult oropharyngeal microcarcinoma detected based on a screening test through the serial measurements of salivary HPV-16 DNA. **(A)** HPV-16 DNA viral load in salivary oral rinse samples over time. B (Baseline); F1 (6 month follow-up); F2 (12 month follow-up); F3 (36 month follow-up); PT (2-week post-tonsillectomy). **(B)** Sections of the left tonsillar tissue found a 2 mm non-keratinising squamous cell carcinoma, with focal stromal invasion <1 mm, excised with clear margins. The remainder of the left tonsil showed follicular lymphoid hyperplasia. Hematoxylin-eosin (H&E x200) **(C)** HPV-16 DNA was only positive in left tonsillar tissue. **(D)** p16INK4a immunohistochemistry staining (IHC) x20: Diffuse positive brown staining for p16INK4a in tumor region comparing non-affected area in the left tonsil.

margins of the left tonsillar malignancy were widely clear. No atypia or malignancy could be identified in the right tonsil and bilateral tongue base specimens all of which were negative for HPV-16 DNA.

DISCUSSION

Long-term persistence of HPV-16 infection is likely to be a prerequisite for the development of malignancy (13, 14). Women with persistent HPV-16 infection in the cervix for <1 year have a 40% risk of developing cervical intraepithelial neoplasia grade 2 or more within 3 years (13). Indeed, the natural history of HPV in the oropharynx from initial infection to carcinogenesis is not known with many questions remaining unanswered. Several studies have evaluated the prevalence of HPV-16 DNA in saliva (15–18) without clinical assessment of positive individuals. Studies aimed at clinical assessment of those with persistence for premalignancy or microscopic carcinoma have failed to detect significant abnormalities (19). This has led to the assertion that screening for early occult or premalignant oropharyngeal lesions is not feasible. Here, we report the first ever histologically confirmed diagnosis of an asymptomatic occult OPC (T1N0M0) discovered by a theoretical screening test through the serial measurements of HPV-16 DNA in salivary oral rinse samples.

The impact of the pattern of salivary HPV persistence including changes in the absolute HPV viral DNA copies over time has never been investigated. The pattern of salivary HPV-DNA detection in this case demonstrates an exponential upward trend with the titer at first sample being 3.43 copies per 50 ng and the final titer before surgery of 1281.7 viral copies per 50 ng. This may represent progression of the lesion with subsequent shedding of increasing levels of HPV-16 DNA into the saliva. In future cases the presence of this pattern should be evaluated, as it may provide a critical marker for the progression of disease and hence a signal for intervention; indeed the pattern of viral copies in serial measurement may have more importance than the persistence itself.

This case also has important implications with regards to the natural history of the disease. The left tonsil was strongly positive for HPV 16 DNA outside the region of malignancy and as anticipated was p16INK4a positive only within carcinoma. This implies that the malignancy is likely to have developed in a wider field of HPV infection with only a component undergoing

malignant change. The existence of a precursor lesion to OPC has long been doubted and is cited as one of the obstacles to OPC screening (16). This case demonstrates that very early lesions can be found in asymptomatic individual, and that they can potentially be eradicated with minimal morbidity.

The quest for a sensitive and specific screening test for HPV-driven OPC is of great importance. The uptake of HPV immunization in developed countries is variable and the developing world remains largely unimmunised. As sexual habits change in the developing world (20, 21) there is likely to be the same rapid expansion in this disease that we have witnessed in the United States and Europe and global burden will continue to rise. As the first singular case, this report does not act as direct evidence of the value of screening in a general population, however, it demonstrates a possible salivary screening test and pathway for the detection of microscopic OPC. It demonstrates that a screened individual can receive significantly less morbid treatment than would be required for the standard presentation at a more advanced stage. This report and previous studies (8, 11, 12, 22), support the value of a salivary oral rinse test as a potential screening tool. Unlike previously published work, our study is the first to demonstrate that continuous monitoring of HPV-16 DNA in salivary oral rinse samples can detect occult OPC.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

This study was approved by institutional ethics committees from the University of Queensland (UQ) [HREC No: 2014000679 and 2014000862]; Queensland University of Technology [HREC No: 1400000617 and 1400000641]; and the Royal Brisbane and Women's Hospital (RBWH) [HREC/16/QRBW/447]. Written informed consent was obtained from this participant for publication of this case report.

AUTHOR CONTRIBUTIONS

All authors have read and agree to the published version of the manuscript. KT and CP: conceptualization. All authors: methodology, validation, formal analysis, data curation, investigation, and writing—review and editing. KT, SV, and CP: writing—original draft preparation. CP: funding acquisition.

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REFERENCES

1. Hong AM, Grulich AE, Jones D, Lee CS, Garland SM, Dobbins TA, et al. Squamous cell carcinoma of the oropharynx in Australian males induced by human papillomavirus vaccine targets. *Vaccine*. (2010) 28:3269–72. doi: 10.1016/j.vaccine.2010.02.098
2. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. (2011) 29:4294–301. doi: 10.1200/JCO.2011.36.4596
3. Habbous S, Chu KP, Lau H, Schorr M, Belayneh M, Ha MN, et al. Human papillomavirus in oropharyngeal cancer in Canada: analysis of 5 comprehensive cancer centres using multiple imputation. *CMAJ*. (2017) 189:E1030–40. doi: 10.1503/cmaj.161379
4. Gillison ML, Broutian T, Pickard RKL, Tong Z-Y, Xiao W, Kahle L, et al. Prevalence of oral HPV Infection in the United States, 2009-2010. *JAMA*. (2012) 307:693–703. doi: 10.1001/jama.2012.101
5. Gravitt PE. The known unknowns of HPV natural history. *J Clin Invest*. (2011) 121:4593–9. doi: 10.1172/JCI57149
6. Bosch FX, Broker TR, Forman D, Moscicki AB, Gillison ML, Doorbar J, et al. Comprehensive control of human papillomavirus infections and related diseases. *Vaccine*. (2013) 31(Suppl. 7):H1–31. doi: 10.1016/j.vaccine.2013.10.003
7. Kreimer AR. Prospects for prevention of HPV-driven oropharynx cancer. *Oral Oncol*. (2014) 50:555–9. doi: 10.1016/j.oraloncology.2013.06.007
8. Rosenthal M, Huang B, Katabi N, Migliacci J, Bryant R, Kaplan S, et al. Detection of HPV related oropharyngeal cancer in oral rinse specimens. *Oncotarget*. (2017) 8:109393–401. doi: 10.18632/oncotarget.22682
9. Sun CX, Bennett N, Tran P, Tang KD, Lim Y, Frazer I, et al. A pilot study into the association between oral health status and human papillomavirus-16 infection. *Diagnostics*. (2017) 7:11. doi: 10.3390/diagnostics7010011
10. Tang KD, Menezes L, Baeten K, Walsh LJ, Whitfield BCS, Batstone MD, et al. Oral HPV16 prevalence in oral potentially malignant disorders and oral cavity cancers. *Biomolecules*. (2020) 10:223. doi: 10.3390/biom10020223
11. Tang KD, Baeten K, Kenny L, Frazer IH, Scheper G, Punyadeera C. Unlocking the potential of saliva-based test to detect HPV-16-driven oropharyngeal cancer. *Cancers*. (2019) 11:473. doi: 10.3390/cancers11040473
12. Tang KD, Kenny L, Frazer IH, Punyadeera C. High-risk human papillomavirus detection in oropharyngeal cancers: comparison of saliva sampling methods. *Head Neck*. (2019) 41:1484–9. doi: 10.1002/hed.25578
13. Castle PE, Rodríguez AC, Burk RD, Herrero R, Wacholder S, Alfaro M, et al. Short term persistence of human papillomavirus and risk of cervical precancer and cancer: population based cohort study. *BMJ*. (2009) 339:b2569. doi: 10.1136/bmj.b2569
14. Bodily J, Laimins LA. Persistence of human papillomavirus infection: keys to malignant progression. *Trends Microbiol*. (2011) 19:33–9. doi: 10.1016/j.tim.2010.10.002
15. Beachler DC, Sugar EA, Margolick JB, Weber KM, Strickler HD, Wiley DJ, et al. Risk factors for acquisition and clearance of oral human papillomavirus infection among HIV-infected and HIV-uninfected adults. *Am J Epidemiol*. (2015) 181:40–53. doi: 10.1093/aje/kwu247
16. Tam S, Fu S, Xu L, Krause KJ, Lairson DR, Miao H, et al. The epidemiology of oral human papillomavirus infection in healthy populations: a systematic review and meta-analysis. *Oral Oncol*. (2018) 82:91–9. doi: 10.1016/j.oraloncology.2018.04.005
17. D'Souza G, McNeel TS, Fakhry C. Understanding personal risk of oropharyngeal cancer: risk-groups for oncogenic oral

HPV infection and oropharyngeal cancer. *Ann Oncol*. (2017) 28:3065–9. doi: 10.1093/annonc/mdx535
18. Antonsson A, Cornford M, Perry S, Davis M, Dunne MP, Whiteman DC. Prevalence and risk factors for oral HPV infection in young Australians. *PLoS ONE*. (2014) 9:e91761. doi: 10.1371/journal.pone.0091761
19. Fakhry C, Rosenthal BT, Clark DP, Gillison ML. Associations between oral HPV16 infection and cytopathology: evaluation of an oropharyngeal "pap-test equivalent" in high-risk populations. *Cancer Prev Res*. (2011) 4:1378–84. doi: 10.1158/1940-6207.CAPR-11-0284
20. Yingying H, Smith K, Suiming P. Changes and correlates in multiple sexual partnerships among Chinese adult women–population-based surveys in 2000 and 2006. *AIDS Care*. (2011) 23(Suppl. 1):96–104. doi: 10.1080/09540121.2010.516350
21. Shisana O, Rehle T, Simbayi LC, Zuma K, Jooste S, Zungu N, et al. *South African National HIV Prevalence, Incidence and Behaviour Survey, 2012*. Cape Town: HSRC Press (2015).
22. Chai RC, Lim Y, Frazer IH, Wan Y, Perry C, Jones L, et al. A pilot study to compare the detection of HPV-16 biomarkers in salivary oral rinses with tumour p16(INK4a) expression in head and neck squamous cell carcinoma patients. *BMC Cancer*. (2016) 16:178. doi: 10.1186/s12885-016-2217-1

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REVIEW
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Significance of Extranodal Extension in Surgically Treated HPV-Positive Oropharyngeal Carcinomas

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Squamous cell carcinomas of the head and neck are the subject of numerous current studies, especially in view of the increasing incidence of tumors induced by human papillomavirus (HPV) and the latest changes to the TNM classification of oropharyngeal squamous cell carcinoma (OPSCC). In addition to HPV status, the presence of extranodal extension of lymph node metastases represents an important risk and prognostic factor, which has now been integrated into the staging algorithm of the eighth edition of TNM classification for HPV-negative OPSCC. In the past numerous studies had shown a lack of prognostic significance of extranodal extension in HPV-associated tumors. However, extranodal extension–as a possible risk factor even in HPV-positive OPSCC–remains an important subject of current studies, which are now particularly characterized by high numbers of cases. In this paper, diagnostic methods and the prognostic significance of extranodal extension in surgically treated HPV-positive OPSCC are presented and discussed based on relevant literature, and the results of current publications are summarized. Further development of diagnostic criteria and procedures as well as international standardization of clinical diagnostics of extranodal extension should be encouraged. Several studies demonstrate that extranodal extension results in worse survival outcomes even in HPV-positive tumors, in contrast to results of previous studies. Consequently, whether the prognostic significance of extranodal extension is not actually relevant to outcome and the staging algorithm of HPV-positive OPSCC should be questioned and further analyzed.

Keywords: extranodal extension, TNM classification, human papilloma virus, oropharyngeal carcinoma, HPV, OPSCC

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INTRODUCTION

The role of HPV in OPSCC has gained a great deal of attention in recent years. In addition to its causative role, HPV infection also proved to have a clear prognostic value (1). With the introduction of the eighth edition of the TNM classification (2017) a distinction is being made for the first time between HPV-positive and HPV-negative squamous cell carcinomas by the use of p16 immunohistochemistry (p16 IHC) as part of the staging of oropharyngeal squamous cell carcinoma (OPSCC). Furthermore, the prognostic influence of extranodal extension (ENE) of lymph node metastases for HPV-negative OPSCC was integrated into the staging algorithm. The prognostic influence of ENE

has been analyzed in several studies and it was recognized as an essential prognostic factor, which should facilitate an even more accurate estimate of the risk of regional disease recurrences or distant metastases (1, 2). In the clinical staging of lymph node metastasis, defined criteria must be fulfilled for the diagnosis of clinical ENE. According to the new edition of the TNM classification, ENE in p16-positive OPSCC compared to HPV-negative tumors does not result in prognostic upstaging with regard to the N category or UICC stage. Several current studies focus on evaluating extensively the prognostic influence of ENE in HPV-positive tumors. The diagnostic methods and significance of ENE—with particular attention to surgically treated HPV-positive OPSCC—are presented and discussed below.

DEFINITION OF EXTRANODAL EXTENSION

Extranodal extension was first described in 1930 by Rupert A. Willis in the context of autopsies on patients with advanced head and neck squamous cell carcinoma (HNSCC) (1, 3). It is generally defined as the spread of tumor tissue or neoplastic cells outside the lymph node capsule with infiltration of perinodal soft tissue (4). By means of histopathologic examination (lymph node metastasis without ENE; **Figure 1A**), ENE can additionally be subdivided into the categories "microscopic" ($\leq$2 mm beyond the lymph node capsule; **Figure 1B**) and "macroscopic" (>2 mm beyond the lymph node capsule; **Figure 1C**) (4). For instance, Bauer et al. in their 2019 study illustrated the importance of the extent of ENE in patients with OPSCC (5). Patients were classified into the categories ENE-negative, microscopic ENE, and macroscopic ENE, and patients with microscopic ENE showed significantly reduced survival compared to patients with negative ENE status (hazard ratio = HR = 1.52; 95% CI = 1.00–2.31; $p = 0.048$) (5). Patients with macroscopic ENE had the worst outcome (HR = 2.50; 95% CI = 1.39–4.51; $p = 0.002$) (5). In addition to that, recent data even differentiate between the categories "no ENE," "minimal ENE" ($\leq$1 mm beyond the lymph node capsule"), and ">1 mm beyond the lymph node capsule" (6).

Although these subcategories have not yet been applied for the purpose of pN classification, they are recommended by the AJCC for data collection and analyses and find application in recent studies (4). In addition to patients with diagnosed lymph node metastases and ENE of metastases, it is reported that a proportion of 10.5–25% of patients exhibit microscopic ENE despite having a clinically unremarkable lymph node status (1, 7). Thus, microscopic ENE, micrometastases, or soft tissue deposits can cause an underestimate of the incidence of ENE—especially with regard to patients with primary radiotherapy that are classified within the cTNM-classification system (1, 7).

IMAGING AND CLINICAL PREDICTORS IN DIAGNOSING EXTRANODAL EXTENSION

In addition to the widely used diagnostic methods involved in postoperative histopathologic examination, various imaging techniques can be applied, such as ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT). Clinical diagnosis of ENE of lymph node metastases presupposes that clear, defined criteria are met (8). Clinical or radiologic signs of tumor invasion alone (including the skin and surrounding soft tissue) as well as clinical symptoms of neural involvement (e.g., paresis of cranial nerves) are defined as clinical ENE in the new TNM classification (9). The following criteria are used for radiologic diagnosis of ENE for both CT and MRI: presence of irregular nodal capsular enhancement, loss of distinct nodal margins and infiltration into adjacent structures (fatty tissue, muscle, blood vessels) (1, 10, 11). Generally speaking, however, the limited sensitivity and specificity of the methods used in relation to the clinical diagnosis of ENE need to be discussed (8). For example, Steinkamp et al. showed in several publications a sensitivity of ~80.9% with specificity of 72.2% for CT investigations and sensitivity of ~74.4% and specificity of 72.2% for MRI imaging (1, 12, 13). Clinical diagnosis of ENE by ultrasound, with a sensitivity of 78.6% and specificity of 81.8%, achieved slightly better results than CT or MRI (1, 14). With regard to diagnosing ENE by contrast-enhanced CT imaging, the values quoted in the literature according to Faraji et al. and others range from 75 to 86% for the accuracy of predicting pathologic ENE, from 65 to 90% for sensitivity and 73–91% for the specificity of the imaging method (12, 15–21). As clinical diagnosis of ENE, for example, does not differentiate between microscopic and macroscopic ENE so far, the data of patient collectives with primary surgery and collectives with primary radiotherapy are not readily comparable.

For a long time there have been strong demands for standardization and further development of investigation methods and internationally recognized diagnostic criteria for the imaging modalities of ultrasound, CT and MRI (1). Only recently Kann et al. published their study on the diagnosis of lymph node metastases and ENE in HNSCC by means of pretreatment CT images and three-dimensional deep learning neural networks (22). In this study they trained the neural network using a data set of 2,875 CT-segmented lymph node specimens and achieved diagnostic results which exceeded those of human clinicians (22). The area under the receiver operating characteristics curve for diagnosing ENE and lymph node metastases was 0.91 (95% CI = 0.85–0.97)—ENE of lymph node metastases could be predicted with a sensitivity of 88% and specificity of 85% (22). The diagnosis of ENE by means of CT was additionally the subject of the recently published work by Faraji et al. (15). Seventy-three patients with HPV-positive OPSCC treated by primary surgery and neck dissection were reviewed for the presence of seven defined criteria of CT imaging (15). The pretreatment CT scans were evaluated by two radiologists who were blinded to the pathologic ENE results (15). In the evaluations, the presence of irregular nodal margins (highest specificity of 94% for examiner A and 95% for examiner B) and the absence of perinodal fatty tissue (highest sensitivity of 87% for examiner A and 96% for examiner B) showed a significant association with ENE (15).

In 2018 Hararah et al. published initial attempts at pretreatment prediction of ENE and positive surgical margins for OPSCC (23). In the course of analyzing prognostic

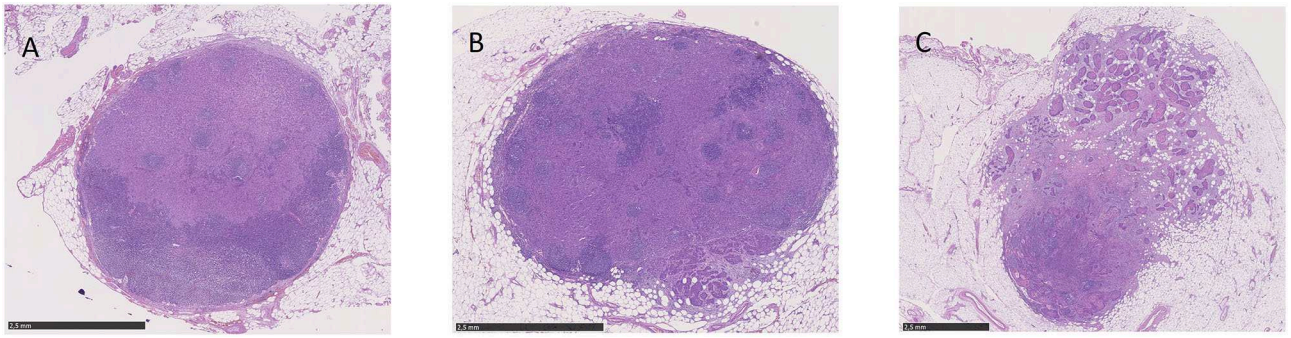

FIGURE 1 | Microscopic view of lymph node metastases without ENE **(A)**, with microscopic ENE **(B)** and with macroscopic ENE **(C)**.

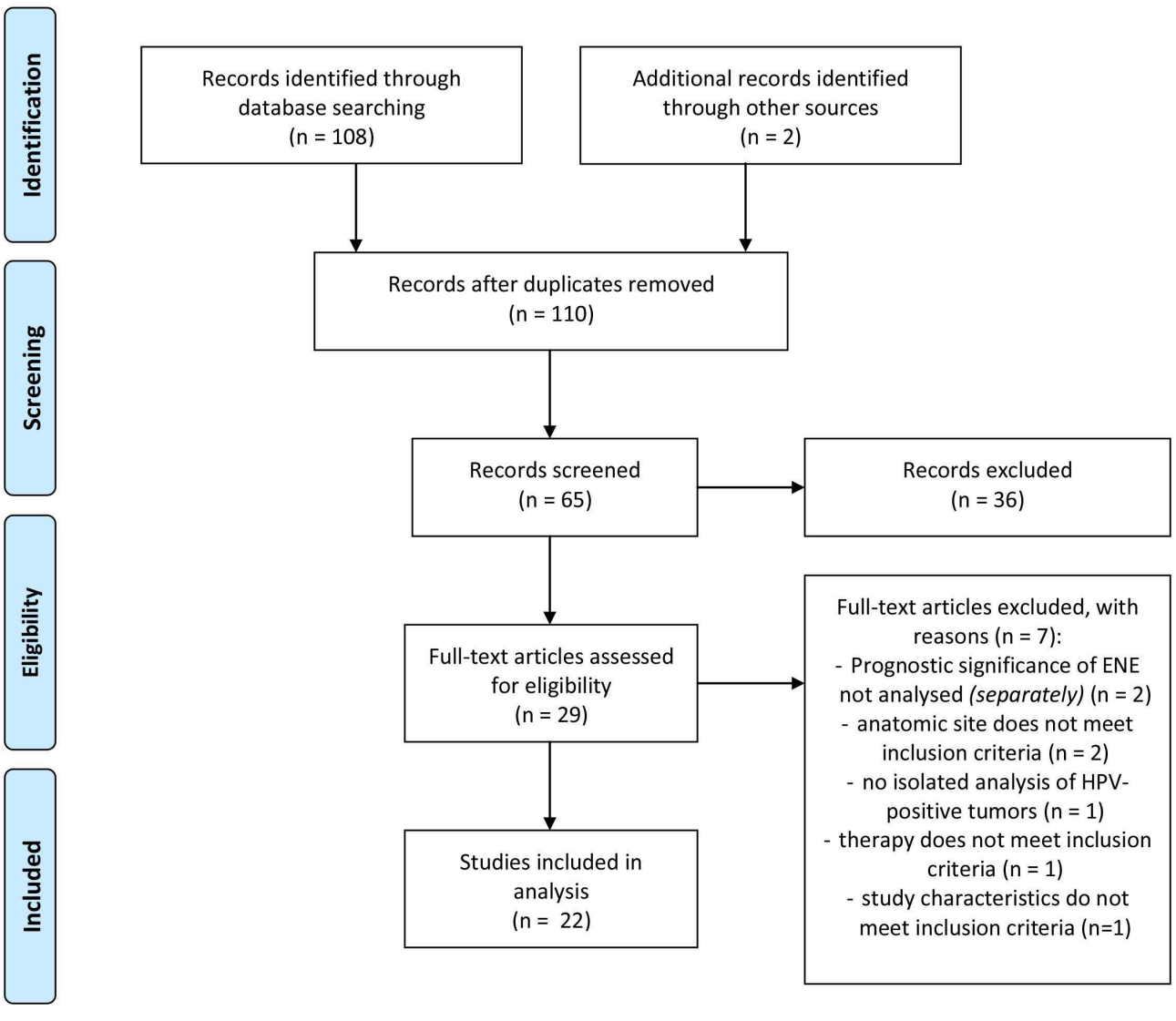

FIGURE 2 | Flow-chart of literature research adapted from PRISMA (24).

parameters of 5,056 patients (3,336 HPV-positive), Hararah et al. developed nomograms for the parameters ENE and/or positive resection status for HPV-negative as well as HPV-positive OPSCC (23). Regarding the prediction of postoperative ENE, for HPV-positive tumors clinical ENE, cN staging, cT staging, age, and tumor grading were integrated into the nomogram as predictive parameters (AUC ROC = 0.66; $p < 0.01$; 95% CI = 0.64–0.68) (23). Hararah et al. are thus presenting additional approaches to diagnosing ENE which, as a whole, could potentially facilitate clinical decision-making regarding primary treatment. However, they particularly stress the current aspiration to further develop clinical (and pathologic) diagnostic methods.

Further studies and more prolonged use of the new TNM classification are required to show how far the new clinical N classification, or particularly the clinical diagnosis of ENE defined therein, can succeed in everyday clinical practice and result in reliable identification of ENE status or whether new diagnostic methods will prevail in future. Understaging or upstaging of patients not surgically treated should be avoided where the prognostic influence of ENE is proven. Improving the modalities for clinical diagnosis of ENE and standardizing ENE diagnosis in general will thus continue to be the objective over the next few years.

METHODS

The aim of this paper is to provide a structured overview of current study results on the topic "Prognostic influence of ENE in surgically treated HPV-positive OPSCC." In order to investigate a possible prognostic influence of ENE in HPV-positive OPSCC, a literature research was conducted with *PubMed*. Using the *PubMed Search Builder* the following search term was created: ((((((((((extracapsular spread[Title/Abstract]) OR perinodal spread[Title/Abstract]) OR transcapsular spread[Title/Abstract]) OR extranodal spread[Title/Abstract]) OR extracapsular extension[Title/Abstract]) OR extranodal extension[Title/Abstract]) OR perinodal extension[Title/Abstract]) OR transcapsular extension[Title/Abstract])) AND (((hpv[Title/Abstract]) OR human papilloma virus[Title/Abstract]) OR p16[Title/Abstract])) AND (((((head and neck squamous cell carcinoma)) OR hnscc) OR oropharyngeal squamous cell carcinoma) OR opscc). The process of literature research is illustrated in **Figure 2**. 109 of 110 results were available in English. The included studies were published before July 2020.

Before screening the records, the following inclusion criteria were defined: (a) OPSCC, (b) patient collective contains HPV-positive tumors, (c) surgically treated collective/Neck Dissection (with or without adjuvant therapy), (d) ENE-Status available, (e) statement on the prognostic influence of ENE, (f) original research paper. Full-text articles assessed for eligibility were screened for further relevant publications. The publications included in analysis were screened for their study results referring to the prognostic impact of ENE in HPV-positive OPSCC. The relevant results as well as characteristics of the studies and number of cases are shown in **Tables 1**, **2** in the following.

EXTRANODAL EXTENSION AS RISK AND PROGNOSTIC FACTOR

Numerous studies in the past have shown that the presence of ENE additionally worsens the prognosis of patients with HNSCC (1, 8, 46–50). In a 2006 meta-analysis by Dunne et al. involving 1,620 patients with diagnosed HNSCC and lymph node metastasization, 5-year overall survival deteriorated to 30.7% in the presence of ENE compared to 58.1% in the absence of ENE (1, 50).

Furthermore, the correlation between ENE and locoregional recurrence and distant metastases has been studied in recent years. For example, Myers et al. showed in their publication of 2001 on 266 patients with squamous cell carcinoma of the tongue that ENE was the most significant prognostic parameter for the risk of regional recurrence and distant metastases in their population (1, 2). The meta-analysis by Mermod et al. from 2016 showed an odds ratio of 2.18 (95% CI = 1.23–3.87) for the correlation between ENE and distant metastases (1).

In 2014 Künzel et al. analyzed, among other things, the influence of ENE on the disease-specific survival of patients with OPSCC not stratified to HPV status. The study analyzed 384 patients first diagnosed between 1980 and 2010 (48). One of the findings was that ENE is associated with significantly worse disease-specific survival of 50% compared to 81% in the absence of ENE ($p < 0.001$) (48).

A few studies, however, demonstrated a lack of significant worsening of outcome due to ENE in HPV-positive OPSCC (1, 25, 27, 29, 30). In particular, the research group of Sinha et al. investigated the influence of ENE in HPV-induced OPSCC in detail (1, 25–27, 30, 31). In 2012, with regard to a group of 171 patients with p16-positive surgically treated OPSCC (adjuvant RT: $n = 73$, adjuvant CRT: $n = 69$), they published a lack of significant impact of ENE on overall and disease-specific survival (1, 26). In their multivariate analysis from 2012 (27), ENE (except for soft tissue deposits) did not prove prognostic in HPV-positive OPSCC in a prospective transoral laser surgery database ($n = 152$ patients—adjuvant RT: $n = 66$, adjuvant CRT: $n = 67$) (1, 27). In 2015 in their multivariate analysis of p16-positive OPSCC treated by surgery and neck dissection ($n = 220$ patients—RT: $n = 97$, CRT: $n = 75$), one of their findings was that the number of lymph node metastases (≥ 5)—but not ENE—was an independent prognostic factor (1, 30).

Their analyses of 2011, 2012, and 2015 additionally investigated the significance of the extent of ENE and its prognostic influence: Thus Lewis et al. also described a lack of significant influence of ENE on overall survival, disease-free survival (DFS), and disease-specific survival (DSS) in surgically treated HPV-positive OPSCC ($n = 101$ patients–postoperative radiation therapy: $n = 100$, postoperative chemotherapy: $n = 44$) (25). In fact, a significant correlation was found between the presence of soft tissue deposits (defined as grade 4 ENE) and overall survival, DSS and DFS. Given a correlation with the

TABLE 1 | Summary of the described studies on the prognostic influence of ENE in HPV-positive OPSCC—part 1.

References	Characteristics	Patients	Prognostic influence of ENE in HPV+ OPSCC
Lewis et al. (25)	– Monocentric – Retrospective – Surgical	$n = 101$ OPSCC (HPV+: $n = 90$)	– Univariate analysis: only grade 4 ENE (soft tissue deposit) associated with OS ($p < 0.001$), DFS ($p = 0.0025$), and DSS ($p = 0.0013$) (but correlates with T stage) – Multivariate analysis: no significant correlation with OS, DFS, or DSS
Haughey et al. (26)	– Monocentric – Retrospective – Surgical	$n = 171$ OPSCC (HPV+)	ENE not significantly associated with OS, DSS, or DFS
Sinha et al. (27)	– Monocentric – Prospective – Surgical	$n = 152$ OPSCC (HPV+)	– Univariate analysis: grade 4 ENE (soft tissue deposit) significantly associated with DFS ($p = 0.02$), DSS ($p = 0.03$), and OS ($p = 0.009$) – Multivariate analysis: grade 4 ENE (soft tissue deposit) significantly associated with DFS ($p = 0.01$), DSS ($p = 0.01$), and OS ($p = 0.03$) – ENE grade 0–3 not prognostic
Klozar et al. (28)	– OPSCC+OSCC – Monocentric – Retrospective – Surgical (except for 3 patients)	$n = 139$ OPSCC (HPV+: $n = 91$) $n = 31$ OSCC (HPV+: $n = 7$)	ENE in univariate and multivariate analysis not significantly associated with DSS
Maxwell et al. (29)	– OPSCC+OSCC – Monocentric – Retrospective – Surgical	$n = 133$ OPSCC (HPV+: $n = 76$) $n = 214$ OSCC	– OPSCC: ENE not significantly associated with DSS ($p = 0.936$) (also for HPV-OPSCC, $p = 0.198$) – ENE as significant independent prognostic factor in OSCC
Sinha et al. (30)	– Monocentric – Prospective – Surgical	$n = 220$ OPSCC (HPV+)	– ENE not prognostic for DFS, DSS, or recurrence – Number of lymph node metastases significantly associated with outcome
Sinha et al. (31)	– Monocentric – Prospective – Surgical	$n = 222$ OPSCC (HPV+)	– Grade 4 ENE (soft tissue deposit) significantly associated with distant metastases and DMFS ($p = 0.004$) only for T3–T4 tumors – No significant correlation with regional recurrence
Iyer et al. (32)	– Monocentric – Retrospective – Surgical	$n = 201$ OPSCC (HPV+: $n = 106$)	ENE not significantly associated with 5Y-OS ($p = 0.300$), 5Y-DSS ($p = 0.116$), or 5Y-RFS ($p = 0.753$)
Kaczmaret al. (33)	– (Monocentric) – Retrospective – Surgical	$n = 114$ OPSCC (HPV+)	Univariate analysis: ENE not significantly associated with increased risk of local and distant progression ($p = 0.575$, $p = 0.793$)
Kumar et al. (34)	– Monocentric – Retrospective – Surgical	$n = 289$ OPSCC (HPV+: n=172)	– Univariate analysis: ENE nearly reached significance ($p = 0.0553$) – Multivariate analysis: ENE not significantly associated with OS ($p = 0.7644$)
Kharytaniuk et al. (35)	– OPSCC+CUP – Monocentric – Retrospective – Surgical (neck dissection)	$n = 62$ OPSCC (HPV+: $n = 36$) $n = 21$ CUP (HPV+: $n = 9$)	– ENE not significantly associated with RFS ($p = 0.93$) or DSS ($p = 0.91$)
Tassone et al. (36)	– Monocentric – Retrospective – Surgical	$n = 85$ OPSCC (HPV+)	– Logistic regression analysis of recurrence (as binary variable): ENE not significantly associated with recurrence (OR = 2.28, $p = 0.383$) – Univariate analysis of DFS: ENE no significant impact on DFS ($p = 0.25$)

CUP, cancer of unknown primary; DFS, disease-free survival; DMFS, distant metastasis-free survival; DSS, disease-specific survival; ENE, extranodal extension; HPV, human papillomavirus; HR, hazard ratio; NCDB, National Cancer Database; OPSCC, oropharyngeal squamous cell carcinoma; OR, odds ratio; OS, overall survival; OSCC, oral squamous cell carcinoma; RFS, recurrence-free survival.

T stage, it was not confirmed in the multivariate analysis (25). In their 2012 publication, Sinha et al. then confirmed a significant influence of soft tissue deposits on overall survival, DFS and DSS (27). In 2015 they again published results which showed a significant correlation between soft tissue deposits and distant metastasis-free survival for T3–T4 tumors only ($n = 222$ patients, adjuvant RT: $n = 97$, adjuvant CRT: $n = 78$) (31).

In this connection Maxwell et al. reached the following conclusion in their 2013 publication (29): In their analysis of 133 patients with OPSCC and 214 patients with carcinoma of the oral cavity (surgically treated in the years 1983–2009), they found no

TABLE 2 | Summary of the described studies on the prognostic influence of ENE in HPV-positive OPSCC—part 2.

References	Characteristics	Patients	Prognostic influence of ENE in HPV+ OPSCC
An et al. (37)	– NCDB (multicenter design) – Retrospective – Surgical	$n = 1,043$ OPSCC (HPV+)	– Univariate analysis: significant correlation with 3Y-OS ($p = 0.01$) – Multivariate analysis: significant correlation with OS (HR = 1.89; 95% CI = 1.01–3.51; $p = 0.046$) – Only patients with 1 lymph node metastasis: significant correlation with 3Y-OS ($p = 0.033$)
Zhan et al. (38)	– NCDB (multicenter design) – Retrospective – Surgical (neck dissection)	$n = 3,745$ OPSCC (HPV+)	– Univariate analysis: significant correlation with 4Y-OS ($p < 0.001$) – Also after stratification according to N classification for pN1 tumors
Shevach et al. (39)	– Monocentric – Retrospective – Surgical (neck dissection)	$n = 75$ OPSCC (HPV+)	– Univariate analysis: significant correlation with 5Y-DC rate ($p = 0.046$) and 5Y-PFS ($p = 0.021$) – Multivariate analysis: independently prognostic of worse DC (HR = 8.26; 95% CI = 1.24–55.21; $p = 0.029$) and PFS (HR = 4.64; 95% CI = 1.18–18.29; $p = 0.028$) – No significant difference in 5Y-LRC or OS
Meyer et al. (40)	– Monocentric – Retrospective – Surgical	$n = 88$ OPSCC (HPV+: $n = 39$)	– Univariate analysis: significant correlation with OS ($p = 0.012$) und RFS ($p = 0.012$) – Multivariate analysis: ENE not included
Bauer et al. (5)	– NCDB (multicenter design) – Retrospective – Surgical	$n = 4,153$ OPSCC (HPV+)	– Univariate analysis: significant correlation with 5Y-OS ($p = 0 < 0.001$) – Stratified according to N stage (8^{th} edition): significant correlation with 5Y-OS ($p = 0 < 0.001$) – Multivariate analysis: significant prognostic parameter (HR = 1.90; 95% CI = 1.35–2.67; $p = 0 < 0.001$)
Miccio et al. (41)	– NCDB (multicenter design) – Retrospective – Surgical	$n = 3,407$ OPSCC (HPV+)	– Univariate analysis: significant correlation with OS (HR = 2.04; 95% CI = 1.59–2.63; $p < 0.001$) – Multivariate analysis: significant correlation with OS (HR = 1.66; 95% CI = 1.26–2.19; $p < 0.001$)
Beltz et al. (42)	– Monocentric – Retrospective – Surgical	$n = 95$ OPSCC (HPV+: $n = 50$)	– Univariate analysis: significant correlation with 5Y-OS, ($p = 0.008$)
Han et al. (43)	– NCDB (multicenter design) – Retrospective – Surgical (alone)	$n = 736$ OPSCC (HPV+)	– Univariate analysis: presence of microscopic ENE ($p = 0.009$) or macroscopic ENE ($p = 0.007$) associated with increased risk of death – Multivariate analysis: macroscopic ENE vs. non-ENE as independent risk factor for death (HR = 4.9; 95% CI = 1.4–18.1; $p = 0.016$)
Freitag et. al. (44)	– Monocentric – Retrospective – Surgical	$n = 92$ OPSCC (HPV+)	– p16+: significant correlation with OS ($p = 0.007$) and TSS ($p = 0.047$) – p16+/HPV16 DNA+: significant correlation with OS ($p = 0.013$) and TSS ($p = 0.026$) – Multivariate analysis: independent predictor for decreased OS ($p = 0.033$), TSS ($p = 0.165$), PFS ($p = 0.42$), and DFS ($p = 0.04$)
Gal et al. (45)	– NCDB (multicenter design) – Retrospective – Surgical	$n = 16,845$ OPSCC (HPV+: $n = 8,780$)	– Pathologic and clinical ENE associated with decreased survival – No significant difference between pathologic and clinical ENE

CI, confidence interval; DC, distant control DFS, disease-free survival; ENE, extranodal extension; HPV, human papillomavirus; HR, hazard ratio; NCDB, National Cancer Database; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; TSS, tumor-specific survival.

significant association between ENE status and DSS for HPV-positive and HPV-negative patients (OPSCC: adjuvant radiation: $n = 111$, adjuvant chemotherapy: $n = 40$).

The investigation of potential prognostic parameters of HPV-positive and HPV-negative OPSCC and oral carcinomas of 170 patients (OPSCC: $n = 139$, 65.5% HPV-positive) was also the purpose of the study by Klozar et al. published in 2013 (28). For HPV-negative tumors, univariate analysis showed UICC stage, Pt, and pN classification, number of positive lymph nodes and ENE to be significant prognostic parameters. Except for ENE, these were confirmed in the multivariate analysis. For HPV-*positive* tumors, by contrast, none of these parameters showed a significant correlation with the DSS of patients (28).

In 2015 Iyer et al. published the following results: While ENE, resection status, lymph vessel invasion, and pN category were independent predictors of survival in the case of HPV-negative OPSCC, they were not prognostic for HPV-positive tumors [in respect of recurrence-free survival (RFS), DSS, and OS] ($n = 201$ patients, adjuvant RT: $n = 138$) (32). In addition to that, Kumar et al. (34) showed that ENE ($p = 0.0021$) and advanced T-classification represent significant predictors of survival in

HPV-negative surgically treated OPSCC (most patients treated with adjuvant RT/RCT based on NCCN guidelines). While ENE nearly reached significance in the univariate analysis of HPV-positive OPSCC ($p = 0.0553$), multivariate analysis revealed that ENE was not significantly associated with survival ($p = 0.7644$) (34). Kharytaniuk et al. in their 2016 analysis of 83 patients ($n = 62$ with OPSCC, $n = 21$ with cancer of unknown primary = CUP) with neck dissection as part of primary definitive treatment—also confirmed that ENE is not a negative prognostic factor for HPV-positive OPSCC (in respect of RFS and DSS) (35) (surgery only: $n = 8$, RT: $n = 50$, CRT: $n = 25$). Kaczmar et al. showed that ENE does not correlate with increased risk of local as well as distant progression in HPV-positive OPSCC (114 surgical patients, 89 with adjuvant radiation, 54 with adjuvant chemotherapy) in univariate analysis (33). In addition to that, Tassone et al. confirmed that ENE is not significantly associated with recurrence and DFS in a retrospective analysis of 85 surgically treated HPV-positive OPSCC (adjuvant RT: $n = 81$, adjuvant systemic therapy: $n = 52$) (36). **Table 1** summarizes the studies described in this chapter showing no or weak prognostic influence of ENE in primarily surgically treated HPV-positive OPSCC.

EXTRANODAL EXTENSION IN THE CONTEXT OF THE 8TH UICC CLASSIFICATION

According to the 8th edition of the TNM classification, the presence of ENE leads to distinct upstaging solely in HPV-negative OPSCC. For HPV-positive tumors, only the number of positive lymph nodes is decisive in terms of pTNM staging. This is why the prognostic influence of ENE in HPV-induced tumors is currently the focus of a number of studies. In a 2019 study by the present authors, the application and prognostic impact of the new TNM classification as well as the factors HPV status and ENE were examined in a group of 255 patients with OPSCC first diagnosed in the years 2008–2015 (42). This included analyzing the overall survival of HPV-positive patients with negative vs. positive ENE status treated with surgery alone or surgery combined with adjuvant radiation/chemoradiation. In this cohort adjuvant therapy was standard in case of pathologically proven ENE. This study addressed, among other things, the question of whether ENE can actually be ignored in HPV-mediated OPSCC (42). Ninety five patients met the inclusion criteria for ENE analysis. The Kaplan-Meier curves presented (**Figure 3**) and the log rank test revealed a statistically significant deterioration of overall survival in the presence of ENE for HPV-positive patients in the univariate analysis (ENE-negative: OS = 92.9%, ENE-positive: 68.0%, $p = 0.008$) (42).

The univariate analysis of the study by Meyer et al. (40), which examined the prognostic influence of the lymph node ratio, also showed a significant influence of ENE on overall survival of HPV-positive patients with surgically treated OPSCC ($p = 0.012$; ENE not included in the multivariate analysis) (surgery: $n = 21$, surgery + R(C)T: $n = 67$).

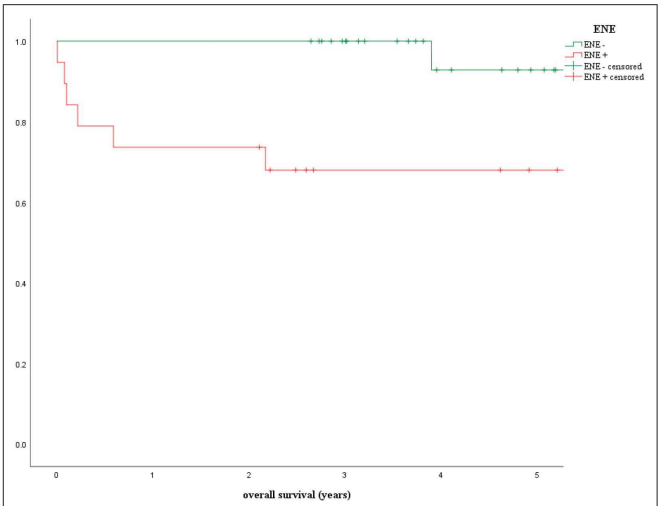

FIGURE 3 | Prognostic influence of extranodal extension in patients with p16-positive oropharyngeal carcinoma (ENE, extranodal extension) (42).

In 2017 Zhan et al. published results of their validation of the new staging system based on 3,745 cases of HPV-positive OPSCC treated by surgery and neck dissection from the National Cancer Database (NCDB) for the years 2010–2014 (surgery only: $n = 642$, surgery + RT: $n = 1{,}005$, surgery + CRT: $n = 1{,}773$) (38). As well as a general evaluation of the new staging algorithm, the study focused on analyzing the prognostic influence of ENE in HPV-positive OPSCC. During the course of their analyses, Zhan et al. demonstrated a statistically significant influence of ENE on 4-year overall survival in HPV-positive OPSCC (ENE-negative: 92% vs. ENE-positive: 85%, $p < 0.001$) (38). Upon stratification according to pN classification, ENE proved to be a significant prognostic parameter for the 4-year overall survival of HPV-positive patients with pN1 stage [pN1: ENE-negative 92%, ENE-positive 87% ($p = 0.004$); pN2: ENE-negative 88%, ENE-positive 77% ($p = 0.061$)] (38).

The described results are consistent with the results of analyses by An et al. published in 2017 (37): In their study the prognostic value of ENE was examined in a group of 1,043 patients with HPV-positive OPSCC (pT1–T4, pN1–N3, M0, R0) who underwent primary surgical treatment (adjuvant RT: $n = 306$, adjuvant CRT: $n = 498$). Patients who met the defined inclusion criteria were identified via the NCDB for the years 2010–2012 (37). In the course of their analyses An et al. demonstrated that a positive ENE status is associated with a significant deterioration of overall survival of HPV-positive patients (3-year overall survival: 89.3 vs. 93.6%, $p = 0.01$) (37). No significant difference in overall survival was found between cases with microscopic vs. macroscopic ENE (37). Furthermore, An et al. also demonstrated in the multivariate analysis that rather than the presence of ≥5 lymph node metastases—as integrated into the TNM classification for HPV-positive OPSCC—it is the presence of ENE that is significantly associated with a deterioration of overall survival (HR = 1.89; 95% CI = 1.01–3.51; $p = 0.046$) (37). Hence the results to some extent contradict the current system of N classification of p16-positive OPSCC (42).

For HPV-positive OPSCC patients who have undergone primary surgical treatment, it is only the absolute number of lymph nodes involved (cut-off between pN1 and pN2: ≥5 involved lymph nodes) which has the decisive prognostic influence on determining the pN category according to the 8th edition (42). Given comparable hazard ratios (≥5 lymph node metastases: HR = 1.81, $p = 0.086$ vs. ENE: HR = 1.89, $p = 0.046$), An et al. do not question the cut-off of ≥5 lymph node metastases as a prognostic parameter for HPV-positive OPSCC. However, they do advocate evaluation of both parameters as potential prognostic factors—especially since a higher number of lymph node metastases is associated with the presence of ENE with a greater probability (37).

In order to eliminate the possible confounding variable of "total number of lymph node metastases" from the analysis and to investigate the relationship between ENE and overall survival in isolation, overall survival was analyzed only in patients with one lymph node metastasis: once again a deterioration of 3-year overall survival to 90.8% compared to 96.0% ($p = 0.033$) was observed (37).

In addition to that, Shevach et al. (39) published results showing that ENE-positive status is significantly associated with a deterioration of distant control and progression-free survival in univariate and multivariate analysis (39). They evaluated a collective of 75 patients with HPV-mediated OPSCC treated with surgery, respectively, neck dissection followed by adjuvant radiotherapy/chemoradiotherapy. However, there was no significant difference in OS and locoregional control between ENE-negative and -positive patients (39).

Bauer et al. recently showed that ENE represents a significant prognostic parameter in respect of overall survival in HPV-mediated OPSCC (5). In their paper published in 2019, they analyzed the prognostic significance of ENE in a group of 4,153 patients with HPV-positive OPSCC from the NCDB who were treated surgically and by neck dissection (N0 = 531, ENE-positive: 1,429, ENE-negative: 2,193—surgery only: $n = 923$, surgery/radiation: $n = 1,403$, surgery/radiation/chemo: $n = 1,827$) (5). The univariate analysis revealed a statistically significant correlation between ENE and overall survival in HPV-positive patients ($p < 0.001$) with 5-year overall survival of 92.6% (95% CI = 90.5–94.7%) for negative ENE status compared to 84.0% (95% CI = 80.7–87.4%) for ENE-positive tumors (5). Furthermore, when stratified according to N stage (8th edition), tumors classified as N1/ENE-negative showed the highest 5-year overall survival rate of 93.4% (95% CI = 91.3–95.5%), whereas N2/ENE-negative and N1/ENE-positive tumors had similar 5-year overall survival of 87.8 and 87.3%, respectively, (5). The multivariate analysis (with age, gender, population group, morbidity, T stage, treatment, and resection status as possible confounding variables) revealed in respect of mortality risk a hazard ratio of 1.90 (95% CI = 1.35–2.67) in the presence of ENE vs. ENE-negative OPSCC ($p < 0.001$). The pathologic N stage—or hence the number of positive lymph nodes—was significantly associated with patients' outcome (pN2 vs. pN1: HR = 1.53) (5). Furthermore, Bauer et al. demonstrated that—when combining pN category and ENE status—tumors classified as *N2/ENE-positive* had the lowest 5-year overall survival rate (HR = 2.93; 95% CI = 1.94–4.43; $p < 0.001$) in comparison with N1/ENE-negative OPSCC (HR = 1.00) (5). OPSCC classified as N1/ENE-positive also showed nearly twice as high mortality risk (HR = 1.88; 95% CI = 1.26–2.80; $p = 0.002$) as ENE-negative pN1 tumors. Bauer et al. thus concluded in the course of their evaluation that ENE is prognostic irrespective of the number of positive lymph nodes and that the combination of ENE status and number of positive lymph nodes (pN category) particularly leads to an improved picture of mortality risk (5). All in all, the work by Bauer et al. including 4,153 patients represents a large study in this field for HPV-positive OPSCC and—as a result of the length of follow-up—also allows the prognostic influence of ENE on 5-year overall survival to be evaluated. As Bauer et al. also stress, this is particularly significant in view of the relatively good prognosis of HPV-positive tumors. Furthermore, a large number of possible confounding variables were integrated into the multivariate analysis. In summary, the large number of cases included and the length of follow-up made it possible for the results to reach statistical significance (5).

Furthermore, Miccio et al. recently published their study on the influence of contralateral lymph node metastasization and ENE on survival in HPV-mediated OPSCC (41). Three thousand four hundred seven patients from the NCDB (2010–2015) with surgically treated, HPV-positive OPSCC and a minimum of 10 lymph nodes removed made up the study population (adjuvant RT: $n = 1,262$, adjuvant CRT: $n = 1,501$, unknown: $n = 78$) (41). In their evaluation, the research group of Miccio et al. concluded that, in both the univariate analysis (HR = 2.04; 95% CI = 1.59–2.63; $p < 0.001$) and the multivariate analysis (HR = 1.66; 95% CI = 1.26–02.19; $p < 0.001$), the presence of ENE is associated statistically highly significantly with a deterioration of overall survival in HPV-positive tumors and it should be included in future staging algorithms (41).

In 2019, Han et al. published a retrospective analysis of 736 patients with only surgically treated HPV-positive OPSCC from the NCDB (2010–2014) (43). Among other things, they showed that microscopic or macroscopic ENE results in a significantly worse OS when compared to positive lymph nodes without ENE (5J-OS: 91% vs. 78%; $p < 0.0001$) (43). In addition, Freitag et al. recently published their analysis of a cohort of 92 patients with surgically treated HPV-mediated OPSCC (IC+OP+RT: $n = 8$, OP: $n = 21$, OP+RT: $n = 23$, OP+RCT: $n = 39$, OP+RT+Cetuximab: $n = 1$) (44). Their multivariate analysis showed that ENE represents an independent predictor for decreased OS ($p = 0.033$), tumor-specific survival ($p = 0.165$), progression-free survival ($p = 0.42$), and DFS ($p = 0.04$) (44). The results of their investigation as a whole led them to the conclusion that ENE (as well as HPV16 DNA status) should be integrated in the prognostic staging algorithm of HPV-mediated OPSCC (44). Furthermore, Gal et al. recently showed a decreased survival in the presence of clinical and pathological ENE compared to the absence of ENE. Their retrospective SEER database study analyzed 16,845 primarily surgical treated patients with tonsillar and base of the tongue primaries (45).

As a whole the results described above contrast with the conclusions of the 2016 review by Mermod et al. which included

an analysis of the prognostic significance of histopathologically proven ENE in HPV-positive OPSCC (1). Individual results of the studies included have already been presented: overall, the analyses had shown a lack of negative influence of ENE in HPV-positive OPSCC (1). Compared with the monocentric design of the studies analyzed by Mermod et al. (1) and the maximum number of 222 patients included Sinha et al. the great strengths of the studies by Zhan et al. (38), An et al. (37), Bauer et al. (5), Miccio et al. (41), and Gal et al. (45) are the case numbers of 3,745, 1,043, 4,153, 3,407, and 16,845 patients, respectively, and hence their power as well as their multicenter design.

Table 2 summarizes the described studies showing significant influence on prognosis of ENE in HPV-positive OPSCC.

The possible influence of tobacco consumption of patients was not analyzed because of the lack of recording in the NCDB. The influence of nicotine consumption on the risk and prognostic profile of HPV-positive OPSCC, however, is a relevant parameter according to the results of Ang et al. (51) and should be considered in future prospective analyses. Kompelli et al. recently published an analysis of patients with HPV-related OPSCC (52): Aim of this study was to investigate the impact of pathologic prognostic factors in the context of chronic tobacco use. The results show that, among other things, presence of ENE did not significantly affect survival in HPV-positive heavy smokers ($\geq$20 pack years) (52). However, HPV-positive ENE-positive heavy smokers had a significant decrease in survival (similar to HPV-negative patients) compared to HPV-positive non-smokers with positive ENE-status (52). In the future, the prognostic impact of ENE should also be evaluated in the context of tobacco consumption and the prognostic influence of tobacco abuse in HPV-positive OPSCC should be examined in detail further on.

All in all, the current study results from various publications presented here emphasize that ENE is a risk and prognostic factor, including HPV-positive OPSCC, which to date has not been integrated into the staging algorithm of the TNM classification. Possible reasons for the different results of the studies mentioned are discussed by Bauer et al. (5), An et al. (37), and Zhan et al. (38). For example, the excellent prognosis of HPV-positive OPSCC could lead to the fact that only studies with a higher number of cases can reveal statistically significant differences between ENE-positive and—negative tumors (5). An et al. stress the greater power achieved by larger patient collectives as well (37). In addition, Zhan et al. support this argumentation with their data–with a moderate effect size on OS (5–11% on 4Y-OS), significant results are only likely in high numbers of cases (38), such as those made possible by the NCDB. Nonetheless, the prognostic influence of ENE in surgically treated HPV-positive OPSCC remains a topic that should be analyzed in further (prospective) multicenter studies.

Limitations of this work are that only studies that explicitly examined ENE in HPV-positive OPSCC (e.g., ENE terms in title/abstract) were included—therefore other possibly relevant research results, which were not identified through literature research or references, could have been missed. The aim of this publication was to provide the reader with an overview of the current status of research in this field in a structured form. Due to a limited number of studies that *explicitly* focus on this issue, as well as partly limited comparability, we did not choose a *systematic* review, but a structured review according to the PRISMA guidelines. We performed our literature research as structured and traceable as possible. Furthermore, no quality assessment of the included studies was carried out. Despite the focus on surgically treated collectives, it can be assumed that the therapy algorithms (especially regarding adjuvant therapy) vary to a certain extent from institute to institute, which influences the comparability of the studies. As histopathologically proven ENE is an accepted risk factor in HNSCC in general and de-escalation strategies in HPV-positive tumors have not been integrated into clinical practice, the majority of patients of all discussed studies should have been treated by adjuvant radiotherapy. As a topic that is currently gaining more and more interest, a future meta-analysis—including possible additional publications of the next months/years—should be considered.

CONCLUSION

Whether the prognostic influence of ENE of lymph node metastases can actually be ignored for HPV-*positive* tumors in the TNM classification system should be reevaluated in detail in the context of prospective multicenter studies. According to the study results presented here, it also seems necessary to record ENE in the tumor documentation for HPV-positive tumors. Furthermore, it is also the extent of ENE (macroscopic vs. microscopic—ENE $\leq/>$1 mm, respectively, ENE $\leq/>$2 mm) that should be examined and documented as it represents an additional prognostic factor. The methods applied (including ultrasound, CT, MRI) in the clinical diagnosing of ENE have limited sensitivity and specificity. In this regard initial attempts at computer-aided analysis of image data should be pursued. Furthermore, clinical diagnostic criteria should be standardized overall at the international level.

AUTHOR CONTRIBUTIONS

JK came up with the idea and planned the manuscript. AB and JK collected the data. AB drafted the manuscript and performed the systematic review and its analysis. AB provided the original data presented. SZ and KE provided expert opinion on histopathology and high resolution histopathologic pictures. IM helped with editing the manuscript. GP, CB, and JK reviewed the manuscript. All authors finally checked the manuscript and provided critical review of its content.

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REFERENCES

1. Mermod M, Tolstonog G, Simon C, Monnier Y. Extracapsular spread in head and neck squamous cell carcinoma: a systematic review and meta-analysis. *Oral Oncol*. (2016) 62:60–71. doi: 10.1016/j.oraloncology.2016.10.003
2. Myers JN, Greenberg JS, Mo V, Roberts D. Extracapsular spread. A significant predictor of treatment failure in patients with squamous cell carcinoma of the tongue. *Cancer*. (2001) 92:3030–6. doi: 10.1002/1097-0142(20011215)92:12<3030::AID-CNCR10148>3.0.CO;2-P
3. Willis RA. Epidermoid carcinoma of the head and neck, with special reference to metastasis. *J. Pathol.* (1930) 33:501–26. doi: 10.1002/path.1700330302
4. Bullock MJ, Beitler JJ, Carlson DL, Fonseca I, Hunt JL, Katabi N, et al. Data set for the reporting of nodal excisions and neck dissection specimens for head and neck tumors: explanations and recommendations of the guidelines from the international collaboration on cancer reporting. *Arch Pathol Lab Med*. (2019) 143:452–62. doi: 10.5858/arpa.2018-0421-SA
5. Bauer E, Mazul A, Chernock R, Rich J, Jackson RS, Paniello R, et al. Extranodal extension is a strong prognosticator in HPV-positive oropharyngeal squamous cell carcinoma. *Laryngoscope*. (2019) 130:939–45. doi: 10.1002/lary.28059
6. Ferris RL, Flamand Y, Weinstein GS, Li S, Quon H, Mehra R, et al. Transoral robotic surgical resection followed by randomization to low- or standard-dose IMRT in resectable p16+ locally advanced oropharynx cancer: a trial of the ECOG-ACRIN cancer research group (E3311). *JCO*. (2020) 38(15 suppl):6500. doi: 10.1200/JCO.2020.38.15_suppl.6500
7. Coatesworth AP, MacLennan K. Squamous cell carcinoma of the upper aerodigestive tract: the prevalence of microscopic extracapsular spread and soft tissue deposits in the clinically N0 neck. *Head Neck*. (2002) 24:258–61. doi: 10.1002/hed.10020
8. Beltz A, Gösswein D, Zimmer S, Stauber RH, Hagemann J, Strieth S, et al. Staging von oropharynxkarzinomen: neue TNM-klassifikation als herausforderung für kopf-hals-tumorzentren. *HNO*. (2018) 66:375–82. doi: 10.1007/s00106-018-0499-0
9. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and neck cancers-major changes in the American joint committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin*. (2017) 67:122–37. doi: 10.3322/caac.21389
10. Som PM. Lymph nodes of the neck. *Radiology*. (1987) 165:593–600. doi: 10.1148/radiology.165.3.3317494
11. King AD, Tse GM, Yuen EH, To EW, Vlantis AC, Zee B, et al. Comparison of CT and MR imaging for the detection of extranodal neoplastic spread in metastatic neck nodes. *Eur J Radiol*. (2004) 52:264–70. doi: 10.1016/j.ejrad.2004.03.004
12. Steinkamp HJ, van der Hoeck E, Böck JC, Felix R. Kapseldurchbrüche zervikaler lymphknotenmetastasen: diagnostischer stellenwert der computertomographie. *Rofo*. (1999) 170:457–62. doi: 10.1055/s-2007-1011073
13. Steinkamp HJ, Beck A, Werk M, Felix R. Kapseldurchbrüche zervikaler lymphknotenmetastasen: diagnostischer stellenwert der magnetresonanztomographie. *Rofo*. (2002) 174:50–5. doi: 10.1055/s-2002-19533
14. Steinkamp HJ, Beck A, Werk M, Rademaker J, Felix R. Kapseldurchbrüche zervikaler lymphknotenmetastasen: diagnostischer stellenwert der sonographie. *Ultraschall Med*. (2003) 24:323–30. doi: 10.1055/s-2003-42914
15. Faraji F, Aygun N, Coquia SF, Gourin CG, Tan M, Rooper LM, et al. Computed tomography performance in predicting extranodal extension in HPV-positive oropharynx cancer. *Laryngoscope*. (2019) 130:1479–86. doi: 10.1002/lary.28237
16. Yousem DM, Som PM, Hackney DB, Schwaibold F, Hendrix RA. Central nodal necrosis and extracapsular neoplastic spread in cervical lymph nodes: MR imaging versus CT. *Radiology*. (1992) 182:753–9. doi: 10.1148/radiology.182.3.1535890
17. Souter MA, Allison RS, Clarkson JH, Cowan IA, Coates MH, Wells JE. Sensitivity and specificity of computed tomography for detection of extranodal spread from metastatic head and neck squamous cell carcinoma. *J Laryngol Otol*. (2009) 123:778–82. doi: 10.1017/S0022215109004332
18. Url C, Schartinger VH, Riechelmann H, Glückert R, Maier H, Trumpp M, et al. Radiological detection of extracapsular spread in head and neck squamous cell carcinoma (HNSCC) cervical metastases. *Eur J Radiol*. (2013) 82:1783–7. doi: 10.1016/j.ejrad.2013.04.024
19. Prabhu RS, Magliocca KR, Hanasoge S, Aiken AH, Hudgins PA, Hall WA, et al. Accuracy of computed tomography for predicting pathologic nodal extracapsular extension in patients with head-and-neck cancer undergoing initial surgical resection. *Int J Radiat Oncol Biol Phys*. (2014) 88:122–9. doi: 10.1016/j.ijrobp.2013.10.002
20. Chai RL, Rath TJ, Johnson JT, Ferris RL, Kubicek GJ, Duvvuri U, et al. Accuracy of computed tomography in the prediction of extracapsular spread of lymph node metastases in squamous cell carcinoma of the head and neck. *JAMA Otolaryngol Head Neck Surg*. (2013) 139:1187–94. doi: 10.1001/jamaoto.2013.4491
21. Carlton JA, Maxwell AW, Bauer LB, McElroy SM, Layfield LJ, Ahsan H, et al. Computed tomography detection of extracapsular spread of squamous cell carcinoma of the head and neck in metastatic cervical lymph nodes. *Neuroradiol J*. (2017) 30:222–9. doi: 10.1177/1971400917694048
22. Kann BH, Aneja S, Loganadane GV, Kelly JR, Smith SM, Decker RH, et al. Pretreatment identification of head and neck cancer nodal metastasis and extranodal extension using deep learning neural networks. *Sci Rep*. (2018) 8:14036. doi: 10.1038/s41598-018-32441-y
23. Hararah MK, Stokes WA, Jones BL, Oweida A, Ding D, McDermott J, et al. Nomogram for preoperative prediction of nodal extracapsular extension or positive surgical margins in oropharyngeal squamous cell carcinoma. *Oral Oncol*. (2018) 83:73–80. doi: 10.1016/j.oraloncology.2018.06.005
24. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med*. (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
25. Lewis JS, Carpenter DH, Thorstad WL, Zhang Q, Haughey BH. Extracapsular extension is a poor predictor of disease recurrence in surgically treated oropharyngeal squamous cell carcinoma. *Mod Pathol*. (2011) 24:1413–20. doi: 10.1038/modpathol.2011.105
26. Haughey BH, Sinha P. Prognostic factors and survival unique to surgically treated p16+ oropharyngeal cancer. *Laryngoscope*. (2012) 122(Suppl. 2):13–33. doi: 10.1002/lary.23493
27. Sinha P, Lewis JS, Piccirillo JF, Kallogjeri D, Haughey BH. Extracapsular spread and adjuvant therapy in human papillomavirus-related, p16-positive oropharyngeal carcinoma. *Cancer*. (2012) 118:3519–30. doi: 10.1002/cncr.26671
28. Klozar J, Koslabova E, Kratochvil V, Salakova M, Tachezy R. Nodal status is not a prognostic factor in patients with HPV-positive oral/oropharyngeal tumors. *J Surg Oncol*. (2013) 107:625–33. doi: 10.1002/jso.23292
29. Maxwell JH, Ferris RL, Gooding W, Cunningham D, Mehta V, Kim S, et al. Extracapsular spread in head and neck carcinoma: impact of site and human papillomavirus status. *Cancer*. (2013) 119:3302–8. doi: 10.1002/cncr.28169
30. Sinha P, Kallogjeri D, Gay H, Thorstad WL, Lewis JS, Chernock R, et al. High metastatic node number, not extracapsular spread or N-classification is a node-related prognosticator in transorally-resected, neck-dissected p16-positive oropharynx cancer. *Oral Oncol*. (2015) 51:514–20. doi: 10.1016/j.oraloncology.2015.02.098
31. Sinha P, Lewis JS, Kallogjeri D, Nussenbaum B, Haughey BH. Soft tissue metastasis in p16-positive oropharynx carcinoma: prevalence and association with distant metastasis. *Oral Oncol*. (2015) 51:778–86. doi: 10.1016/j.oraloncology.2015.05.004
32. Iyer NG, Dogan S, Palmer F, Rahmati R, Nixon IJ, Lee N, et al. Detailed analysis of clinicopathologic factors demonstrate distinct difference in outcome and prognostic factors between surgically treated HPV-positive and negative oropharyngeal cancer. *Ann Surg Oncol*. (2015) 22:4411–21. doi: 10.1245/s10434-015-4525-0
33. Kaczmar JM, Tan KS, Heitjan DF, Lin A, Ahn PH, Newman JG, et al. HPV-related oropharyngeal cancer: risk factors for treatment failure in patients managed with primary transoral robotic surgery. *Head Neck*. (2016) 38:59–65. doi: 10.1002/hed.23850
34. Kumar B, Cipolla MJ, Old MO, Brown NV, Kang SY, Dziegielewski PT, et al. Surgical management of oropharyngeal squamous cell carcinoma: survival and functional outcomes. *Head Neck*. (2016) 38(Suppl. 1):E1794–802. doi: 10.1002/hed.24319
35. Kharytaniuk N, Molony P, Boyle S, O'Leary G, Werner R, Heffron C, et al. Association of extracapsular spread with survival according to human

papillomavirus status in oropharynx squamous cell carcinoma and carcinoma of unknown primary site. *JAMA Otolaryngol Head Neck Surg*. (2016) 142:683–90. doi: 10.1001/jamaoto.2016.0882

36. Tassone P, Crawley M, Bovenzi C, Zhan T, Keane W, Cognetti D, et al. Pathologic markers in surgically treated HPV-associated oropharyngeal cancer: retrospective study, systematic review, and meta-analysis. *Ann Otol Rhinol Laryngol*. (2017) 126:365–74. doi: 10.1177/0003489417693014
37. An Y, Park HS, Kelly JR, Stahl JM, Yarbrough WG, Burtness BA, et al. The prognostic value of extranodal extension in human papillomavirus-associated oropharyngeal squamous cell carcinoma. *Cancer*. (2017) 123:2762–72. doi: 10.1002/cncr.30598
38. Zhan KY, Eskander A, Kang SY, Old MO, Ozer E, Agrawal AA, et al. Appraisal of the AJCC 8th edition pathologic staging modifications for HPV-positive oropharyngeal cancer, a study of the national cancer data base. *Oral Oncol*. (2017) 73:152–9. doi: 10.1016/j.oraloncology.2017.08.020
39. Shevach J, Bossert A, Bakst RL, Liu J, Misiukiewicz K, Beyda J, et al. Extracapsular extension is associated with worse distant control and progression-free survival in patients with lymph node-positive human papillomavirus-related oropharyngeal carcinoma. *Oral Oncol*. (2017) 74:56–61. doi: 10.1016/j.oraloncology.2017.09.014
40. Meyer MF, Meinrath J, Seehawer J, Lechner A, Odenthal M, Quaas A, et al. The relevance of the lymph node ratio as predictor of prognosis is higher in HPV-negative than in HPV-positive oropharyngeal squamous cell carcinoma. *Clin Otolaryngol*. (2018) 43:192–8. doi: 10.1111/coa.12938
41. Miccio JA, Verma V, Kelly J, Kann BH, An Y, Park HS, et al. Impact of contralateral lymph nodal involvement and extranodal extension on survival of surgically managed HPV-positive oropharyngeal cancer staged with the AJCC eighth edition. *Oral Oncol*. (2019) 99:104447. doi: 10.1016/j.oraloncology.2019.104447
42. Beltz A, Gösswein D, Zimmer S, Limburg I, Wünsch D, Gribko A, et al. Staging of oropharyngeal squamous cell carcinoma of the head and neck: prognostic features and power of the 8th edition of the UICC staging manual. *Eur J Surg Oncol*. (2019) 45:1046–53. doi: 10.1016/j.ejso.2019.02.032
43. Han M, Stanford-Moore GB, Larson AR, Schoppy DW, Cognetti DM, Joshi AS, et al. Predictors of mortality in HPV-associated oropharynx carcinoma treated with surgery alone. *Laryngoscope*. (2019) 130:E423–35. doi: 10.1002/lary.28344
44. Freitag J, Wald T, Kuhnt T, Gradistanac T, Kolb M, Dietz A, et al. Extracapsular extension of neck nodes and absence of human papillomavirus 16-DNA are predictors of impaired survival in p16-positive oropharyngeal squamous cell carcinoma. *Cancer*. (2020) 126:1856–72. doi: 10.1002/cncr.32667
45. Gal TJ, O'Brien KJ, Chen Q, Huang B. Clinical vs microscopic extranodal extension and survival in oropharyngeal carcinoma in the human papillomavirus era. *Otolaryngol Head Neck Surg*. (2020) 162:693–701. doi: 10.1177/0194599820910431
46. Alvi A, Johnson JT. Extracapsular spread in the clinically negative neck (N0): implications and outcome. *Otolaryngol Head Neck Surg*. (1996) 114:65–70. doi: 10.1016/S0194-5998(96)70285-1
47. Johnson JT, Myers EN, Bedetti CD, Barnes EL, Schramm VL, Thearle PB. Cervical lymph node metastases. Incidence and implications of extracapsular carcinoma. *Arch Otolaryngol*. (1985) 111:534–7. doi: 10.1001/archotol.1985.00800100082012
48. Künzel J, Psychogios G, Mantsopoulos K, Grundtner P, Waldfahrer F, Iro H. Lymph node ratio as a predictor of outcome in patients with oropharyngeal cancer. *Eur Arch Otorhinolaryngol*. (2014) 271:1171–80. doi: 10.1007/s00405-013-2513-1
49. Künzel J, Mantsopoulos K, Psychogios G, Grundtner P, Koch M, Iro H. Lymph node ratio as a valuable additional predictor of outcome in selected patients with oral cavity cancer. *Oral Surg Oral Med Oral Pathol Oral Radiol*. (2014) 117:677–84. doi: 10.1016/j.oooo.2014.02.032
50. Dünne AA, Müller HH, Eisele DW, Kessel K, Moll R, Werner JA. Meta-analysis of the prognostic significance of perinodal spread in head and neck squamous cell carcinomas (HNSCC) patients. *Eur J Cancer*. (2006) 42:1863–8. doi: 10.1016/j.ejca.2006.01.062
51. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. (2010) 363:24–35. doi: 10.1056/NEJMoa0912217
52. Kompelli AR, Morgan P, Li H, Harris W, Day TA, Neskey DM. Prognostic impact of high-risk pathologic features in hpv-related oropharyngeal squamous cell carcinoma and tobacco use. *Otolaryngol Head Neck Surg*. (2019) 160:855–61. doi: 10.1177/0194599818818446

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Co-incidence of Human Papillomaviruses and Epstein–Barr Virus Is Associated With High to Intermediate Tumor Grade in Human Head and Neck Cancer in Syria

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High-risk human papillomaviruses (high-risk HPVs) have been recently reported to be co-present with Epstein–Barr virus (EBV) in different types of human cancers including head and neck (HN), where they can cooperate in the initiation and/or progression of this cancer. Accordingly, we herein explored the prevalence of high-risk HPVs and EBV in 80 HN cancer tissues from the Syrian population using polymerase chain reaction, immunohistochemistry, and tissue microarray methodologies. We report that high-risk HPVs and EBV are present in 35/80 (43.7%) and 41/80 (51.2%) of our samples, respectively, and the most frequent HPV types are 33, 16, 18, 45, 52, 58, 35, 51, and 31, in this order. More significantly, our data reveal that 25/80 (31.2%) of cancer cases are positive for high-risk HPVs as well as EBV, and their co-presence is associated with high/intermediate-grade squamous cell carcinomas. These data confirm the co-presence of high-risk HPVs and EBV in HN cancers in the Syrian population of the Middle East and demonstrate that their co-incidence is linked to a more aggressive cancer phenotype. Thus, future studies are required to confirm these data and elucidate the exact role of high-risk and EBV cooperation in human HN carcinogenesis.

Keywords: head and neck cancers, human papillomaviruses, Epstein–Barr virus, tumor grade, Syrian population

INTRODUCTION

Head and neck (HN) cancer is a broad term that incorporates epithelial malignancies located in the paranasal sinuses, oral cavity, nasal cavity, pharynx, and larynx (1). HN cancer is one of the most common among both male and female worldwide, with around 650,000 new cases and 330,000 deaths each year assessed by the World Health Organization (2); notably, most of these deaths occur in developing countries (3). When it comes to cancer-related mortality, it is generally either directly attributed to metastasis, as in tumor involvement of critical organs, or caused indirectly due to therapeutic resistance and the adverse effect of treatment on human organs (4, 5).

Today, it is well-known that more than 20% of human cancers are estimated to be linked with microorganism infections including oncoviruses infection especially high-risk human

papillomaviruses (high-risk HPV) and Epstein–Barr virus (EBV) (6–8). More specifically, it has been well-established that high-risk HPV infections are critical etiological factors in the development of human HN cancers, especially oral, as ∼40% of oral cancer cases are positive for high-risk HPVs, particularly types 16, 18, 31, 33, 35, 45, 52, and 58 worldwide including the Middle East (ME) region (7, 9). Additionally, it was pointed out that their presence is linked with vascular invasion and lymph node metastases in different types of human carcinomas including cervical and HN (10–12).

Likewise, EBV is a human gamma herpesvirus that commonly infects more than 90% of the adult population (13). Persistent infection with EBV can cause infectious mononucleosis, and its latent state can lead to several types of human B-cell lymphomas and certain solid cancers, especially nasopharyngeal (14–17); additionally, EBV has been shown to be strongly associated with undifferentiated nasopharyngeal carcinomas (NPCs). Several studies have detected the presence of EBV in HN squamous cell tumors implying its possible role in the development of malignancies throughout the upper aerodigestive tract (7, 18, 19). Moreover, it has been recently revealed that EBNA1 and LMP1 of EBV oncoproteins can enhance invasion of human cancer cells via the induction of epithelial-to-mesenchymal transition (EMT) (20, 21).

On the other hand, several recent studies revealed that high-risk HPVs and EBV are co-present in human HN cancers especially oral (22–24). Moreover, it has been reported that the co-occurrence of high-risk HPVs and EBV in oral cancer is associated with a significant increase in the invasiveness ability of cancer cells (25). We recently demonstrated that the co-presence of high-risk HPVs and EBV is linked to high/intermediate grade in different types of human carcinomas including HN (5, 26, 27). Thus, it is evident that the co-presence of high-risk HPVs and EBV in high-grade human carcinomas could suggest a possible cooperation between their oncoproteins; however, there are only few studies regarding the co-presence of high-risk HPVs and EBV in the ME region focusing only on NPCs.

Therefore, in this investigation, we assessed the presence of high-risk HPVs and EBV and their association with tumor phenotype in human HN cancer samples from Syria. Our study pointed out that high-risk HPVs and EBV are present in 43.7 and 51.2% of our samples, respectively, while co-incidence of these oncoviruses is 32.2%. More significantly, we noted that the co-incidence of these oncoviruses is associated with high/intermediate-grade squamous cell carcinomas in the majority of positive cases.

MATERIALS AND METHODS

High-Risk HPV and EBV Detection

Eighty formalin-fixed paraffin-embedded blocks of HN cancer (57 larynx, 19 lower lip, 3 upper lip, and 1 nasopharynx) from Syrian patients, 73 males and 7 females, with an average age of 54.51 years were used. The samples were obtained from the Pathology Department, Faculty of Medicine of Aleppo University, Syria. Tissue blocks and data used in this report were approved, in March 22, 2009, by the Ethics Committee of the Faculty of Medicine of Aleppo University, # 2009-007, Aleppo, Syria. One hundred nanograms of DNA was extracted from each sample using Qiagen GmbH kit (Hilden, Germany). These samples were analyzed for high-risk HPVs and EBV by PCR using primers for E6/E7 genes of high-risk HPV types (16, 18, 31, 33, 35, 45, 51, 52, and 58) in addition to primers for LMP1 and EBNA1 genes of EBV; meanwhile, primers for the GAPDH gene were utilized as an internal control (26, 28). This analysis was achieved as illustrated earlier by our group (5, 26, 28).

Tissue Microarray

Tissue microarray (TMA) building was realized as elucidated previously by our group (28, 29). Briefly, HN cancer samples were inserted into a virgin paraffin TMA block using a non-automated tissue arrayer (Beecher Instruments, Silver Spring, MD) irrespective of pathological staging information. Three TMA cores of 1.0 mm in diameter were sampled from a cohort of 80 block tissue samples of Syrian HN cancer patients. Afterwards, to verify the histological diagnosis, 4-μm sections were cut and stained with hematoxylin and eosin (H&E). Then, slides of the completed blocks were used for immunohistochemistry assay.

Immunohistochemistry

Immunohistochemical (IHC) procedures examining the expression patterns of E6 and LMP1, of HPV and EBV, were done using standard practices. Briefly, slides were deparaffinized in graded alcohol, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Then, TMA slides were incubated for 35 min at 37°C with primary monoclonal and polyclonal antibodies for E6 of HPV and LMP1 of EBV (clones 1–4 and clone C1P5 from Dako and Calbiochem, Canada, respectively) using an automated immunostainer (Ventana Medical System, Tuscon, AZ). Afterwards, staining procedures were achieved according to the manufacturer's recommendations as slides were counterstained with hematoxylin prior to mounting. Negative controls were achieved by omitting primary antibody for E6 and LMP1. Following immunohistochemistry, two independent observers examined all TMA slides. The tumors were considered positive for E6 and LMP1 oncoproteins if cancer cells exhibited positivity ≥1% at any intensity (≥1+, scale 0–3+).

All TMAs also contained various cores representing positive and negative controls (e.g., cervical carcinoma and lymphatic tissues served as positive controls for HPV and EBV stains, respectively; normal HN tissues and epithelium were used as negative controls).

Statistical Analysis

Statistical assessments were achieved using IBM SPSS Statistics (version 22; SPSS Inc., Chicago, IL, USA) and R. Data were analyzed as non-parametric files. We used χ^2-test with Yates correction to explore the significance of the association between tumor grade and the co-incidence of high-risk HPVs and EBV.

RESULTS

In order to classify the presence of high-risk HPVs and EBV in human HN cancer tissues in the ME region, we explored the

TABLE 1 | High-risk HPVs and EBV detection in human head and neck cancers.

Number of samples[#]	HPVs+	EBV+	HPVs+/EBV+
Positive cases (%)[##]	35/80 (43.7)	41/80 (51.2)	25/80 (31.2)

The presence of HPVs and EBV was found in 35 (43.7%) and 41 (51.2%) of the 80 cancer samples, respectively, while we observed that 25 (31.2%) of cancer cases are positive for both high-risk HPVs and EBV. The presence of these oncoviruses was confirmed by PCR and IHC using specific primers for E6/E7 and LMP1 as well as EBNA1 genes of high-risk HPVs and EBV, in addition to monoclonal antibodies for E6 and LMP1, as illustrated in the Materials and Methods section.

[#]The total number of samples examined in this study is 80.

[##]These two methodologies, PCR and IHC, were used to detect the presences of high-risk HPVs and EBV.

TABLE 2 | Presence of HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 58 in human HN cancer.

No. of cases	High-risk HPVs								
	16	18	31	33	35	45	51	52	58
80	31/80	28/80	13/80	34/80	18/80	25/80	15/80	22/80	20/80

We note that HPV types 33, 16, 18, and 45 are the most common in HN cancer in the Syrian population.

incidence of high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 58 in a cohort of 80 HN cancer specimens from the Syrian population by PCR analysis, using specific primers for E6/E7 and LMP1 as well as EBNA1 genes of HPVs and EBV, respectively (5, 26, 28). Our data revealed that 35 (43.7%) and 41 (51.2%) of the 80 cancer samples are positive for high-risk HPVs and EBV, respectively (**Table 1**), and all of HPVs-positive specimens are co-infected with more than one HPV type. Regarding high-risk HPVs genotyping in these samples, our results pointed out that the most prevalent high-risk HPVs among the positive samples are types 33 (34/80), 16 (31/80), 18 (28/80), 45 (25/80), 52 (22/80), 58 (21/80), 35 (18/80), 51 (15/80), and 31 (13/80), as shown in **Table 2**.

Next, we investigated the co-presence of high-risk HPVs and EBV in our HN cancer samples by IHC and PCR analysis using monoclonal antibodies, for E6 and LMP1, as well as specific primers for these oncoproteins/genes, respectively; we found that 25 (31.2%) of the 80 cancer cases are positive for both high-risk HPVs and EBV with $P < 0.001$ (**Table 1**). Furthermore, we examined the relation between the co-presence of these oncoviruses and tumor grade in these samples. Our data revealed that the co-expression of E6 and LMP1 oncoproteins of high-risk HPVs and EBV, respectively, in the majority of cases (88%) is associated with high/intermediate (G3/G2)-grade invasive carcinoma form in comparison with high-risk HPVs+ or EBV+ alone cases or HPVs/EBV-negative cases, which are 4/10 (40%), 5/16 (31.2%), and 3/29 (10.2%) with $P = 0.00328$, 0.00018, and <0.001, respectively (**Table 3** and **Figures 1**, **2**), while it is important to highlight that cancer phenotype in the HPV^+/EBV^+ was not linked to a specific HPV type since all our positive cases were infected with more than one type of high-risk HPVs. Finally, normal HN tissues and epithelial cells, which served as controls, were shown to be negative for both high-risk HPVs as well as EBV.

TABLE 3 | High-risk HPVs and EBV status in relation to tumor grade in HN cancer samples.

EBV/HPVs status	$EBV^+/HPVs^+$ (%)	$EBV^+/HPVs^-$ (%)	$EBV^-/HPVs^+$ (%)	$EBV^-/HPVs^-$ (%)
TUMOR GRADE				
High	5 (20.0)	1 (10.0)	2 (12.5)	1 (3.4)
Intermediate	17 (68.0)	3 (30.0)	3 (18.7)	2 (6.8)
Low	3 (12.0)	6 (60.0)	11 (68.7)	26 (89.6)
Number of samples[#]	25 (31.2)	10 (12.5)	16 (20.0)	29 (36.2)

We notice that 88% of HPVs- and EBV-positive cases are high/intermediate-grade carcinomas in comparison with EBV^+/HPV^-, EBV^-/HPV^+, and EBV^-/HPV^-, which are 40, 31.2, and 10.2% with a P-value of 0.00328, 0.00018, and <0.001, respectively.

[#]The total number of samples examined in this study is 80.

Values inside parentheses denote percentage.

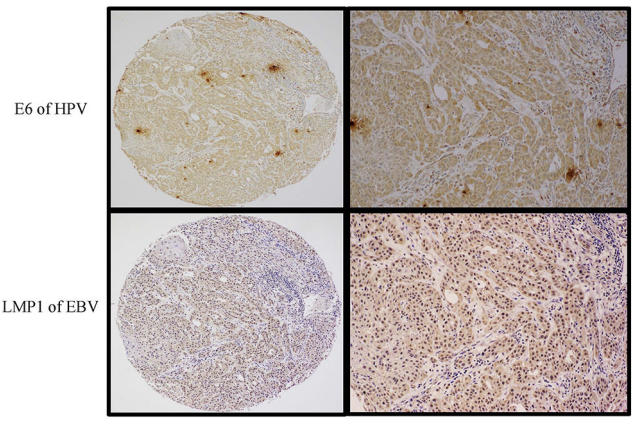

FIGURE 1 | High-grade (Grade 3), non-keratinizing squamous cell carcinomas with basaloid appearance. Tumor cells were diffusely (>75%) and strongly (2+ and 3+ intensity on the scale 0–3) positive for both HPV (E6, upper images) and EBV (LMP1 protein) (lower images). The left-sided images are captured at 4× magnification, while the right-side ones are at 10× magnification (Olympus BX53). Expression of both proteins was assessed by immunohistochemistry using a semiquantitative approach.

DISCUSSION

In this investigation, we explored, for the first time, the incidence/co-incidence of high-risk HPVs and EBV in human HN cancer and the association of their co-presence with tumor grade in Syria, which can also be considered the first study of this type in the ME region. We report that high-risk HPVs and EBV are present in 43.7 and 51.2%, respectively, in our Syrian samples, and the most frequent HPV types in HN cancer in Syria are 33, 16, 18, 45, 52, 58, 35, 51, and 31, correspondingly. Meanwhile, our data pointed out that 31.2% of the samples are positive for both high-risk HPVs and EBV. More significantly, we report that the co-presence of these oncoviruses is associated with high/intermediate tumor grade in 88% of the samples in

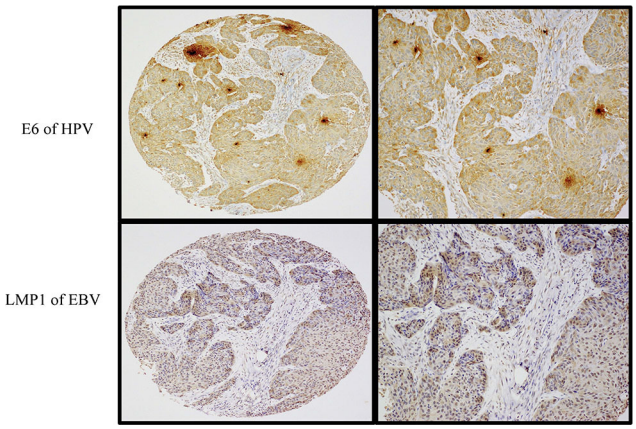

FIGURE 2 | A case of moderately differentiated (Grade 2) squamous cell carcinoma exhibiting co-expression of E6 (upper images) and LMP1 (lower images) oncoproteins of HPV and EBV, respectively. Left-sided images are captured at 4× magnification, while the right-sided ones are at 10× magnification (Olympus BX53). Note that the expression of E6 protein was observed in >90% of cancer cells while LMP1 protein was expressed in ∼70% of cancer cells. Expression of both oncoproteins was assessed by immunohistochemistry using a semiquantitative approach.

comparison to HPVs or EBV-positive alone and HPVs/EBV-negative samples. Regarding the most common HPV types in the Syrian population, the present data concur with our previous studies on HPVs in different types of human carcinomas including cervical and breast in Syria where we found that HPV type 33 is the most frequent in these cancer tissues. In this context, HPV type 33 was reported to be the most common in breast cancer in Turkey (30). Herein, it is important to highlight that the Syrian samples were collected from Aleppo province, which is located in the northern part of the country bordering Turkey. Accordingly, our data confirm that specific types of high-risk HPV infection, in human cancers, are related to certain geographic locations, as it was demonstrated by a large number of investigations worldwide (7, 31–36).

Concerning the co-presence of HPVs and EBV in HN cancer in the ME region, Tatli Dogan et al. (37) published a study regarding the incidence of HPVs and EBV in NPCs in Turkey, they found that 72 of their 82 samples are positive for EBV and only one case revealed positive for HPVs; meanwhile, they reported that the highest rate of EBV positivity correspond with undifferentiated NPCs. However, and in agreement with our investigation, one report from North Africa assessed the presence and co-presence of high-risk HPVs and EBV in 70 cases of NPCs from the Moroccan population (38). Their study revealed that 24 of the samples are positive for high-risk HPVs, and the most frequent HPV types are 31, 59, 16, 18, 33, and 35. They found that all their cancer cases are positive for EBV. Consequently, 24 (34%) Moroccan samples were positive for both high-risk HPVs and EBV, which are in their majority NPCs grade III and II. It is important to highlight that the Turkish and Moroccan studies in addition to another investigation from Iran with only 20 cancer cases (39) focused only on the co-presence of HPVs and EBV in NPCs; therefore, our investigation can be considered the first study regarding the co-presence of these oncoviruses in HN cancer in the ME region since our samples include tissues from several HN locations.

On the other hand, in 2012, Jalouli et al. (40) examined the incidence and co-incidence of high-risk HPVs and EBV in 155 oral squamous cell carcinomas (OSCCs) from eight different countries from Europe, Asia, Africa, and North America with a limited number of cases ∼20 cancer cases from each country including Sudan and Yemen from the ME region. They found that 35 and 55% of the samples are positive for HPVs and EBV, respectively. They reported that HPVs and EBV are co-present in 21% of all OSCCs. However, no clear conclusions can be drawn from this study due to the limited number of cancer cases from participant countries. Meanwhile, there are two recently published investigations from Europe regarding the presence of HPVs and EBV in human oral cancer; one from Poland showed that HPVs and EBV are co-present in 34.1% of cancer cases in comparison with HPV and EBV infections alone, which are 28.1 and 54.7%, respectively (23). In the second study from Finland, the authors reported that the co-incidence of HPV and EBV is 14% in the population of Finland (41). In comparison, our study focused on the presence/co-presence of HPVs and EBV in HN cancer in Syria with an acceptable number of samples, which allowed us to make an adequate conclusion about these oncoviruses in HN cancer in this country, revealing that 31.2% of the samples are $HPVs^{+}/EBV^{+}$, which is comparable with the study published from Poland.

The co-presence of high-risk HPVs and EBV and their association with tumor phenotype in HN cancer is clearly demonstrated in our present study regardless of HPV type since all our HPV/EBV-positive cases are infected with more than one HPV virus strain. Our findings are in agreement with several investigations worldwide, including three from our group in addition to the Turkish and Moroccan studies; data of these reports pointed out that the co-presence of HPVs and EBV is associated with high-grade carcinomas in addition to positive axillary lymph nodes (5, 23, 25–27, 42, 43). Indeed, it has been reported that prevalence of poorly differentiated tumors is four times more frequent in HPV/EBV co-infection in comparison with EBV or HPV infection alone in oral cancer samples from Poland (23); in addition, the study pointed out that there is a significant correlation between tumor dimensions in co-infected patients compared with single infection. However, a recent investigation in NPCs reported that 5-year overall survival is significantly higher in HPV/EBV-positive patients in comparison with HPV/EBV-negative ones (41). Well, this could be due to radiation sensitivity as demonstrated by several investigations. Actually, earlier studies in HPV-positive cases of HNSCC found that the virus takes control of the cellular machinery for DNA repair, altering cell cycle distribution and causing hypoxia during radiation treatment (44, 45). On the other hand, numerous studies on the alteration of radiation response by EBV reported that LMP-1 blocks DNA repair by suppressing the phosphorylation and activity of DNA-dependent protein kinase, a key enzyme of non-homologous end-joining pathway in NPCs, and by repressing ATM, which ultimately modulates resistance of ionizing radiation-induced apoptotic cell death (46).

Apropos the mechanism of HPVs and EBV interaction, based on the fact that high-risk HPVs and EBV oncoproteins share different downstream pathways, we assumed that oncoproteins (E5, E6/E7, LMP1, and EBNA1) of these oncoviruses can cooperate in the initiation and/or progression of several types of human carcinomas where the EMT event can play a crucial role in this procedure (47). Indeed, earlier investigations showed that E5 and E6/E7 oncoproteins of high-risk HPVs can enhance cell invasion and cancer progression via the induction of EMT in several types of human cancers including cervical and oral as well as NPCs (48–53). On the other hand, it has been reported by several investigations that LMP1, LMP2A, EBNA3C, and EBNA1 oncoproteins of EBV can enhance cancer progression via the modulation of EMT in human carcinomas including NPCs (20, 53–56). Meanwhile, our preliminary data showed that E6/E7 of HPV type 16 can cooperate with LMP1 of EBV to enhance EMT progression and consequently cell motility via the activation (phosphorylation) of Erk1/Erk2 and β-catenin (in preparation). Nevertheless, further studies are needed to elucidate the complete pathogenesis and role of the co-incidence of high-risk HPVs and EBV in human carcinomas including HN, especially since HPVs and EBV vaccines are currently available and under clinical trial, respectively (57–59). This is a key step, which could possibly limit HPV and EBV infection and their associated cancers including HN malignancy initiation and development to a metastatic form, thus diminishing cancer-related mortalities especially in developing countries where oncoviruses-associated cancers are still considered major causes of death among both males and females in these countries.

Lastly, with regard to the number of specimens that we were able to amass from Aleppo, Syria, it is essential to confirm our data using a larger number of samples from different areas in this country and in combination with numerous investigations from the ME in general.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Faculty of Medicine of Aleppo University, # 2009-007. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

A-EA, SV, and HA-T conceived the study. LG provided the samples. IG, LG, SV, MM, AY, HA-T, and A-EA analyzed the data. All authors wrote and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Daraei P, Moore CE. Racial disparity among the head and neck cancer population. *J Cancer Educ*. (2015) 30:546–51. doi: 10.1007/s13187-014-0753-4
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. (2015) 136:E359–86. doi: 10.1002/ijc.29210
3. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *J Clin Oncol*. (2013) 31:4550–9. doi: 10.1200/JCO.2013.50.3870
4. Al Moustafa A, Yasmeen A, Ghabreau L, Mohamed AH, Achkhar A. Brain metastases progression of breast cancer, breast cancer - carcinogenesis, cell growth and signalling pathways. In: Gunduz M, editor. London, UK: IntechOpen (2011).
5. Al-Thawadi H, Ghabreau L, Aboulkassim T, Yasmeen A, Vranic S, Batist G, et al. Co-Incidence of Epstein-Barr virus and high-risk human papillomaviruses in cervical cancer of Syrian women. *Front Oncol*. (2018) 8:250. doi: 10.3389/fonc.2018.00250
6. de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol*. (2012) 13:607–15. doi: 10.1016/S1470-2045(12)70137-7
7. Al Moustafa AE, Al-Awadhi R, Missaoui N, Adam I, Durusoy R, Ghabreau L, et al. Human papillomaviruses-related cancers. Presence and prevention strategies in the middle east and north African regions. *Hum Vaccin Immunother*. (2014) 10:1812–21. doi: 10.4161/hv.28742
8. Al Moustafa AE, Cyprian FS, Al-Antary N, Yasmeen A. High-risk human papillomaviruses and Epstein-Barr virus presence and crosstalk in human oral carcinogenesis. In: Al Moustafa AE, editor. *Development of Oral Cancer: Risk Factors and Prevention Strategies*. Cham: Springer International Publishing (2017). p. 83–94.
9. de Sanjosé S, Serrano B, Tous S, Alejo M, Lloveras B, Quirós B, et al. Burden of Human Papillomavirus (HPV)-related cancers attributable to HPVs 6/11/16/18/31/33/45/52 and 58. *JNCI Cancer Spectrum*. (2019) 2:pky045. doi: 10.1093/jncics/pky045
10. Graflund, M. SB, Sigurdardóttir S, Karlsson M. HPV-DNA, vascular space invasion, and their impact on the clinical outcome in early-stage cervical carcinomas. *Int J Gynecol Cancer*. (2004) 14:896–902. doi: 10.1111/j.1048-891X.2004.014527.x
11. Zuna R, Allen RA, Moore WE, Mattu R, Dunn ST. Comparison of human papillomavirus genotypes in high-grade squamous intraepithelial lesions and invasive cervical carcinoma: evidence for differences in biologic potential of precursor lesions. *Mod Pathol*. (2004) 17:1314–22. doi: 10.1038/modpathol.3800223
12. Umudum H, Rezanko T, Dag F, Dogruluk T. Human papillomavirus genome detection by in situ hybridization in fine-needle aspirates of metastatic lesions

from head and neck squamous cell carcinomas. *Cancer*. (2005) 105:171–7. doi: 10.1002/cncr.21027
13. Niedobitek G, Meru N, Delecluse HJ. Epstein-Barr virus infection and human malignancies. *Int J Exp Pathol*. (2001) 82:149–70. doi: 10.1046/j.1365-2613.2001.iep0082-0149-x
14. Middeldorp JM, Brink AA, van den Brule AJ, Meijer CJ. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Crit Rev Oncol Hematol*. (2003) 45, 1–36. doi: 10.1016/S1040-8428(02)00078-1
15. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer*. (2004) 4:757–68. doi: 10.1038/nrc1452
16. Murata T, Tsurumi T. Switching of EBV cycles between latent and lytic states. *Rev Med Virol*. (2014) 24:142–53. doi: 10.1002/rmv.1780
17. Elgui de Oliveira D, Müller-Coan BG, Pagano JS. Viral carcinogenesis Beyond malignant transformation: EBV in the progression of human cancers. *Trends Microbiol*. (2016) 24:649–64. doi: 10.1016/j.tim.2016.03.008
18. Gonzalez-Moles MA, Gutierrez J, Rodriguez MJ, Ruiz-Avila I, Rodriguez-Archilla A. Epstein-Barr Virus Latent Membrane Protein-1 (LMP-1) expression in oral squamous cell carcinoma. *Laryngoscope*. (2002) 112:482–7. doi: 10.1097/00005537-200203000-00014
19. Goldenberg D, Benoit NE, Begum S, Westra WH, Cohen Y, Koch WM, et al. Epstein-Barr Virus in head and neck cancer assessed by quantitative polymerase chain reaction. *Laryngoscope*. (2004) 114:1027–31. doi: 10.1097/00005537-200406000-00013
20. Gaur N, Gandhi J, Robertson ES, Verma SC, Kaul R. Epstein-Barr virus latent antigens EBNA3C and EBNA1 modulate epithelial to mesenchymal transition of cancer cells associated with tumor metastasis. *Tumour Biol*. (2015) 36:3051–60. doi: 10.1007/s13277-014-2941-6
21. Zuo LL, Zhang J, Liu LZ, Zhou Q, Du SJ, Xin SY, et al. Cadherin 6 is activated by Epstein-Barr virus LMP1 to mediate EMT and metastasis as an interplay node of multiple pathways in nasopharyngeal carcinoma. *Oncogenesis*. (2017) 6:402–402. doi: 10.1038/s41389-017-0005-7
22. Deng Z, Uehara T, Maeda H, Hasegawa M, Matayoshi S, Kiyuna A, et al. Epstein-Barr virus and human papillomavirus infections and genotype distribution in head and neck cancers. *PLoS ONE*. (2014) 9:e113702. doi: 10.1371/journal.pone.0113702
23. Drop B, Strycharz-Dudziak M, Kliszczewska E, Polz-Dacewicz M. Coinfection with Epstein-Barr Virus (EBV), Human Papilloma Virus (HPV) and Polyoma BK Virus (BKPyV) in laryngeal, oropharyngeal and oral cavity cancer. *Int J Mol Sci*. (2017) 18:2752. doi: 10.3390/ijms18122752
24. Huang WB, Chan JYW, Liu DL. Human papillomavirus and World Health Organization type III nasopharyngeal carcinoma: multicenter study from an endemic area in Southern China. *Cancer*. (2018) 124:530–6. doi: 10.1002/cncr.31031
25. Jiang R, Ekshyyan O, Moore-Medlin T, Rong X, Nathan S, Gu X, et al. Association between human papilloma virus/Epstein–Barr virus coinfection and oral carcinogenesis. *J Oral Pathol Med*. (2015) 44:28–36. doi: 10.1111/jop.12221
26. Al Moustafa AE, Al-Antary N, Aboulkassim T, Akil N, Batist G, Yasmeen A. Co-prevalence of Epstein-Barr virus and high-risk human papillomaviruses in Syrian women with breast cancer. *Hum Vaccin Immunother*. (2016) 12:1936–9. doi: 10.1080/21645515.2016.1139255
27. Malki MI, Gupta I, Fernandes Q, Aboulkassimc T, Yasmeen A, Vranic S, et al. Co-presence of Epstein–Barr virus and high-risk human papillomaviruses in Syrian colorectal cancer samples. *J Hum Vaccin Immunother*. (2020). doi: 10.1080/21645515.2020.1726680. [Epub ahead of print].
28. Darnel AD, Wang D, Ghabreau L, Yasmeen A, Sami S, Akil N, et al. Correlation between the presence of high-risk human papillomaviruses and Id gene expression in Syrian women with cervical cancer. *Clin Microbiol Infect*. (2010) 16:262–6. doi: 10.1111/j.1469-0691.2009.02774.x
29. Akil N, Yasmeen A, Kassab A, Ghabreau L, Darnel AD, Al Moustafa AE. High-risk human papillomavirus infections in breast cancer in Syrian women and their association with Id-1 expression: a tissue microarray study. *Br J Cancer*. (2008) 99:404–7. doi: 10.1038/sj.bjc.6604503
30. Gumus M, Yumuk PF, Salepci T, Aliustaoglu M, Dane F, Ekenel M, et al. HPV DNA frequency and subset analysis in human breast cancer patients' normal and tumoral tissue samples. *J Exp Clin Cancer Res*. (2006) 25, 515–21.
31. Abramowitz L, Jacquard A-C, Jaroud F, Haesebaert J, Siproudhis L, Pradat P, et al. Human papillomavirus genotype distribution in anal cancer in France: the EDiTH V study. *Int J Cancer*. (2011) 129:433–9. doi: 10.1002/ijc.25671
32. García Muentes GD, García Rodríguez LK, Burgos Galarraga RI, Almeida Carpio F, Ruiz Cabezas JC. Genotypes distribution of human papillomavirus in cervical samples of Ecuadorian women. *Revista Brasileira de Epidemiologia*. (2016) 19:160–6. doi: 10.1590/1980-5497201600010014
33. Li Z, Liu F, Cheng S, Shi L, Yan Z, Yang J, et al. Prevalence of HPV infection among 28,457 Chinese women in Yunnan Province, southwest China. *Sci Rep*. (2016) 6:21039. doi: 10.1038/srep21039
34. Wang L, Wang P, Ren Y, Du J, Jiang J, Jia X, et al. Prevalence of High-Risk Human Papillomavirus (HR-HPV) genotypes and multiple infections in cervical abnormalities from Northern Xinjiang, China. *PLoS ONE*. (2016) 11:e0160698. doi: 10.1371/journal.pone.0160698
35. Gheit T, Anantharaman D, Holzinger D, Alemany L, Tous S, Lucas E, et al. Role of mucosal high-risk human papillomavirus types in head and neck cancers in central India. *Int J Cancer*. (2017) 141:143–51. doi: 10.1002/ijc.30712
36. Tang Y, Zheng L, Yang S, Li B, Su H, Zhang LP. Epidemiology and genotype distribution of human papillomavirus (HPV) in Southwest China: a cross-sectional five years study in non-vaccinated women. *Virol J*. (2017) 14:84. doi: 10.1186/s12985-017-0751-3
37. Tatli Dogan H, Kiliçarslan A, Dogan M, Süng,ü N, Güler Tezel G, Güler G. Retrospective analysis of oncogenic human papilloma virus and Epstein-Barr virus prevalence in Turkish nasopharyngeal cancer patients. *Pathol Res Pract*. (2016) 212:1021–6. doi: 10.1016/j.prp.2016.08.013
38. Laantri N, Attaleb M, Kandil M, Naji F, Mouttaki T, Dardari R, et al. Human papillomavirus detection in moroccan patients with nasopharyngeal carcinoma. *Infect Agents Cancer*. (2011) 6:3–3. doi: 10.1186/1750-9378-6-3
39. Mirzamani N, Salehian P, Farhadi M, Tehran EA. Detection of EBV and HPV in nasopharyngeal carcinoma by in situ hybridization. *Exp Mol Pathol*. (2006) 81:231–4. doi: 10.1016/j.yexmp.2006.04.006
40. Jalouli J, Jalouli MM, Sapkota D, Ibrahim SO, Larsson PA., Sand L. Human papilloma virus, herpes simplex virus and Epstein Barr virus in oral squamous cell carcinoma from eight different countries. *Anticancer Res*. (2012) 32:571–80.
41. Ruuskanen M, Irjala H, Minn H, Vahlberg T, Randen-Brady R, Hagström J, et al. Epstein-Barr virus and human papillomaviruses as favorable prognostic factors in nasopharyngeal carcinoma: a nationwide study in Finland. *Head Neck*. (2019) 41:349–57. doi: 10.1002/hed.25450
42. Glenn WK, Heng B, Delprado W, Iacopetta B, Whitaker NJ, Lawson JS. Epstein-Barr virus, human papillomavirus and mouse mammary tumour virus as multiple viruses in breast cancer. *PLoS ONE*. (2012) 7:e48788–e48788. doi: 10.1371/journal.pone.0048788
43. Broccolo F, Ciccarese G, Rossi A, Anselmi L, Drago F, Toniolo A. Human papillomavirus (HPV) and Epstein-Barr virus (EBV) in keratinizing versus non- keratinizing squamous cell carcinoma of the oropharynx. *Infect Agents Cancer*. (2018) 13:32. doi: 10.1186/s13027-018-0205-6
44. Weaver AN, Cooper TS, Rodriguez M, Trummell HQ, Bonner JA, Rosenthal EL, et al. DNA double strand break repair defect and sensitivity to poly ADP-ribose polymerase (PARP) inhibition in human papillomavirus 16-positive head and neck squamous cell carcinoma. *Oncotarget*. (2015) 6:26995–7007. doi: 10.18632/oncotarget.4863
45. Swick AD, Chatterjee A, De Costa A-MA, Kimple RJ. Modulation of therapeutic sensitivity by human papillomavirus. *Radiother Oncol*. (2015) 116:342–5. doi: 10.1016/j.radonc.2015.09.002
46. Rödel F, Martin D, Balermpas P, Wieland U, Winkelmann R, Riekmann T, et al. Modulation of radiation sensitivity and antitumor immunity by viral pathogenic factors: Implications for radio-immunotherapy. *Biochim Biophys Acta Rev Cancer*. (2019) 1871:126–37. doi: 10.1016/j.bbcan.2018.12.006
47. Cyprian FS, Al-Farsi HF, Vranic S, Akhtar S, Al Moustafa A-E. Epstein-Barr virus and human papillomaviruses interactions and their roles in the initiation of epithelial-mesenchymal transition and cancer progression. *Front Oncol*. (2018) 8:111. doi: 10.3389/fonc.2018.00111
48. Cheng Y-M, Chou C-Y, Hsu Y-C, Chen M-J, Wing L-YC. The role of human papillomavirus type 16 E6/E7 oncoproteins in cervical epithelial-mesenchymal transition and carcinogenesis. *Oncol Lett*. (2012) 3:667–71. doi: 10.3892/ol.2011.512

49. Chamulitrat W, Sattayakhom A, Herold-Mende C, Stremmel W. Human papillomavirus 16 E6/E7-immortalized human gingival keratinocytes with epithelial mesenchymal transition acquire increased expression of cIAP-1, Bclx and p27Kip1. *Exp Dermatol.* (2009) 18:1067–9. doi: 10.1111/j.1600-0625.2009.00888.x
50. Jung YS, Kato I, Kim HR. A novel function of HPV16-E6/E7 in epithelial-mesenchymal transition. *Biochem Biophys Res Commun.* (2013) 435:339–44. doi: 10.1016/j.bbrc.2013.04.060
51. Hatakeyama H, Mizumachi T, Sakashita T, Kano S, Homma A, Fukuda S. Epithelial-mesenchymal transition in human papillomavirus-positive and -negative oropharyngeal squamous cell carcinoma. *Oncol Rep.* (2014) 32:2673–79.
52. Wakisaka N, Yoshida S, Kondo S, Kita M, Sawada-Kitamura S, Endo K, et al. Induction of epithelial-mesenchymal transition and loss of podoplanin expression are associated with progression of lymph node metastases in human papillomavirus-related oropharyngeal carcinoma. *Histopathology.* (2015) 66:771–80. doi: 10.1111/his.12496
53. Chen X, Bode AM, Dong Z, Cao Y. The epithelial-mesenchymal transition (EMT) is regulated by oncoviruses in cancer. *Faseb j.* (2016) 30:3001–10. doi: 10.1096/fj.201600388R
54. Horikawa T, Yang J, Kondo S, Yoshizaki T, Joab I, Furukawa M, et al. Twist and Epithelial-mesenchymal transition are induced by the EBV oncoprotein latent membrane protein 1 and are associated with metastatic nasopharyngeal carcinoma. *Cancer Research.* (2007) 67:1970–8. doi: 10.1158/0008-5472.Can-06-3933
55. Horikawa T, Yoshizaki T, Kondo S, Furukawa M, Kaizaki Y, Pagano JS. Epstein-Barr Virus latent membrane protein 1 induces Snail and epithelial-mesenchymal transition in metastatic nasopharyngeal carcinoma. *Br J Cancer.* (2011) 104:1160–7. doi: 10.1038/bjc.2011.38
56. Kong Q-L, Hu L-J, Cao J-Y, Huang Y-J, Xu L-H, Liang Y, et al. Epstein-Barr virus-encoded LMP2A induces an epithelial-mesenchymal transition and increases the number of side population stem-like cancer cells in nasopharyngeal carcinoma. *PLoS Pathog.* (2010) 6:e1000940. doi: 10.1371/journal.ppat.1000940
57. Lee LY, Garland SM. Human papillomavirus vaccination: the population impact. *F1000Research.* (2017) 6:866. doi: 10.12688/f1000research.10691.1
58. Luxembourg A, Moeller E. 9-Valent human papillomavirus vaccine: a review of the clinical development program. *Expert Rev Vaccin.* (2017) 16:1119–39. doi: 10.1080/14760584.2017.1383158
59. Rajčáni J, Bánáti F, Szenthe K, Szathmary S. The potential of currently unavailable herpes virus vaccines. *Expert Rev Vaccin.* (2018) 17:239–48. doi: 10.1080/14760584.2018.1425620

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REVIEW
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HPV Detection in Head and Neck Squamous Cell Carcinomas: What Is the Issue?

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Besides classic tobacco and alcohol risk factors, human papillomavirus (HPV) plays a role in the development of a subset of head and neck squamous cell carcinomas (HNSCCs), and notably oropharynx squamous cell carcinomas (OPSCCs). HPV-induced OPSCCs have a different biological behavior and a better prognosis compared to non-HPV-induced OPSCCs and the eighth-edition TNM classification now separates these two entities. Therefore, determining the HPV status of patients with OPSCC is now essential for treatment, prognosis, and development of clinical trials. In this review, after reminding essential steps of HPV implication in the cell cycle, we describe the existing tools that are currently feasible in routine practice according to facilities available in health structures, with their benefits and drawbacks: HPV PCR, E6/E7 mRNA RT-PCR, E6/E7 mRNA *in situ* hybridization, HPV DNA *in situ* hybridization, and P16 immunochemistry. Besides these traditional HPV detection tools, novel diagnostic approaches are being evaluated for HPV-induced OPSCC "ultrastaging." E6 humoral response and ddPCR-detecting HPVct DNA are two techniques performed on blood and are therefore non-invasive. Baseline E6 humoral levels could have a prognostic value, and HPVct DNA could be helpful for HPV OPSCC recurrence monitoring. At last, next-generation sequencing (NGS)-based "capture HPV" is a technique feasible on biopsies and circulating DNA material. It helps characterize HPV integration status and sites, and it could define prognostic subgroups in HPV-induced OPSCC. These novel precision detection tools could be further integrated in the care of patients with HPV-induced OPSCC.

Keywords: HPV, DNA hybridization, RNA hybridization, p16, RNAscope, PCR, head and neck, squamous cell carcinoma

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) constitute a group of malignant tumors located in the oropharynx, larynx, hypopharynx, nasopharynx, and oral cavity. All together, they represent approximately 800,000 new cases and 400,000 deaths per year (1). Classic risk factors include tobacco and alcohol exposure, but it is now established that human papillomavirus (HPV)

plays a major role in the development of oropharyngeal squamous cell carcinomas (OPSCCs) (2–5). This role is not so clear in non-oropharyngeal squamous cell carcinomas (non-OPSCCs), but some reports suggest a possible association between HPV infection and nasopharyngeal carcinomas (6, 7). HPV infection is found in 20–60% of the OPSCCs depending on the countries (8) with, for example, approximately 20% of HPV-induced OPSCCs in Bangladesh and South China (9, 10) and higher rates of HPV-induced OPSCCs in Western Europe and North America (11). Subdividing the HNSCCs in oropharyngeal and non-oropharyngeal carcinomas is therefore well integrated now, because of their different carcinogenesis. HPV-induced OPSCCs tend to occur more often in non-smokers and are associated with more frequent nodal involvement (4, 5). Previous studies reporting the HPV-induced OPSCC occurrence mostly in younger patients seem now to be countered by recent reports revealing that they can also develop at a later age under certain geographic and sociosexual conditions (12–14). Moreover, HPV-induced OPSCCs have a better prognosis than non-HPV-induced OPSCCs, with a better sensitivity to radiations and a better overall survival (5, 15). More broadly, HPV-induced OPSCCs have a better prognosis regardless of the modality of treatment (16–18). For HPV-positive non-OPSCCs, some subgroups might also have a better prognosis, but studies are heterogeneous and controversial (19). Because of these significant biological and clinical differences, HPV-induced OPSCCs have their own classification in the eighth edition of the UICC TNM classification (Union for International Cancer Control) (20). In this context, determining HPV status in HNSCCs and especially in OPSCCs has become mandatory. Besides, several trials based on radiation de-escalation programs or on immunotherapy are evaluating performances of treatments according to HPV status in OPSCCs, and it is essential to adequately classify patients (16, 21–23). Interestingly, the College of American Pathologists has recently published guidelines for HPV testing in HNSCCs (24). These recommendations focus on diagnostic tests in routine practice, and many of them are based on expert consensus opinion. According to these guidelines, all OPSCC samples should be tested for HPV. In this review, we will present the different tests currently used and give an insight into novel diagnostic approaches currently available in research but that could be further used in routine practice.

HPV INVOLVEMENT IN THE CELL CYCLE

HPV involved in mucosal cancer can be divided into two main groups, depending on their oncogenic associated risk. Low-risk HPV are very rarely associated with the development of cancers, and HPV-induced OPSCCs are usually developed after a high-risk HPV infection. Conversely, high-risk HPV genotypes encompass HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68. The high-risk genotypes produce E6 and E7 oncoproteins. E6 protein binds to tumor suppressor p53 by the formation of a trimeric complex E6/E6AP/p53 (25), leading to the proteolytic degradation of p53 (26, 27). E7 protein binds to pRb (phosphorylated retinoblastoma protein), releasing E2F transcription factor and then promoting cell-cycle progression, and consecutively to p16 overexpression. Briefly, p16 is a CDK (cyclin-dependent kinase) inhibitor. This protein is involved in the pRB pathway, implicated in cell-cycle regulation. p16 protein has a cell-cycle regulation role by inhibiting the S phase. It is important to underline that interaction between p16 with CDK4/6 avoids CDK4/6-cyclin D complex formation and phosphorylation of Rb. Overall, p16 overexpression avoids phosphorylation of Rb family members, leading to capture of E2F by Rb proteins and thus to cell-cycle arrest into the G1 phase (28). Low-risk HPV produce E6 and E7 proteins which have lower affinity for p53 and pRb proteins (29) and thus are not theoretically associated with cell-cycle progression, nor with p16 overexpression. Nevertheless, no study has systematically studied the patterns of p16 expression in OPSSCs associated with low-risk HPVs. The reason why high-risk HPV-induced cancers overexpress p16 protein has been partially answered by studying epigenetic changes in HPV16 E7-expressing human epithelial cell lines (30). Independent of its function to inhibit pRB, E7 oncoprotein is responsible of KDM6B demethylase upregulation, leading to decreased levels of repressive H3K27me3 marks in the p16INK4a-encoding CDKN2A promoter region, responsible for the overexpression of the p16 protein. At last, maintenance of an HPV malignant phenotype (e.g., promotion of proliferation and prevention of apoptosis) in established HPV16-positive human OPSCC cell lines requires E6 and E7 proteins, as shown by Rampias et al. using shRNA targeting E6 and E7 transcripts (31).

MAIN TECHNIQUES USED TO DETECT HPV IN OPSCC

According to recent studies, based on this well-known molecular characteristic of the HPV virus to drive the cell toward a tumoral phenotype, different techniques have been developed. They tend to certify the HPV implication in OPSCC tissues: PCR (HPV DNA detection), RT-PCR (E6 and E7 mRNA detection), p16 immunohistochemistry, *in situ* hybridization targeting DNA (DNA ISH), and *in situ* hybridization targeting RNA (RNA ISH) (**Table 1**). All these assays have different advantages, diagnostic performances, and counterparts that we will detail further. These recommended routine diagnostic tests are completed to classify OPSCC HPV-positive (HPV+) or HPV-negative (HPV-) and other new performant biomarkers seem to be adapted for HPV-induced OPSCC ultrastaging. Indeed, as we already described before, the E6 and E7 HPV oncoproteins are responsible for cell transformation and carcinogenesis and have been proven to be indispensable for the maintenance of tumor phenotype (32). According to the CAP guidelines, every diagnosis of OPSCC should be followed by an assay evaluating HPV infection status in the tissue (24). Several techniques are available, depending on diagnostic performances and resources available in the laboratory. Optimal HPV detection should consider assays detecting (i) transcriptionally active infections, because transient

TABLE 1 | Description of the benefits and drawbacks of different HPV detection techniques.

Detection technique	Benefits	Drawbacks	References
HPV PCR	High sensitivity HPV genotype information FFPE manageable Easy and inexpensive technique	No information about viral transcription High risk of contamination (intrinsic and extrinsic)	(49–60)
E6/E7 mRNA RT-PCR	High sensitivity and specificity Detects active viral infection Gold standard for research	Time-consuming Non-FFPE manageable (fresh or frozen tissue only) RNA fragility	(39–45)
E6/E7 mRNA *in situ* hybridization	High specificity and good sensitivity *In situ* detection of a transcriptionally active HPV infection FFPE manageable	RNA degradation over time Expensive technique	(62–65, 69–72)
HPV DNA *in situ* hybridization	*In situ* detection of HPV DNA High specificity FFPE manageable	Reduced sensitivity (needs a minimum DNA copy number)	(54, 62–67)
P16 immunochemistry	High sensitivity Inexpensive technique FFPE manageable	Moderate specificity Surrogate marker of HPV infection	(8, 62, 63, 70, 71, 81, 82) (87, 88, 92–95)
Serology for antibodies against E6 protein	Present in more than 90% of patient with OPSCC related to HPV16 Easy to set up	Lack of clinical data and hindsight	(119–124, 126)
HPV circulating tumoral DNA by ddPCR	Correlation with clinical outcome Early detection of recurrences in posttreatment monitoring High sensitivity and specificity Low cost	Need to be validated on larger cohorts	(52, 117, 130, 133)

HPV, human papillomavirus; RT-PCR, reverse-transcriptase polymerase chain reaction; ddPCR, droplet digital PCR; FFPE, formalin-fixed paraffin-embedded tissues.

infection does not seem sufficient to develop a carcinoma (33–35) and (ii) consistency with high-risk HPV, because those are associated with malignant processes (3, 4, 36). The 2017 revised WHO/IARC (World Health Organization/International Agency for Research on Cancer) recommendations introduced direct HPV testing based on *in situ* hybridization and/or PCR and/or anti-p16 immunochemistry to classify the OPSCC according to HPV status (37).

Molecular Assays

mRNAE6/E7 Detection

The maintenance of the transformed phenotype of HPV-driven tumor cells is based on the expression of E6 and E7 proteins (33–35). Therefore, detecting E6 and/or E7 protein expression constitutes the best tool to define a tumoral sample as an HPV-driven tumoral tissue or not. However, for the time being, performant techniques based on reliable immunohistochemical probes to detect such viral protein on tissue sample are not current. A recent study compared the results of E6 protein detection in lymph-node fine-needle aspirates, and oral samples (saliva or swabs) by OncoE6^{TM} Oral Test (Arbor Vita Corp©) to reference tests performed on FFPE material: p16 and high-risk HPV mRNA. Agreement between fine-needle aspirates OncoE6^{TM} and FFPE p16 was good (kappa = 0.53). Agreement between oral samples and FFPE p16 and high-risk HPV mRNA was poor (kappa = 0.02 for both), probably due to lower concentrations of E6 protein in these analytes (38). Thus, using such commercial assays on minimally invasive lymph-node fine-needle aspirates could be helpful to diagnose high-risk HPV infection in routine practice. Detection of E6 and/or E7 mRNA by RT-PCR on fresh/frozen samples is considered by some authors as the gold standard to diagnose an HPV-related OPSCC, particularly based on its capacity to represent an eventual prognosis biomarker (39). Nevertheless, it is important to be cautious about the accuracy and reliability of techniques detecting mRNA by RT PCR regarding available samples. Even if the accuracy of this technique has been tested on formalin-fixed paraffin-embedded (FFPE) samples (40), such assays should be used on fresh/frozen tissues given the better diagnostic performances obtained with these types of samples when compared to FFPE ones (41–44). This may be mostly explained by higher RNA destruction and fragmentation of FFPE samples and subsequent decreased sensibility of RNA detection by RT-PCR techniques. Therefore, the gold standard E6/E7 mRNA detection for HPV-related OPSCC diagnosis requires fresh samples (45) and is not useful for routine screening as it is technically demanding. However, a recent study about the development and the validation of a novel and rapid molecular detection method for HR-HPV in FFPE tumor tissues based on combined HPV DNA and E6 mRNA detection reached an accuracy of 97 and 100%, respectively, in OPSCC and oral cavity squamous cell carcinoma (46).

PCR and HPV Genotyping

Firstly and until now, several commercially available assays have been clinically validated on cervical swabs to detect high-grade preneoplastic lesions (47, 48). However, none of these commercial molecular assays have been specifically validated

for clinical routine practice on OPSCC samples. Most of these assays target the L1 gene and amplify a region from 65 to >400 bp according to the technique. Different studies in small cohorts of patients have demonstrated the possibility of using these assays for HPV detection in OPSCC on fresh tissues. These techniques are known to be stable and reproducible, and a recent meta-analysis found the pooled sensitivity and specificity of HPV DNA PCR to be respectively 98 and 84% for HPV detection in OPSCCs (49). However, FFPE samples are often the only material available for molecular testing after pathological examination in the OPSCC context and only few studies have evaluated different commercial molecular assays on head and neck FFPE biopsies (50–55). Regarding the frequent proportion of degraded DNA in FFPE samples, some authors such as Steinau et al. suggest to pretreat FFPE tissues using specific protocols to enhance DNA extraction yields before PCR assay (56). However, it is well reported that DNA recovering in FFPE specimens may be influenced by several factors, such as formalin quality and concentration, length of fixation, paraffin quality, and temperature (57) leading to nucleic-acid fragmentation (56, 58, 59). As a consequence, DNA in FFPE biopsy is either completely or partially degraded into DNA fragments of 200 bp or less (58). Low HPV viral load in FFPE biopsy samples associated with a large region targeted by the molecular assay used (>200 pb) could be a limiting factor, and in medical practice, this decreased sensitivity could sometimes hamper HPV detection in OPSCC. Since PCR is a very sensitive technique, the risk of a false positive due to contamination is not negligible. It may occur within the specimen by a fragment of normal epithelium infected with an HPV unrelated to the cancer. Contamination may also occur during specimen processing with another sample (cross-contamination) or with a soiled object in the laboratory (60). For all these reasons, HPV diagnosis and genotyping on FFPE biopsy from OPSCC using commercially available HPV molecular assays require a good expertise, particularly for preanalytical treatment. This step could require complementary technical approaches to increase sensitivity, as we recently described (52). For example, since HPV16 is known to be the most prevalent HPV genotype in OPSCC, diagnosed in more than 85% of HPV-driven OPSCC (61), we think that it is better to confirm negative results obtained with certain commercial tests through an HPV16-specific home-made PCR able to detect smaller fragments of DNA (<100 pb) from FFPE samples (52).

In situ Hybridization Targeting DNA (DNA ISH)

Many studies have evaluated the use of DNA ISH to diagnose HPV infection in oropharynx carcinomas (54, 62–66). This technique is based on the hybridization of probes against specific sequences of DNA, and conventional light microscopy is sufficient to read the assay result. It has the advantage of being cheaper than RNA *in situ* hybridization, but it seems that sensitivities and specificities of this assay strongly depend on the type of probes used to target HPV (e.g., different manufacturers, probe designs). Depending on the DNA targets, DNA ISH can focus only on high-risk 16 and 18 genotypes, or on broader high-risk HPV-like genotypes 16, 18, 31, 33, 51 (Enzo©'s high-risk cocktail here for example; Enzo, NY, United States). Ventana© Inform HPV III Family 16 Probe cocktail is also able to detect 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 types. Finally, some screening probe cocktails can detect most frequent high-risk HPV (16, 18, 31, 33, 51), as well as some low-risk HPV (6 and 11 types for Enzo©'s screening probe for example).

Data about consistency of DNA ISH results in OPSCCs are quite controversial. For Schlecht et al., comparison of high-risk HPVs probe cocktail (Ventana©, AZ, United States) and HPV16/18 DNA probe cocktail (Dako©, CA, United States) showed better performances by the first manufacturer (66). Conversely, Keung et al. did not find significant differences between performances of three different manufacturers' probe: Enzo© (NY, United States), Leica© (Germany), and Ventana© (AZ, United States) (67). It seems that DNA ISH quality is highly dependent on quality control procedures, and experience of the laboratory with this technique should be taken into account (68). Importantly, Bishop et al. reported that an important background signal could hinder the visualization of the punctuate signal corresponding to target DNA and thus lead to false-negative cases (69). More precisely, it seems that when less than 100 copies of target HPV are present in tumor cells, approximately 25–45% of cases would be reported falsely negative (67). For all these technical reasons, the popularity of DNA ISH appears to have come to a standstill whereas RNA ISH interest is surely growing. **Figure 1A** shows an example of positive DNA ISH targeting HPV in OPSCC.

In situ Hybridization Targeting RNA (RNA ISH)

Studies about RNA ISH have been rising in the last 10 years and showing excellent diagnostic performances. Sensitivities vary from 87 to 100%, and specificities vary from 88 to 100%, being more frequently around 95% (62–65, 69–72). Importantly, studies using RT-PCR as the reference test found the best diagnostic performances, making RNA ISH the method of choice for detecting high-risk HPV infections (62, 65, 71). The RNAscope© (ACD©, DC, United States) technology is the most used one and gives excellent results. This technology can detect E6 and E7 transcripts from 18 high-risk HPV genotypes (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). RNA ISH has the advantage of being feasible on paraffin-embedded tissues. In short, RNAscope© has a good specificity thanks to paired "Z" probes system and a good sensitivity thanks to the amplification system. Moreover, the small size of probes used for this assay enables hybridization to partially degraded mRNA, notably in paraffin-embedded tissues (**Figure 1B**). Another advantage of this method is to be readable on conventional optic light microscope (73). Biologically, RNA ISH is relevant because it addresses several points: (i) the presence of signal indicates the presence of one of the 18 high-risk HPVs included in the probe cocktail, (ii) whether there is a transcriptionally active infection, and (iii) the location of the signal within the tumor cells. Some studies suggest that the analysis of signal could be quantitative or semiquantitative (63, 74, 75), but more studies are necessary to confirm these data. Combining RNA ISH with other assays

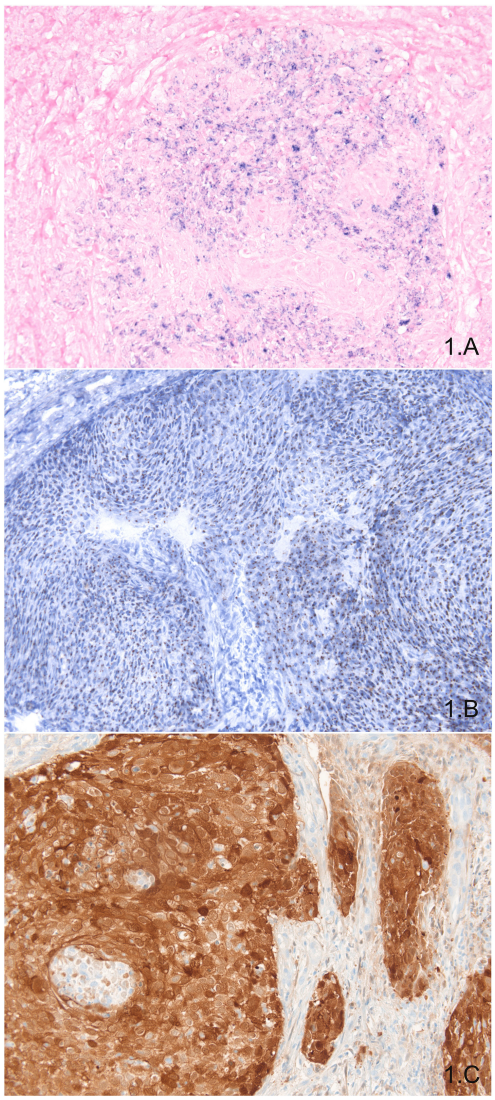

FIGURE 1 | *In situ* exploration of HPV presence: DNA ISH showing blue punctate staining in tumor cells **(A)**; RNA ISH showing brown punctate staining in tumor cells **(B)**; P16 immunohistochemistry showing diffuse and intense nuclear and cytoplasmic staining in almost all tumor cells **(C)**.

does not seem to be worthwhile, because it has great diagnostic performances and it would hamper the workflow of specimens using two assays. Nevertheless, to answer that question, a study testing RNA ISH and p16 immunostaining using RT-PCR as the gold standard would be required. In a perspective of clinical routine use of RNA ISH, Kerr et al. compared the diagnostic performances of manual and automated assays in a series of 45 HNSCCs, approximately two thirds being OPSCCs (76). Concordance between manual and automated assays was high (96%). Another study showed the same results with a high concordance between automated and manual RNA ISH, with only 3 cases out of 42 HNSCCs (35 OPSCCs) being discrepant (kappa = 0.915) (77). These data are in favor of the utilization of RNA ISH on automated platforms. This would enhance workflow efficiency in a routine practice with a high volume of specimens.

The main inconvenient of RNA ISH is its cost, rendering this diagnostic option poorly available for numerous pathology laboratories. A secondary limit of this assay is its incapacity to assess which one of the high-risk HPV types is present in the tumor tissue, whereas this information could be useful to precise prognosis of HPV-positive OPSCCs (78).

It has been shown that oropharynx cancers with transcriptionally active HPV infections are genetically different entities and have a better prognosis (79, 80). In practice, it was necessary to ascertain that RNA ISH was able to predict survival of patients, as well as RT-PCR. Studies have shown that *in situ* hybridization is equivalent for appreciation of prognosis compared to RT-PCR. They showed better survival for patients with HPV-driven OPSCC sought by E6/E7 *in situ* hybridization (65, 81–83). Additionally, our team has shown a difference in prognosis within HPV-related OPSCC depending on the intensity of the RNA ISH staining. Over 50 histologically confirmed p16 positive oropharyngeal squamous cell carcinomas, we applied HPV RNA ISH with a E6/E7 high-risk RNA probe. The staining was assessed semiquantitatively to define two scores: RNA ISH "low" and RNA ISH "high." This series contained 29 RNA ISH low cases (58%) and 21 RNA ISH high cases (42%). RNA ISH high staining was associated with a better overall survival in both univariate and multivariate analyses ($p = 0.033$ and $p = 0.042$, respectively) (84). Nowadays, this technique is not yet recommended to be used routinely and is only applied for research purposes.

p16 Immunostaining

Immunostaining against p16 protein is a cost-effective method to diagnose a high-risk HPV infection within tissues. Overexpression of p16 protein may be an indirect sign of expression of E6 and E7 proteins with cell-cycle upregulation (24, 30). Nevertheless, other processes can lead to p16 overexpression: inflammation, regeneration, and p53 mutations (85, 86). Diagnostic performances of p16 immunostaining are considered high enough to diagnose a high-risk HPV infection in oropharyngeal squamous cells carcinomas, and according to the College of American Pathologists and to the eighth edition of the TNM classification, this assay can be used as a surrogate marker of high-risk HPV infection (24). Sensitivity and specificity of p16 immunostaining for high-risk HPV infection vary from approximately 80–98% according to studies. Among other causes, these differences may be explained by the number of cases included for comparison, by the reference test used as gold standard (RT-PCR, PCR, RNA ISH), and by whether tissue microarray (TMA) were used or not. Interestingly, studies using TMA to evaluate diagnostic performances of p16 immunostaining tend to report lower sensitivities (54, 87). This might be explained by intratumoral heterogeneity of p16 immunostaining (88). Chen et al. have shown that a diffuse nuclear and cytoplasmic staining is significantly associated with HPV positivity in OPSCCs regardless of the intensity of staining, contrary to focal nuclear and cytoplasmic staining (89). Nevertheless, this information

is difficult to evaluate on biopsies. Concerning subcellular localization, according to Lai et al. and Zhao et al., it seems that OPSCCs associated with a highly intense nuclear and slightly intense cytoplasmic p16 immunostaining have poor prognosis, similarly to p16-negative OPSCCs. In both studies, OPSCCs with a high nuclear and high cytoplasmic p16 immunostaining are confirmed to be significantly associated with a better prognosis (90, 91). **Figure 1C** shows an example of positive p16 immunohistochemistry with a diffuse and intense nuclear and cytoplasmic staining of most of tumor cells.

Sensitivity of p16 immunostaining in the oropharynx is around 80–90% (8, 62, 63, 70, 71, 81, 82, 87, 88, 92–95). One study compared the performances of p16 immunostaining according to the threshold of positivity used to assess p16 immunostaining positivity (96). The authors show that determining p16 positivity using a 75% threshold is associated with a poor reproducibility, whereas a 50% threshold is more reproducible. Besides, although a 70% threshold is recommended by most institutions (24, 97, 98), several teams have shown that 50–70% of positivity is often consistent with high-risk HPV infection (88). Thus, one could wonder if using a 50% positivity threshold to assess p16 positivity in routine practice might be an effective diagnostic approach. Further studies led in different OPSCC populations and comparing different thresholds of positivity are required. Considering that specificity of p16 immunostaining varies from 80 to 90% (8, 62, 63, 70, 71, 81, 82, 87, 88, 92–95), some patients with OPSCC may be diagnosed as having a transcriptionally active HPV infection when it is not the case. Rietbergen et al. showed especially that OPSCCs with a p16 immunostaining, and no transcripts of E6 and E7 proteins have a poorer prognosis compared to those with E6 and E7 transcripts (86). Using RNA ISH, we have found similar results (63). Using p16 immunostaining alone could misclassify some patients, but in a large scale of OPSCC management, this option makes sense because the assay is affordable and available for many pathologic departments. However, for trials evaluating impact of treatments according to HPV status, this diagnostic option does not seem performant enough for us, and an assay detecting E6 and E7 transcripts could then be used (RT-qPCR if frozen samples are available, RNA ISH if only formalin-fixed paraffin-embedded tissues are available).

Several studies and meta-analyses have shown that p16-positive OPSCCs have a better overall survival and a better disease-free survival compared to p16-negative OPSCCs (5, 99–101), whatever the age of patients (102). Within p16 + OPSCC, it is unclear whether the prognosis is solely related to HPV or whether p16 expression could be a prognostic factor in itself. Indeed, few studies compare the prognosis of p16 + /HPV- OPSCC patients to p16 + /HPV + or p16-/HPV- OPSCC patients: this p16 + /HPV- subgroup in OPSCC most often has a small number of patients, and the results are therefore not representative. The studies are moreover contradictory, demonstrating for some that there is a better prognosis in spite of the expression of p16 alone in OPSCC (100), and for others that there is no difference in prognosis in OPSCC between the p16 + /HPV- and p16-/HPV- subgroups (70, 101, 103). Studies with a higher number of patients are needed to clarify this issue. One caveat about p16 immunostaining is that it does not provide any data about HPV types involved in the oncogenic process, although this information may be important because a recent study suggested that some high-risk HPV types might be associated with a worse prognosis than others. Indeed, Chatfield-Reed et al. showed that compared to HPV16 type, HPV33 type could be independently associated with a shorter survival, making p16 immunostaining suboptimal to predict survival differences within high-risk HPV-positive OPSCCs (78).

As p16 immunostaining is not a good surrogate marker of high risk HPV infection in non-OPSCC (104), it is rational to ask whether this marker is of prognostic interest in these cancers. Studies are contradictory, but those with larger cohorts seem to support an absence of prognostic difference. In over 1362 HNSCC from the United States, Brazil, and Europe, D'souza et al. found that p16-positive cases had a lower risk of death compared to p16-negative cases among non-OP HNSSCs in univariate analysis (HR = 0.74, 95% CI = 0.57–0.96), but it was not confirmed after adjusting for other risk factors (aHR = 0.83, 95% CI = 0.60–1.14) (101). In another cohort of 621 non-OPSCC, Fakhry et al. found a similar result: overall survivals of patients with p16−positive non−OP HNSCC (n = 62) and with p16−negative non−OP HNSCC (n = 559) were not significantly different (p = 0.26) (105). More specifically, regarding laryngeal and hypopharyngeal SCC in a small cohort of 31 patients, there was no significant difference in overall survivals (p = 0.34) between the p16-positive and p-16 negative patients (106).

There are few data concerning the response to anti-EGFR treatment according to the p16 status. In locally advanced OPSCC, patients with p16−positive tumors had significant superior OS than those with p16−negative tumors in both cetuximab plus radiotherapy (RT) and RT-alone treatment arms (107). Regarding recurrent or metastatic HNSCC, p16−positive status was associated with better overall survival in both the cetuximab plus platinum plus 5−FU and platinum plus 5−FU treatment arms (108). On the contrary, with the panitumumab in the SPECTRUM study, median overall survival in patients with p16-negative HNSCC was longer in the panitumumab group than in the control group (p = 0.0115). This difference was not shown for p16-positive patients (p = 0.998) (109).

Finally, concerning the response to immunotherapies there are again few data available, but p16 status is quite consistently used. In KEYNOTE-012, for the head-and-neck cohorts, the percentage of p16 + patients was relatively small with 45 (23%) being p16 + and 147 (77%) being p16- (110). When stratified by p16 status, response rates were higher in p16 + patients compared to p16- patients, with demonstrated ORRs of 24% (95% CI, 13–40%) and 16% (95% CI, 10–23), respectively (110, 111). These results are contradictory with the CheckMate 141 study in which 63 (26%) patients were p16-positive, 50 (21%) were p16-negative, and 127 (53%) were not tested (112). Analyses revealed nivolumab to be beneficial compared to standard-of-care chemotherapy, irrespective of p16 (112). This was confirmed in a recent update, with significant benefit in both p16- patients and p16 + patients (113).

NEW HPV BIOMARKERS IN THE MANAGEMENT OF HPV-DRIVEN OPSCC

Completing these recommended routine diagnostic tests used to properly classify OPSCC due or not to HPV infection, other new performant biomarkers seem to be useful for HPV-induced OPSCC ultrastaging. Indeed, as we already described before, the E6 and E7 HPV oncoproteins are involved in cell transformation and carcinogenesis and have been proven to be indispensable for maintenance of tumor phenotype (32). Moreover, recent *in vitro* data suggest that E6 and E7 oncoproteins and spliced isoforms of E6 oncoprotein would be associated with higher levels of IL6, responsible of an immunosuppressive environment within cancer (114). This immunosuppressive context could be targeted by therapies associating IL6 and PD-1/PD-L1 blockade (115). To our knowledge, HPV-derived nucleic acids, and particularly the E6 and E7 genes, have not been detected in blood samples in case of simple HPV mucosal infection but only in HPV-related cancer cases (116). Therefore, HPV circulating tumoral DNA (ctDNA) based on detection of HPV DNA in plasma with new ultrasensitive methods appears to have a clinical interest in HPV OPSCC (52, 117). The detection of humoral response against HPV early proteins, especially antibodies against E6, has also been associated with an increased risk to develop oropharyngeal cancer (118).

E6 Humoral Response

The detection of humoral response against HPV early proteins, particularly antibodies against E6 protein, has been associated with a 132-fold increase risk to develop oropharyngeal cancer (118). Rather interestingly, these antibodies seem to develop more than 10 years before HPV-driven OPSCC diagnosis (119). Meanwhile, these E6 antibodies are detectable in <1% of healthy controls (120, 121). Finally, different studies have shown that the vast majority of HPV-positive OPSCC patients (>90%) present an HPV16 E6 antibody response in blood at the time of their HPV16-OPSCC diagnosis (119–124). Even if some authors argue that E6 serology could be helpful for HPV OPSCC monitoring, particularly to track residual disease or recurrence (125), its interest must be confirmed and validated before considering its general use in clinical routine. Even if baseline HPV16 E6 antibodies may have a potential clinical utility for the diagnosis and/or prognosis of HPV-induced OPSCC because HPV16 E6 seropositivity is associated with significant reduced risk of recurrence, E6 serology does not represent a good biomarker for posttreatment monitoring and early identification of relapses. Indeed, HPV16 E6 antibody level remains stable in patients after treatment and eventual variations in antibodies level were not associated with recurrence (126).

HPVct DNA by ddPCR

As we previously mentioned, HPV circulating tumoral DNA (ctDNA) based on detection of plasmatic HPV DNA (E6 or E7 genes) with new ultrasensitive methods appears to have a clinical interest in HPV OPSCC. Indeed, the liquid-biopsy approach using the detection of ctDNA released from tumor cells and detectable in blood has garnered growing interest (127) particularly in HNSCC (128). Detection of ctDNA has demonstrated its relevance in lung or colorectal cancer with the detection of *EGFR* and *KRAS* mutations for non-invasive tumors genotyping, treatment response follow-up, and relapse prediction (129). HPV-related cancers are an ideal model to monitor ctDNA by detecting HPV oncogenes E6 or E7. The feasibility and the interest of HPV ctDNA detection in the plasma of HPV-related OPSCC patients using new ultrasensitive molecular tools such as droplet-based digital PCR (ddPCR) assays have been recently reported and correlated with clinical outcome (52, 117) and early detection of recurrences in posttreatment monitoring (130). This quantitative method of ddPCR is characterized by its high sensitivity, its accuracy, and its reproducibility inter- and intra-laboratories (131). Our team has recently highlighted the interest of quantifying HPVctDNA in plasma samples of OPSCC patients at baseline (52). Indeed, it is the first time that pre-therapeutic HPVctDNA using ddPCR technology was evaluated as a biomarker for OPSCC staging correlated with the new AJCC staging algorithm for HR HPV-associated OPSCC (132) and for patients' clinical outcome. We demonstrated a positive correlation between the level of HPVctDNA load quantified by ddPCR and T status, N status, and the specific stages of the new HPV OPSCC staging algorithm. Moreover, in our series, we observed a positive correlation between HPVctDNA detection by ddPCR and patient clinical outcome. Even if further studies need to be performed in larger cohorts to confirm the prognostic interest of this biomarker before considering its use in routine practice, HPVctDNA appears to be a very interesting biomarker to monitor for optimization of HPV-related OPSCC management with potential interest to select patients for whom treatment de-escalation could eventually be offered.

Finally, the performance of HPVctDNA has also been evaluated to monitor treatment response early, showing that HPVctDNA kinetics are clearly correlated with treatment failure or success and this feature would be more precocious than classical Response Evaluation Criteria in Solid Sumours (RECIST) criteria (52, 117, 133). In the future, the monitoring of HPVctDNA could also be considered as an easy-to-use plasmatic biomarker to determine treatment efficacy early considering the increasing use of very specific and expensive treatment such as immunotherapies in OPSCC medical support. According to the different studies already published on HPVctDNA in HPV-driven OPSCC, this biomarker has a very high sensitivity and specificity, recently estimated at 89 and 97%, respectively by Chera et al. (133). Finally, another great interest of the quantification of HPVctDNA by ddPCR is its very low cost compared to other innovative technologies.

HPV Capture Technology and Viral Molecular Signatures

In cervical carcinomas, integration of HPV DNA into the host genome seems to be the main critical etiological event in the progression from normal cervix to intraepithelial neoplasm, and

finally to invasive cervical cancer. This HPV oncogenic process is considered to be identical in OPSCC, but with no scientific certainties as pretumoral lesions are not yet characterized in head and neck cancer. However, for cervical cancers, different studies have already shown that a part of HPV-driven tumor does not present any integration and is associated only with episomal HPV (134–136). Therefore, HPV molecular status (integrated or not) in the tumor cells could represent an interesting profile to clarify and to correlate with clinical data. Moreover, if integration occurred, the site of HPV integration could also have a real impact on cancer progression (disruption of cancer suppressor genes, immunomodulatory genes, etc.). Finally, HPV genotype variant description could also be of interest as HPV variants have been shown to differ biologically and functionally, thereby affecting persistence and potentially the risk of progression (137, 138). Identifying HPV genotype variants could be pertinent to classify them according to their tumoral aggressiveness.

Recently, using a next-generation sequencing (NGS) technology called "Capture HPV" (135) on biopsies and circulating DNA material, five molecular signatures of HPV integration have been identified in HPV cervical cancer and correlated with survival (but not significantly). To describe the molecular HPV profile and variants in tumor samples, this new and innovative "HPV capture" technology is based on a generic and comprehensive HPV genome capture (235 genotypes and variants) followed by NGS. Exhaustive data will be obtained as HPV whole-genome sequencing/HPV molecular status (integrated or episomal)/HPV integration site, both in virus and human genomes/HPV genotype variant sequences.

"HPV capture" technology has already been performed on HPV cervical and anal cancers (135, 139) to determine a potential prognosis value of the HPV molecular signatures described. Investigations based on this new technology are actually in process in HPV oropharyngeal cancer. The deep information obtained with such technology such as viral molecular status, genotype variants, integration of viral genes deletion, and sites of integration could be extremely informative regarding the viral oncogenic process and could allow the possibility to ultrastratify HPV-driven OPSCC based on virological information.

Which Sample for Which Test?

Depending on the material obtained from patients, different HPV assays are feasible. Some samples require more invasive procedures than others. For this reason, except for the specific context of a clinical trial, performing a second "fresh" biopsy for RT-PCR is not standard because it requires invasive procedures. The new generation of HPV assays is highly sensitive and can be performed on non-invasive or minimally invasive samples, such as blood puncture and oral rinse. These approaches will undoubtedly be complementary to current classical routine practice HPV assays and will help to stratify and monitor HPV-positive HNSCCs. Considering the availability of human samples and technical aspects of assays cited above, we have briefly, through this review, given an overview of the techniques feasible on each kind of sample.

CONCLUSION

In this review, we have explored main HPV detection tools available in routine practice on fresh, frozen, and formalin-fixed tissues in the HNSCC context. If p16 immunostaining is the most affordable technique, it seems that the threshold of 70% of positive tumor cells recommended by the College of American Pathologists might be a little too high because a fraction of cases with a nuclear and cytoplasmic staining in 50–70% of tumor cells are clearly associated with high-risk HPV infection. Of the two *in situ* hybridization assays, the popularity of RNA ISH stems from its excellent diagnostic performances and the biological value of the assay, because positive cases show evidence of transcriptionally active HPV infection. Nevertheless, the price of this assay hampers its use in routine practice. DNA ISH is more difficult to read, and the technique process is highly dependent on the level of expertise of pathology laboratories. This variability leads to moderate diagnostic performances, and this assay is becoming unpopular. RT-PCR and PCR are non-spatial assays but are powerful tools to detect HPV infection. RT-PCR is more performant on fresh and frozen tissues which are often not available in routine practice. For PCR, several commercial assays have been developed for cervical cancers and could be used for HNSCCs, but an important work of comparative evaluation of these tools is needed in HNSCCs and some pre-PCR steps might be optimized to enhance the yield of the technique. Pragmatically, the high sensitivity of p16 immunostaining and the value of PCR to specify HPV type make these tools really interesting in routine practice. Indeed, using p16 immunostaining as a screening tool than PCR constitutes a performant way to diagnose and specify the HPV type since this information is important because of its prognostic value even among high-risk HPV types. In case RNA ISH is feasible, using it as a standalone test might be a seductive solution but it does not provide any precision on the HPV type. We think that further studies evaluating the impact of high-risk HPV type in the prognosis of patients should be conducted to be sure that this information requires a second PCR assay. Among new HPV biomarkers, HPVctDNA detection could be a useful monitoring tool to detect early disease recurrence. This latter tool also seems to have prognostic value, since quantification of HPVctDNA is correlated with T and N stages in OPSCCs. Finally, HPV capture, based on next-generation sequencing, gives insights into the integration process of various genotypes of HPV. In the near future, this assay could be a stratification and prognostic tool for patients with HPV-induced OPSCC.

AUTHOR CONTRIBUTIONS

JA, CL, and CBa designed the manuscript. JA, CL, and HP wrote the first draft. AM and AB illustrated the manuscript. JA, CL, CBa, AM, AB, DV, CBr, HP, and HM edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. WHO *Global Cancer Observatory*. (2020). Available online at: http://gco.iarc.fr/ (accessed February 19, 2020).
2. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. (2011) 29:4294–301. doi: 10.1200/JCO.2011.36.4596
3. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, 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. doi: 10.1093/jnci/92.9.709
4. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med*. (2007) 356:1944–56. doi: 10.1056/NEJMoa065497
5. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. (2010) 363:24–35. doi: 10.1056/NEJMoa0912217
6. Wotman M, Oh EJ, Ahn S, Kraus D, Costantino P, Tham T. HPV status in patients with nasopharyngeal carcinoma in the United States: a SEER database study. *Am J Otolaryngol*. (2019) 40:705–10. doi: 10.1016/j.amjoto.2019.06.007
7. Verma V, Simone CB, Lin C. Human papillomavirus and nasopharyngeal cancer. *Head Neck*. (2018) 40:696–706. doi: 10.1002/hed.24978
8. Castellsagué X, Alemany L, Quer M, Halec G, Quirós B, Tous S, et al. HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients. *J Natl Cancer Inst*. (2016) 108:djv403. doi: 10.1093/jnci/djv403
9. Lam EWH, Chan JYW, Chan ABW, Ng CS, Lo STH, Lam VSC, et al. Prevalence, clinicopathological characteristics, and outcome of human papillomavirus-associated oropharyngeal cancer in Southern Chinese patients. *Cancer Epidemiol Biomarkers Prev*. (2016) 25:165–73. doi: 10.1158/1055-9965.EPI-15-0869
10. Shaikh MH, Khan AI, Sadat A, Chowdhury AH, Jinnah SA, Gopalan V, et al. Prevalence and types of high-risk human papillomaviruses in head and neck cancers from Bangladesh. *BMC Cancer*. (2017) 17:792. doi: 10.1186/s12885-017-3789-0
11. Lim MY, Dahlstrom KR, Sturgis EM, Li G. Human papillomavirus integration pattern and demographic, clinical, and survival characteristics of patients with oropharyngeal squamous cell carcinoma. *Head Neck*. (2016) 38:1139–44. doi: 10.1002/hed.24429
12. Fakhry C, Waterboer T, Westra WH, Rooper LM, Windon M, Troy T, et al. Distinct biomarker and behavioral profiles of human papillomavirus-related oropharynx cancer patients by age. *Oral Oncol*. (2020) 101:104522. doi: 10.1016/j.oraloncology.2019.104522
13. Del Mistro A, Frayle H, Menegaldo A, Favaretto N, Gori S, Nicolai P, et al. Age-independent increasing prevalence of Human Papillomavirus-driven oropharyngeal carcinomas in North-East Italy. *Sci Rep*. (2020) 10:9320. doi: 10.1038/s41598-020-66323-z
14. Rettig EM, Zaidi M, Faraji F, Eisele DW, El Asmar M, Fung N, et al. Oropharyngeal cancer is no longer a disease of younger patients and the prognostic advantage of human Papillomavirus is attenuated among older patients: analysis of the National cancer database. *Oral Oncol*. (2018) 83:147–53. doi: 10.1016/j.oraloncology.2018.06.013
15. Lassen P, Lacas B, Pignon J-P, Trotti A, Zackrisson B, Zhang Q, et al. MARCH collaborative group. prognostic impact of HPV-associated p16-expression and smoking status on outcomes following radiotherapy for oropharyngeal cancer: the MARCH-HPV project. *Radiother Oncol*. (2018) 126:107–15. doi: 10.1016/j.radonc.2017.10.018
16. Misiukiewicz K, Gupta V, Miles BA, Bakst R, Genden E, Selkridge I, et al. Standard of care vs reduced-dose chemoradiation after induction chemotherapy in HPV+ oropharyngeal carcinoma patients: the Quarterback trial. *Oral Oncol*. (2019) 95:170–7. doi: 10.1016/j.oraloncology.2019.06.021
17. Wang MB, Liu IY, Gornbein JA, Nguyen CT. HPV positive oropharyngeal carcinoma: a systematic review of treatment and prognosis. *Otolaryngol Head Neck Surg*. (2015) 153:758–69. doi: 10.1177/0194599815592157
18. Posner MR, Lorch JH, Goloubeva O, Tan M, Schumaker LM, Sarlis NJ, et al. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. *Ann Oncol*. (2011) 22:1071–7. doi: 10.1093/annonc/mdr006
19. Chung CH, Zhang Q, Kong CS, Harris J, Fertig EJ, Harari PM, et al. p16 protein expression and human papillomavirus status as prognostic biomarkers of nonoropharyngeal head and neck squamous cell carcinoma. *J Clin Oncol*. (2014) 32:3930–8. doi: 10.1200/JCO.2013.54.5228
20. Wiley *TNM Classification of Malignant Tumours*. 8th ed. (2020). Available online at: https://www.wiley.com/en-gb/TNM+Classification+of+Malignant+Tumours%2C+8th+Edition-p-9781119263579 (accessed February 17, 2020).
21. Howard J, Dwivedi RC, Masterson L, Kothari P, Quon H, Holsinger FC. De-intensified adjuvant (chemo)radiotherapy versus standard adjuvant chemoradiotherapy post transoral minimally invasive surgery for resectable HPV-positive oropharyngeal carcinoma. *Cochrane Database Syst Rev*. (2018) 12:CD012939. doi: 10.1002/14651858.CD012939.pub2
22. Jones DA, Mistry P, Dalby M, Fulton-Lieuw T, Kong AH, Dunn J, et al. Concurrent cisplatin or cetuximab with radiotherapy for HPV-positive oropharyngeal cancer: medical resource use, costs, and quality-adjusted survival from the De-ESCALaTE HPV trial. *Eur J Cancer*. (2020) 124:178–85. doi: 10.1016/j.ejca.2019.10.025
23. Beaty BT, Moon DH, Shen CJ, Amdur RJ, Weiss J, Grilley-Olson J, et al. PIK3CA mutation in HPV-associated OPSCC patients receiving deintensified chemoradiation. *J Natl Cancer Inst*. (2019) 112:855–8. doi: 10.1093/jnci/djz224
24. Lewis JS, Beadle B, Bishop JA, Chernock RD, Colasacco C, Lacchetti C, et al. Human papillomavirus testing in head and neck carcinomas: guideline from the college of American pathologists. *Arch Pathol Lab Med*. (2018) 142:559–97. doi: 10.5858/arpa.2017-0286-CP
25. Martinez-Zapien D, Ruiz FX, Poirson J, Mitschler A, Ramirez J, Forster A, et al. Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53. *Nature*. (2016) 529:541–5. doi: 10.1038/nature16481
26. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*. (1990) 63:1129–36. doi: 10.1016/0092-8674(90)90409-8
27. Hoppe-Seyler K, Bossler F, Braun JA, Herrmann AL, Hoppe-Seyler F. The HPV E6/E7 oncogenes: key factors for viral carcinogenesis and therapeutic targets. *Trends Microbiol*. (2018) 26:158–68. doi: 10.1016/j.tim.2017.07.007
28. Serrano M. The tumor suppressor protein p16INK4a. *Exp Cell Res*. (1997) 237:7–13. doi: 10.1006/excr.1997.3824
29. Oh ST, Longworth MS, Laimins LA. Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. *J Virol*. (2004) 78:2620–6. doi: 10.1128/jvi.78.5.2620-2626.2004
30. McLaughlin-Drubin ME, Crum CP, Münger K. Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc Natl Acad Sci USA*. (2011) 108:2130–5. doi: 10.1073/pnas.1009933108
31. Rampias T, Sasaki C, Weinberger P, Psyrri A. E6 and E7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells. *JNCI J Natl Cancer Inst*. (2009) 101:412–23. doi: 10.1093/jnci/djp017
32. Alvarez-Salas LM, Cullinan AE, Siwkowski A, Hampel A, DiPaolo JA. Inhibition of HPV-16 E6/E7 immortalization of normal keratinocytes by hairpin ribozymes. *Proc Natl Acad Sci USA*. (1998) 95:1189–94. doi: 10.1073/pnas.95.3.1189
33. 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) 11:5694–9. doi: 10.1158/1078-0432.CCR-05-0587
34. Jones DL, Alani RM, Münger 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) 11:2101–11. doi: 10.1101/gad.11.16.2101
35. Münger 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) 63:4417–21.

36. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. *Lancet Oncol*. (2010) 11:781–9. doi: 10.1016/S1470-2045(10)70017-6
37. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ eds *WHO Classification of Head and Neck Tumours*. 4th ed. Lyon: International Agency for Research on Cancer. (2017).
38. Chernesky M, Jang D, Schweizer J, Arias M, Doerwald-Munoz L, Gupta M, et al. HPV E6 oncoproteins and nucleic acids in neck lymph node fine needle aspirates and oral samples from patients with oropharyngeal squamous cell carcinoma. *Papillomavirus Res*. (2018) 6:1–5. doi: 10.1016/j.pvr.2018.05.003
39. Schache AG, Liloglou T, Risk JM, Filia A, Jones TM, Sheard J, et al. Evaluation of human papilloma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity, and prognostic discrimination. *Clin Cancer Res*. (2011) 17:6262–71. doi: 10.1158/1078-0432.CCR-11-0388
40. Smeets SJ, Hesselink AT, Speel E-JM, Haesevoets A, Snijders PJF, Pawlita M, 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. doi: 10.1002/ijc.22980
41. Wimmer I, Tröscher AR, Brunner F, Rubino SJ, Bien CG, Weiner HL, et al. Systematic evaluation of RNA quality, microarray data reliability and pathway analysis in fresh, fresh frozen and formalin-fixed paraffin-embedded tissue samples. *Sci Rep*. (2018) 8:6351. doi: 10.1038/s41598-018-24781-6
42. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res*. (1999) 27:4436–43. doi: 10.1093/nar/27.22.4436
43. von Ahlfen S, Missel A, Bendrat K, Schlumpberger M. Determinants of RNA quality from FFPE samples. *PLoS One*. (2007) 2:e1261. doi: 10.1371/journal.pone.0001261
44. Benchekroun M, DeGraw J, Gao J, Sun L, von Boguslawsky K, Leminen A, et al. Impact of fixative on recovery of mRNA from paraffin-embedded tissue. *Diagn Mol Pathol*. (2004) 13:116–25. doi: 10.1097/00019606-200406000-00008
45. Bussu F, Ragin C, Boscolo-Rizzo P, Rizzo D, Gallus R, Delogu G, et al. 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. *Head Neck*. (2019) 41:1104–11. doi: 10.1002/hed.25591
46. Mes SW, Heideman DAM, Bloemena E, Brink A, Bogaarts M, Leemans CR, et al. Development and validation of a novel and rapid molecular detection method for high-risk human papillomavirus in formalin-fixed, paraffin-embedded tumor tissue. *J Mol Diagn*. (2020) 22:262–71. doi: 10.1016/j.jmoldx.2019.10.009
47. Arbyn M, Castle PE. Offering self-sampling kits for HPV testing to reach women who do not attend in the regular cervical cancer screening program. *Cancer Epidemiol Biomarkers Prev*. (2015) 24:769–72. doi: 10.1158/1055-9965.EPI-14-1417
48. Hesselink AT, Sahli R, Berkhof J, Snijders PJF, van der Salm ML, Agard D, et al. Clinical validation of Anyplex™ II HPV HR detection according to the guidelines for HPV test requirements for cervical cancer screening. *J Clin Virol*. (2016) 76:36–9. doi: 10.1016/j.jcv.2016.01.009
49. Prigge E-S, 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) 140:1186–98. doi: 10.1002/ijc.30516
50. Donà MG, Rollo F, Pichi B, Spriano G, Pellini R, Covello R, et al. Evaluation of the Xpert® HPV assay in the detection of human Papillomavirus in formalin-fixed paraffin-embedded oropharyngeal carcinomas. *Oral Oncol*. (2017) 72:117–22. doi: 10.1016/j.oraloncology.2017.07.016
51. Pettus JR, Wilson TL, Steinmetz HB, Lefferts JA, Tafe LJ. Utility of the Roche Cobas 4800 for detection of high-risk human papillomavirus in formalin-fixed paraffin-embedded oropharyngeal squamous cell carcinoma. *Exp Mol Pathol*. (2017) 102:47–9. doi: 10.1016/j.yexmp.2016.12.004
52. Veyer D, Wack M, Grard O, Bonfils P, Hans S, Bélec L, et al. HPV detection and genotyping of head and neck cancer biopsies by molecular testing with regard to the new oropharyngeal squamous cell carcinoma classification based on HPV status. *Pathology*. (2019) 51:421–5. doi: 10.1016/j.pathol.2019.02.002
53. Rollo F, Donà MG, Pichi B, Pellini R, Covello R, Benevolo M. Evaluation of the anyplex II HPV28 assay in the detection of human Papillomavirus in archival samples of oropharyngeal carcinomas. *Arch Pathol Lab Med*. (2019) 144:620–5. doi: 10.5858/arpa.2019-0199-OA
54. Craig SG, Anderson LA, Moran M, Graham L, Currie K, Rooney K, et al. Comparison of molecular assays for HPV testing in oropharyngeal squamous cell carcinomas: a population-based study in Northern Ireland. *Cancer Epidemiol Biomarkers Prev*. (2020) 29:31–8. doi: 10.1158/1055-9965.EPI-19-0538
55. Huho AN, Yadak N, Bocklage TJ, Yang S. Evaluation of diagnostic utility of a high-risk human Papillomavirus PCR test on formalin-fixed, paraffin-embedded head and neck tumor tissues. *J Mol Diagn*. (2018) 20:232–9. doi: 10.1016/j.jmoldx.2017.11.008
56. Steinau M, Patel SS, Unger ER. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*. (2011) 13:377–81. doi: 10.1016/j.jmoldx.2011.03.007
57. Klopfleisch R, Weiss ATA, Gruber AD. Excavation of a buried treasure–DNA, mRNA, miRNA and protein analysis in formalin fixed, paraffin embedded tissues. *Histol Histopathol*. (2011) 26:797–810. doi: 10.14670/HH-26.797
58. Gilbert MTP, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, et al. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? *PLoS One*. (2007) 2:e537. doi: 10.1371/journal.pone.0000537
59. Kocjan BJ, Hošnjak L, Poljak M. Detection of alpha human papillomaviruses in archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens. *J Clin Virol*. (2016) 76(Suppl. 1):S88–97. doi: 10.1016/j.jcv.2015.10.007
60. Boscolo-Rizzo P, Pawlita M, Holzinger D. From HPV-positive towards HPV-driven oropharyngeal squamous cell carcinomas. *Cancer Treat Rev*. (2016) 42:24–9. doi: 10.1016/j.ctrv.2015.10.009
61. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. *J Clin Oncol*. (2015) 33:3235–42. doi: 10.1200/JCO.2015.61.6995
62. Schache AG, Liloglou T, Risk JM, Jones TM, Ma X-J, Wang H, et al. Validation of a novel diagnostic standard in HPV-positive oropharyngeal squamous cell carcinoma. *Br J Cancer*. (2013) 108:1332–9. doi: 10.1038/bjc.2013.63
63. Augustin J, Outh-Gauer S, Mandavit M, Gasne C, Grard O, Denize T, et al. Evaluation of the efficacy of the 4 tests (p16 immunochemistry, polymerase chain reaction, DNA, and RNA in situ hybridization) to evaluate a human papillomavirus infection in head and neck cancers: a cohort of 348 French squamous cell carcinomas. *Hum Pathol*. (2018) 78:63–71. doi: 10.1016/j.humpath.2018.04.006
64. Randén-Brady R, Carpén T, Jouhi L, Syrjänen S, Haglund C, Tarkkanen J, et al. In situ hybridization for high-risk HPV E6/E7 mRNA is a superior method for detecting transcriptionally active HPV in oropharyngeal cancer. *Hum Pathol*. (2019) 90:97–105. doi: 10.1016/j.humpath.2019.05.006
65. Mirghani H, Casiraghi O, Amen F, He M, Ma X-J, Saulnier P, et al. Diagnosis of HPV-driven head and neck cancer with a single test in routine clinical practice. *Mod Pathol*. (2015) 28:1518–27. doi: 10.1038/modpathol.2015.113
66. Schlecht NF, Brandwein-Gensler M, Nuovo GJ, Li M, Dunne A, Kawachi N, et al. A comparison of clinically utilized human papillomavirus detection methods in head and neck cancer. *Mod Pathol*. (2011) 24:1295–305. doi: 10.1038/modpathol.2011.91
67. Keung ES, Souers RJ, Bridge JA, Faquin WC, Graham RP, Hameed MR, et al. Comparative performance of high-risk human papillomavirus RNA and DNA in situ hybridization on college of american pathologists proficiency tests. *Arch Pathol Lab Med*. (2019) 144:344–9. doi: 10.5858/arpa.2019-0093-CP
68. Unger ER. In situ diagnosis of human papillomaviruses. *Clin Lab Med*. (2000) 20:289–301, vi.
69. Bishop JA, Ma X-J, Wang H, Luo Y, Illei PB, Begum S, et al. Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. *Am J Surg Pathol*. (2012) 36:1874–82. doi: 10.1097/PAS.0b013e318265fb2b
70. Craig SG, Anderson LA, Schache AG, Moran M, Graham L, Currie K, et al. Recommendations for determining HPV status in patients with oropharyngeal cancers under TNM8 guidelines: a two-tier approach. *Br J Cancer*. (2019) 120:827–33. doi: 10.1038/s41416-019-0414-9

71. Gao G, Chernock RD, Gay HA, Thorstad WL, Zhang TR, Wang H, et al. A novel RT-PCR method for quantification of human papillomavirus transcripts in archived tissues and its application in oropharyngeal cancer prognosis. *Int J Cancer*. (2013) 132:882–90. doi: 10.1002/ijc.27739
72. Rahimi S, Akaev I, Brennan PA, Virgo A, Marani C, Gomez RS, et al. A proposal for classification of oropharyngeal squamous cell carcinoma: morphology and status of HPV by immunohistochemistry and molecular biology. *J Oral Pathol Med*. (2020) 49:110–6. doi: 10.1111/jop.12956
73. Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*. (2012) 14:22–9. doi: 10.1016/j.jmoldx.2011.08.002
74. Wang H, Su N, Wang L-C, Wu X, Bui S, Nielsen A, et al. Quantitative ultrasensitive bright-field RNA in situ hybridization with RNAscope. *Methods Mol Biol*. (2014) 1211:201–12. doi: 10.1007/978-1-4939-1459-3_16
75. Chang H-H, Tseng Y-F, Lu M-Y, Yang Y-L, Chou S-W, Lin D-T, et al. MYCN RNA levels determined by quantitative in situ hybridization is better than MYCN gene dosages in predicting the prognosis of neuroblastoma patients. *Mod Pathol*. (2019) 33:531–40. doi: 10.1038/s41379-019-0410-x
76. Kerr DA, Arora KS, Mahadevan KK, Hornick JL, Krane JF, Rivera MN, et al. Performance of a branch chain RNA in situ hybridization assay for the detection of high-risk human papillomavirus in head and neck squamous cell carcinoma. *Am J Surg Pathol*. (2015) 39:1643–52. doi: 10.1097/PAS.0000000000000516
77. Rooper LM, Gandhi M, Bishop JA, Westra WH. RNA in-situ hybridization is a practical and effective method for determining HPV status of oropharyngeal squamous cell carcinoma including discordant cases that are p16 positive by immunohistochemistry but HPV negative by DNA in-situ hybridization. *Oral Oncol*. (2016) 55:11–6. doi: 10.1016/j.oraloncology.2016.02.008
78. Chatfield-Reed K, Gui S, O'Neill WQ, Teknos TN, Pan Q. HPV33+ HNSCC is associated with poor prognosis and has unique genomic and immunologic landscapes. *Oral Oncol*. (2020) 100:104488. doi: 10.1016/j.oraloncology.2019.104488
79. Jung AC, Briolat J, Millon R, de Reyniès A, Rickman D, Thomas E, et al. Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. *Int J Cancer*. (2010) 126:1882–94. doi: 10.1002/ijc.24911
80. Braakhuis BJM, Snijders PJF, Keune W-JH, Meijer CJLM, Ruijter-Schippers HJ, Leemans CR, 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. doi: 10.1093/jnci/djh183
81. Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, et al. Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. *Am J Surg Pathol*. (2012) 36:945–54. doi: 10.1097/PAS.0b013e318253a2d1
82. Ukpo OC, Flanagan JJ, Ma X-J, Luo Y, Thorstad WL, Lewis JS. High-risk human papillomavirus E6/E7 mRNA detection by a novel in situ hybridization assay strongly correlates with p16 expression and patient outcomes in oropharyngeal squamous cell carcinoma. *Am J Surg Pathol*. (2011) 35:1343–50. doi: 10.1097/PAS.0b013e318220e59d
83. Shi W, Kato H, Perez-Ordonez B, Pintilie M, Huang S, Hui A, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *J Clin Oncol*. (2009) 27:6213–21. doi: 10.1200/JCO.2009.23.1670
84. Augustin J, Mandavit M, Outh-Gauer S, Grard O, Gasne C, Lépine C, et al. HPV RNA CISH score identifies two prognostic groups in a p16 positive oropharyngeal squamous cell carcinoma population. *Mod Pathol*. (2018) 31:1645–52. doi: 10.1038/s41379-018-0090-y
85. Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, et al. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. *Proc Natl Acad Sci USA*. (1996) 93:4350–4. doi: 10.1073/pnas.93.9.4350
86. Rietbergen MM, Snijders PJF, Beekzada D, Braakhuis BJM, Brink A, Heideman DAM, et al. Molecular characterization of p16-immunopositive but HPV DNA-negative oropharyngeal carcinomas. *Int J Cancer*. (2014) 134:2366–72. doi: 10.1002/ijc.28580
87. Holzinger D, Schmitt M, Dyckhoff G, Benner A, Pawlita M, Bosch FX. Viral RNA patterns and high viral load reliably define oropharynx carcinomas with active HPV16 involvement. *Cancer Res*. (2012) 72:4993–5003. doi: 10.1158/0008-5472.CAN-11-3934
88. Hong A, Jones D, Chatfield M, Lee CS, Zhang M, Clark J, et al. HPV status of oropharyngeal cancer by combination HPV DNA/p16 testing: biological relevance of discordant results. *Ann Surg Oncol*. (2013) 20(Suppl. 3):S450–8. doi: 10.1245/s10434-012-2778-4
89. Chen ZW, Weinreb I, Kamel-Reid S, Perez-Ordoñez B. Equivocal p16 immunostaining in squamous cell carcinoma of the head and neck: staining patterns are suggestive of HPV status. *Head Neck Pathol*. (2012) 6:422–9. doi: 10.1007/s12105-012-0382-3
90. Lai S, Wenaas AE, Sandulache VC, Hartman C, Chiao E, Kramer J, et al. Prognostic significance of p16 cellular localization in oropharyngeal squamous cell carcinoma. *Ann Clin Lab Sci*. (2016) 46:132–9.
91. Zhao N, Ang M-K, Yin X-Y, Patel MR, Fritchie K, Thorne L, et al. Different cellular p16(INK4a) localisation may signal different survival outcomes in head and neck cancer. *Br J Cancer*. (2012) 107:482–90. doi: 10.1038/bjc.2012.264
92. Isayeva T, Xu J, Dai Q, Whitley AC, Bonner J, Nabell L, et al. African Americans with oropharyngeal carcinoma have significantly poorer outcomes despite similar rates of human papillomavirus-mediated carcinogenesis. *Hum Pathol*. (2014) 45:310–9. doi: 10.1016/j.humpath.2013.09.006
93. Nauta IH, Rietbergen MM, van Bokhoven AAJD, Bloemena E, Lissenberg-Witte BI, Heideman DAM, et al. Evaluation of the eighth TNM classification on p16-positive oropharyngeal squamous cell carcinomas in the Netherlands and the importance of additional HPV DNA testing. *Ann Oncol*. (2018) 29:1273–9. doi: 10.1093/annonc/mdy060
94. Näsman A, Nordfors C, Holzhauser S, Vlastos A, Tertipis N, Hammar U, et al. Incidence of human papillomavirus positive tonsillar and base of tongue carcinoma: a stabilisation of an epidemic of viral induced carcinoma? *Eur J Cancer*. (2015) 51:55–61. doi: 10.1016/j.ejca.2014.10.016
95. Plath M, Broglie MA, Förbs D, Stoeckli SJ, Jochum W. Prognostic significance of cell cycle-associated proteins p16, pRB, cyclin D1 and p53 in resected oropharyngeal carcinoma. *J Otolaryngol Head Neck Surg*. (2018) 47:53. doi: 10.1186/s40463-018-0298-3
96. Shelton J, Purgina BM, Cipriani NA, Dupont WD, Plummer D, Lewis JS. p16 immunohistochemistry in oropharyngeal squamous cell carcinoma: a comparison of antibody clones using patient outcomes and high-risk human papillomavirus RNA status. *Mod Pathol*. (2017) 30:1194–203. doi: 10.1038/modpathol.2017.31
97. Fakhry C, Lacchetti C, Perez-Ordonez B. Human papillomavirus testing in head and neck carcinomas: ASCO clinical practice guideline endorsement summary of the CAP guideline. *J Oncol Pract*. (2018) 14:613–7. doi: 10.1200/JOP.18.00433
98. Mehanna H, Evans M, Beasley M, Chatterjee S, Dilkes M, Homer J, et al. Oropharyngeal cancer: United Kingdom national multidisciplinary guidelines. *J Laryngol Otol*. (2016) 130:S90–6. doi: 10.1017/S0022215116000505
99. Sedghizadeh PP, Billington WD, Paxton D, Ebeed R, Mahabady S, Clark GT, et al. Is p16-positive oropharyngeal squamous cell carcinoma associated with favorable prognosis? A systematic review and meta-analysis. *Oral Oncol*. (2016) 54:15–27. doi: 10.1016/j.oraloncology.2016.01.002
100. Albers AE, Qian X, Kaufmann AM, Coordes A. Meta analysis: HPV and p16 pattern determines survival in patients with HNSCC and identifies potential new biologic subtype. *Sci Rep*. (2017) 7:16715. doi: 10.1038/s41598-017-16918-w
101. D'Souza G, Anantharaman D, Gheit T, Abedi-Ardekani B, Beachler DC, Conway DI, et al. Effect of HPV on head and neck cancer patient survival, by region and tumor site: a comparison of 1362 cases across three continents. *Oral Oncol*. (2016) 62:20–7. doi: 10.1016/j.oraloncology.2016.09.005
102. Windon MJ, D'Souza G, Rettig EM, Westra WH, van Zante A, Wang SJ, et al. Increasing prevalence of human papillomavirus-positive oropharyngeal cancers among older adults. *Cancer*. (2018) 124:2993–9. doi: 10.1002/cncr.31385
103. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, et al. Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favorable prognosis. *J Clin Oncol*. (2006) 24:736–47. doi: 10.1200/JCO.2004.00.3335

104. D'Souza G, Westra WH, Wang SJ, van Zante A, Wentz A, Kluz N, et al. Differences in the prevalence of human papillomavirus (HPV) in head and neck squamous cell cancers by sex, race, anatomic tumor site, and HPV detection method. *JAMA Oncol*. (2017) 3:169–77. doi: 10.1001/jamaoncol.2016.3067
105. Fakhry C, Westra WH, Wang SJ, van Zante A, Zhang Y, Rettig E, et al. The prognostic role of sex, race, and human papillomavirus in oropharyngeal and nonoropharyngeal head and neck squamous cell cancer. *Cancer*. (2017) 123:1566–75. doi: 10.1002/cncr.30353
106. Meshman J, Wang P-C, Chin R, John MS, Abemayor E, Bhuta S, et al. Prognostic significance of p16 in squamous cell carcinoma of the larynx and hypopharynx. *Am J Otolaryngol*. (2017) 38:31–7. doi: 10.1016/j.amjoto.2016.09.007
107. Rosenthal DI, Harari PM, Giralt J, Bell D, Raben D, Liu J, et al. Association of human papillomavirus and p16 status with outcomes in the IMCL-9815 phase III registration trial for patients with locoregionally advanced oropharyngeal squamous cell carcinoma of the head and neck treated with radiotherapy with or without cetuximab. *J Clin Oncol*. (2016) 34:1300–8. doi: 10.1200/JCO.2015.62.5970
108. Vermorken JB, Psyrri A, Mesía R, Peyrade F, Beier F, de Blas B, et al. Impact of tumor HPV status on outcome in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck receiving chemotherapy with or without cetuximab: retrospective analysis of the phase III EXTREME trial. *Ann Oncol*. (2014) 25:801–7. doi: 10.1093/annonc/mdt574
109. Vermorken JB, Stöhlmacher-Williams J, Davidenko I, Licitra L, Winquist E, Villanueva C, et al. Cisplatin and fluorouracil with or without panitumumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck (SPECTRUM): an open-label phase 3 randomised trial. *Lancet Oncol*. (2013) 14:697–710. doi: 10.1016/S1470-2045(13)70181-5
110. Mehra R, Seiwert TY, Gupta S, Weiss J, Gluck I, Eder JP, et al. Efficacy and safety of pembrolizumab in recurrent/metastatic head and neck squamous cell carcinoma: pooled analyses after long-term follow-up in KEYNOTE-012. *Br J Cancer*. (2018) 119:153–9. doi: 10.1038/s41416-018-0131-9
111. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *Lancet Oncol*. (2016) 17:956–65. doi: 10.1016/S1470-2045(16)30066-3
112. Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *N Engl J Med*. (2016) 375:1856–67. doi: 10.1056/NEJMoa1602252
113. Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. *Oral Oncol*. (2018) 81:45–51. doi: 10.1016/j.oraloncology.2018.04.008
114. Artaza-Irigaray C, Molina-Pineda A, Aguilar-Lemarroy A, Ortiz-Lazareno P, Limón-Toledo LP, Pereira-Suárez AL, et al. E6/E7 and E6* From HPV16 and HPV18 Upregulate IL-6 expression independently of p53 in keratinocytes. *Front Immunol*. (2019) 10:1676. doi: 10.3389/fimmu.2019.01676
115. Tsukamoto H, Fujieda K, Miyashita A, Fukushima S, Ikeda T, Kubo Y, et al. Combined blockade of IL6 and PD-1/PD-L1 signaling abrogates mutual regulation of their immunosuppressive effects in the tumor microenvironment. *Cancer Res*. (2018) 78:5011–22. doi: 10.1158/0008-5472.CAN-18-0118
116. Jeannot E, Becette V, Campitelli M, Calméjane M-A, Lappartient E, Ruff E, et al. Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma. *J Pathol Clin Res*. (2016) 2:201–9. doi: 10.1002/cjp2.47
117. Hanna GJ, Lizotte P, Cavanaugh M, Kuo FC, Shivdasani P, Frieden A, et al. Frameshift events predict anti-PD-1/L1 response in head and neck cancer. *JCI Insight*. (2018) 3:e98811. doi: 10.1172/jci.insight.98811
118. Anantharaman D, Gheit T, Waterboer T, Abedi-Ardekani B, Carreira C, McKay-Chopin S, et al. Human papillomavirus infections and upper aero-digestive tract cancers: the ARCAGE study. *J Natl Cancer Inst*. (2013) 105:536–45. doi: 10.1093/jnci/djt053
119. Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *J Clin Oncol*. (2013) 31:2708–15. doi: 10.1200/JCO.2012.47.2738
120. Kreimer AR, Johansson M, Yanik EL, Katki HA, Check DP, Lang Kuhs KA, et al. Kinetics of the human papillomavirus type 16 E6 antibody response prior to oropharyngeal cancer. *J Natl Cancer Inst*. (2017) 109:djx005. doi: 10.1093/jnci/djx005
121. Lang Kuhs KA, Anantharaman D, Waterboer T, Johansson M, Brennan P, Michel A, et al. Human papillomavirus 16 E6 antibodies in individuals without diagnosed cancer: a pooled analysis. *Cancer Epidemiol Biomarkers Prev*. (2015) 24:683–9. doi: 10.1158/1055-9965.EPI-14-1217
122. Holzinger D, Wichmann G, Baboci L, Michel A, Höfler D, Wiesenfarth M, et al. Sensitivity and specificity of antibodies against HPV16 E6 and other early proteins for the detection of HPV16-driven oropharyngeal squamous cell carcinoma. *Int J Cancer*. (2017) 140:2748–57. doi: 10.1002/ijc.30697
123. Lang Kuhs KA, Pawlita M, Gibson SP, Schmitt NC, Trivedi S, Argiris A, et al. Characterization of human papillomavirus antibodies in individuals with head and neck cancer. *Cancer Epidemiol*. (2016) 42:46–52. doi: 10.1016/j.canep.2016.03.003
124. Broglie MA, Jochum W, Michel A, Waterboer T, Foerbs D, Schoenegg R, et al. Evaluation of type-specific antibodies to high risk-human papillomavirus (HPV) proteins in patients with oropharyngeal cancer. *Oral Oncol*. (2017) 70:43–50. doi: 10.1016/j.oraloncology.2017.05.010
125. Mirghani H, Lang Kuhs KA, Waterboer T. Biomarkers for early identification of recurrences in HPV-driven oropharyngeal cancer. *Oral Oncol*. (2018) 82:108–14. doi: 10.1016/j.oraloncology.2018.05.015
126. Lang Kuhs KA, Kreimer AR, Trivedi S, Holzinger D, Pawlita M, Pfeiffer RM, et al. Human papillomavirus 16 E6 antibodies are sensitive for human papillomavirus-driven oropharyngeal cancer and are associated with recurrence. *Cancer*. (2017) 123:4382–90. doi: 10.1002/cncr.30966
127. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. *N Engl J Med*. (2018) 379:1754–65. doi: 10.1056/NEJMra1706174
128. Kulasinghe A, Kapeleris J, Kimberley R, Mattarollo SR, Thompson EW, Thiery J-P, et al. The prognostic significance of circulating tumor cells in head and neck and non-small-cell lung cancer. *Cancer Med*. (2018) 7:5910–9. doi: 10.1002/cam4.1832
129. Phallen J, Leal A, Woodward BD, Forde PM, Naidoo J, Marrone KA, et al. Early noninvasive detection of response to targeted therapy in non-small cell lung cancer. *Cancer Res*. (2019) 79:1204–13. doi: 10.1158/0008-5472.CAN-18-1082
130. Chera BS, Kumar S, Shen C, Amdur R, Dagan R, Green R, et al. Plasma circulating tumor HPV DNA for the surveillance of cancer recurrence in HPV-associated oropharyngeal cancer. *J Clin Oncol*. (2020) 38:1050–8. doi: 10.1200/JCO.19.02444
131. Whale AS, Fernandez-Gonzalez A, Gutteridge A, Devonshire AS. Control materials and digital PCR methods for evaluation of circulating cell-free DNA extractions from plasma. *Methods Mol Biol*. (2018) 1768:45–65. doi: 10.1007/978-1-4939-7778-9_4
132. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin*. (2017) 67:122–37. doi: 10.3322/caac.21389
133. Chera BS, Kumar S, Beaty BT, Marron D, Jefferys S, Green R, et al. Rapid clearance profile of plasma circulating tumor HPV type 16 DNA during chemoradiotherapy correlates with disease control in HPV-associated oropharyngeal cancer. *Clin Cancer Res*. (2019) 25:4682–90. doi: 10.1158/1078-0432.CCR-19-0211
134. Cancer Genome Atlas Research Network, Albert Einstein College of Medicine, Analytical Biological Services, Barretos Cancer Hospital, Baylor College of Medicine, Beckman Research Institute of City of Hope, et al. Integrated genomic and molecular characterization of cervical cancer. *Nature*. (2017) 543:378–84. doi: 10.1038/nature21386
135. Holmes A, Lameiras S, Jeannot E, Marie Y, Castera L, Sastre-Garau X, et al. Mechanistic signatures of HPV insertions in cervical carcinomas. *NPJ Genom Med*. (2016) 1:16004. doi: 10.1038/npjgenmed.2016.4
136. Vinokurova S, Wentzensen N, Kraus I, Klaes R, Driesch C, Melsheimer P, et al. Type-dependent integration frequency of human papillomavirus

genomes in cervical lesions. *Cancer Res*. (2008) 68:307–13. doi: 10.1158/0008-5472.CAN-07-2754

137. Sichero L, Ferreira S, Trottier H, Duarte-Franco E, Ferenczy A, Franco EL, et al. High grade cervical lesions are caused preferentially by non-European variants of HPVs 16 and 18. *Int J Cancer*. (2007) 120:1763–8. doi: 10.1002/ijc.22481
138. Touze A, El Mehdaoui S, Sizaret PY, Mougin C, Muñoz N, Coursaget P. The L1 major capsid protein of human papillomavirus type 16 variants affects yield of virus-like particles produced in an insect cell expression system. *J Clin Microbiol*. (1998) 36:2046–51.
139. Morel A, Neuzillet C, Wack M, Lameiras S, Vacher S, Deloger M, et al. Mechanistic signatures of human papillomavirus insertions in anal squamous cell carcinomas. *Cancers (Basel)*. (2019) 11:1846. doi: 10.3390/cancers11121846

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REVIEW
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Immunological Network in Head and Neck Squamous Cell Carcinoma—A Prognostic Tool Beyond HPV Status

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Head and neck squamous cell carcinoma (HNSCC) is a highly heterogeneous disease that affects more than 800,000 patients worldwide each year. The variability of HNSCC is associated with differences in the carcinogenesis processes that are caused by two major etiological agents, namely, alcohol/tobacco, and human papillomavirus (HPV). Compared to non-virally induced carcinomas, the oropharyngeal tumors associated with HPV infection show markedly better clinical outcomes and are characterized by an immunologically "hot" landscape with high levels of tumor-infiltrating lymphocytes. However, the standard of care remains the same for both HPV-positive and HPV-negative HNSCC. Surprisingly, treatment de-escalation trials have not shown any clinical benefit in patients with HPV-positive tumors to date, most likely due to insufficient patient stratification. The in-depth analysis of the immune response, which places an emphasis on tumor-infiltrating immune cells, is a widely accepted prognostic tool that might significantly improve both the stratification of HNSCC patients in de-escalation trials and the development of novel immunotherapeutic approaches.

Keywords: head and neck squamous cell carcinoma, human papillomavirus (HPV), tumor microenvironment, immune infiltrate, antitumor immune response, treatment de-escalation

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INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are a heterogeneous group of epithelial tumors that are localized in the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx with an estimated global incidence of more than 800,000 new cases per year (1). In general, heavy tobacco and alcohol exposure have been determined to be the most important risk factors for HNSCC. In the 1990s, human papillomavirus (HPV) was described as an emerging etiological agent of oropharyngeal cancer [oropharyngeal squamous cell carcinoma (OPSCC)]. In the following years, the incidence of HPV-associated tumors of the tonsils and base of the tongue has markedly increased, especially in the developed world. Recently, the proportion of patients with HPV-associated OPSCC may be as high as 70–90%, depending on the patients' region of origin (2, 3).

HPVs are small double-stranded DNA viruses from the family Papillomaviridae. At present, more than 200 different HPV types have been identified, including 16 "high-risk" types that are preferentially found in precancerous and cancerous lesions (4, 5). In OPSCC, the most commonly detected type is HPV16 (>80%) followed by HPV18 (3%) (6). In contrast to tobacco- and alcohol-related mutagens, which induce mutagenesis in broad areas of the cells that form the

stratified squamous cell epithelium of the upper aerodigestive tract, the carcinogenic activity of HPV is localized to the reticulated epithelium of the tonsillar crypts, thereby promoting the malignant transformation of epithelial cells within the oropharyngeal region (**Figure 1**) (7). Additionally, whereas >80% of HPV-negative tumors bear mutations in *TP53*, HPV-associated tumors mostly harbor wild-type *TP53* (8). During HPV infection, the HPV-derived oncoprotein E6 binds to host tumor suppressor protein p53, inducing its ubiquitin-mediated degradation, whereas the oncoprotein E7 inactivates pRb (9, 10). Inactivation of pRb results in overexpression of p16 (11), which is used as a valid marker for HPV status assessment in OPSCC patients.

Although the process of carcinogenesis differs markedly between HPV-associated OPSCC and HNSCC of other etiology, both types of tumors have a high tumor mutational burden (TMB). In general, tumors with high TMB express higher levels of neoantigens that can be recognized by the immune system (12). Surprisingly, high TMB correlated in HNSCC patients with unfavorable immune expression signatures and poor clinical outcome (13). Besides carcinogen exposure, a significant part of mutations in HNSCC can be attributed to the activity of apolipoprotein B mRNA editing enzyme, a catalytic polypeptide-like 3 (APOBEC3) family of cytosine deaminases. In accordance with the well-defined role of the APOBEC family in viral restriction, APOBEC3 mutations are particularly prominent in HPV-associated OPSCC. Contrary to the general TMB mentioned above, immune cell infiltration was positively associated with APOBEC mutational burden in HNSCC (14, 15).

Smoking and alcohol consumption on the one hand and HPV infection on the other hand can also markedly affect the composition of the salivary microbiome. It has been reported that microbes and their products can influence cancer development and progression, antitumor immune response, and in the upshot patients' survival (16–18). Therefore, the specific impact of the shifts in the oral salivary microbiome during HNSCC progression needs further evaluation.

Patients with HPV-associated tumors are typically diagnosed with large, cystic metastatic cervical lymph nodes; however, they are highly responsive to standard treatment approaches and have significantly better prognoses compared to HPV-negative patients (19–21). Due to the discrepancy between the predictive value of the standard staging algorithm in patients with HPV-negative and HPV-positive HNSCC, the eighth edition of the American Joint Committee on Cancer Staging Manual proposed a new, independent staging system for HPV- associated OPSCC (22). Consequently, since 2018, HPV-associated OPSCC and HPV-negative HNSCC have been considered distinct diseases with independent classification and multiple, significant differences in their clinicopathological features (**Table 1**). In contrast to squamous cell carcinoma of the oropharynx, the clinical impact of HPV and its detection in non-oropharyngeal HNSCC have not been confirmed to date and need to be further evaluated. *In silico* study published by Chakravarthy et al. (30) showed that although HPV-positive non-oropharyngeal HNSCC shared a gene expression signature and basaloid morphology with HPV-positive OPSCC, HPV-positivity in non-oropharyngeal HNSCC was not associated with improved patients' prognosis. The major difference between HPV-associated non-oropharyngeal and oropharyngeal HNSCC was in the level of tumor-infiltrating immune cells, suggesting a crucial role of immune response in the disease outcome.

The excellent prognosis of HPV-positive OPSCC patients also initiated discussions about treatment de-escalation strategies, which may achieve similar efficacy with decreased toxicity in this particular group of patients (31, 32). The standard treatment regimens, which mainly include curative chemoradiotherapy or surgery followed by adjuvant radiotherapy or chemoradiotherapy, are highly effective; however, they are associated with substantial long-term morbidity, which escalates with treatment intensity and negatively impacts the quality of the patients' lives (32). However, due to the existence of a subgroup of "high-risk" HPV-positive OPSCC patients with a poor prognosis, patient stratification according to HPV status alone is insufficient for successful treatment deintensification. A positive correlation between heavy smoking and poor clinical outcome, as reported by several authors (25, 31, 33), led to the use of smoking status as a cofactor in some de-escalation clinical trials (25, 34). In addition to smoking history, the immune signature might be another important cofactor for the precise selection of patients for de-escalation regimens. Although pan-cancer analyses reveal both HPV-negative and HPV-positive HNSCC as malignancies with a high level of immune cell infiltration (35), HPV-positive OPSCCs show in general markedly higher densities of tumor-infiltrating lymphocytes (TILs) and belong to the immunologically "hottest" of all cancer types (29, 35–37). This feature was reported to be positively correlated with patient survival in a wide range of malignancies (36, 38–42). However, HPV-positive tumors are heterogeneous, and some of the patients with confirmed HPV-associated OPSCC were shown to have immunologically "colder" tumors with low levels of TILs and markedly worse clinical outcome (26, 42, 43). Indeed, Ward et al. (26) described a prognostic model based on the TIL density, smoking status and T stage, and this model can effectively identify the subgroup of HPV-positive patients with poor survival who should be excluded from treatment deintensification trials.

It is widely accepted that the shape of the antitumor immune response is a significant factor that determines a patient's clinical outcome. Thus, it is thought that the detailed characterization of the tumor microenvironment will translate into targeted therapeutic approaches and significant improvements in both overall survival and quality of life following treatment. This review will summarize the knowledge about the immune cell infiltration of the remarkable HNSCC tumor microenvironment with respect to HPV status.

IMMUNE MICROENVIRONMENT OF HEAD AND NECK SQUAMOUS CELL CARCINOMA TUMORS

In the 1950s, the theory of immune surveillance was proposed by Burnet (44). According to this concept, the immune system

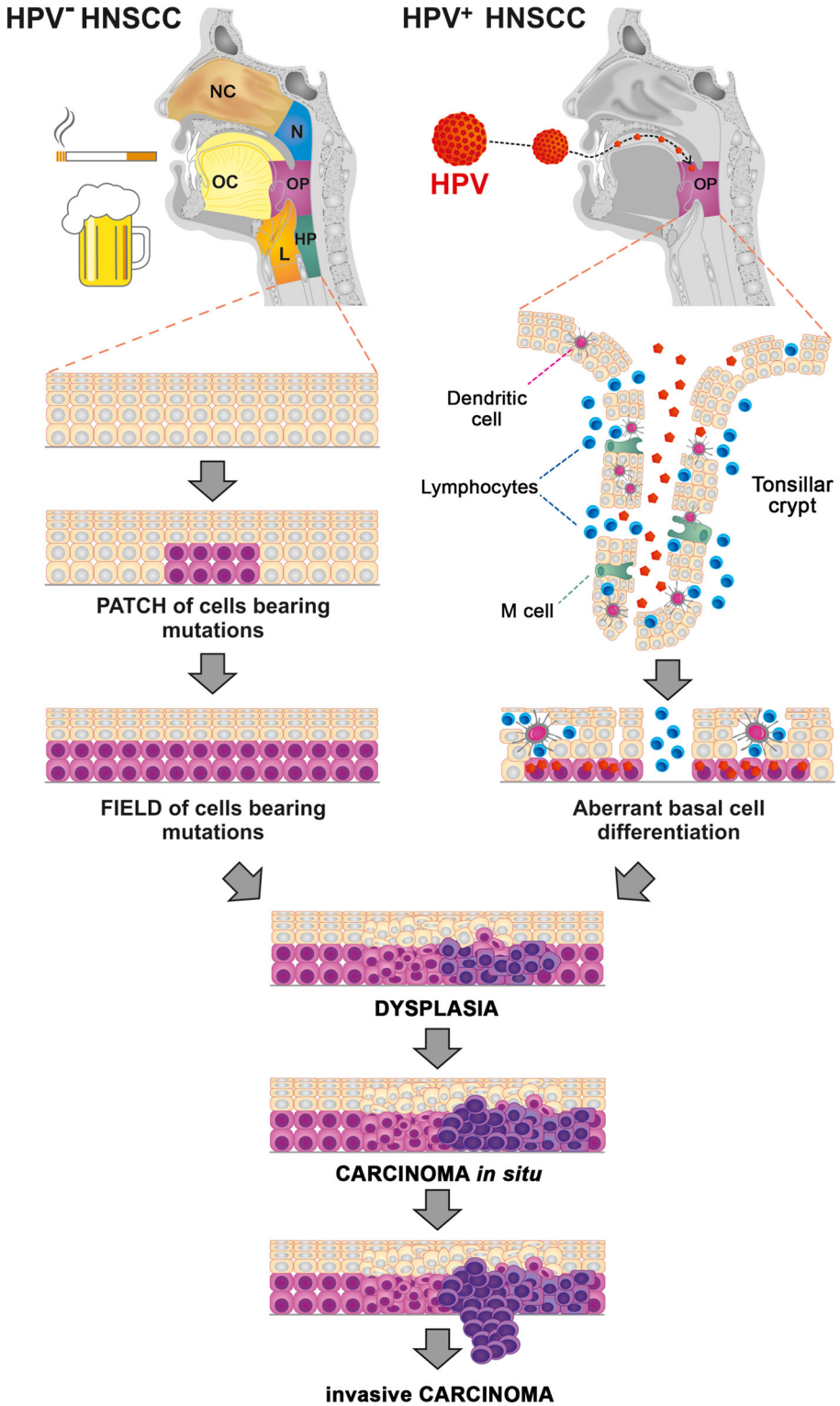

FIGURE 1 | Processes of carcinogenesis in human papillomavirus (HPV)-negative and HPV-associated head and neck squamous cell carcinoma (HNSCC). Tobacco- and alcohol-related mutagens induce widespread mutagenesis in the cells that form the stratified squamous cell epithelium of the upper aerodigestive tract, including the nasal cavity (NC), oral cavity (OC), nasopharynx (N), oropharynx (OP), hypopharynx (HP), and larynx (L). HPV preferentially infects the basal cell layer of the reticulated epithelium of the tonsillar crypts, thus promoting the malignant transformation of epithelial cells within the oropharyngeal region (OP).

TABLE 1 | Features of HPV-negative and HPV-positive HNSCC.

Feature	HPV−	HPV+	References
Risk factors	Tobacco, Alcohol	HPV	(23)
Incidence	Decreasing	Increasing	(24)
Most common anatomic site	Oral cavity, Larynx	Oropharynx	(20)
Age	Older	Younger	(25)
Race	Non-Caucasian	Caucasian	(20)
Education level	Lower	Higher	(24)
5-years overall survival	48%	80%	(26)
Histological subtype	Keratinizing	Non-keratinizing	(27)
LN metastases	55.7%	86%	(20)
Mutational spectrum	*TP53, CDKN2A, MLL2, CUL3, NSD1, PIK3CA, NOTCH*	*PIK3CA, DDX3X, CYLD, FGFR*	(28)
Density of tumor-infiltrating immune cells	Lower	Higher	(29)

HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; LN, lymph node.

constantly recognizes and destroys emerging malignant cells before they can develop into detectable tumors. This theory is supported by the fact that cancers, including HNSCC, are more prevalent in immunosuppressed patients (45, 46). To escape the control of the immune system, tumor cells develop multiple strategies that make them unrecognizable by immune cells or that efficiently suppress the immune response. The mechanisms of tumor immune evasion include the reduction of antigen presentation due to the loss of major histocompatibility complex (MHC) class I expression, the production of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β, the resistance to apoptosis, and the expression of Fas ligand (FasL), which is capable of inducing the death of TILs (47). Together with the recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) into the tumor, these mechanisms help to establish an immunosuppressive microenvironment, which supports tumor growth (47, 48). Despite the prevailing immunosuppressive character, the pattern of immune cell infiltrate markedly differs between HPV-associated and HPV-negative tumors (29, 37) (**Figure 2**). Indeed, not only the density of tumor-infiltrating immune cells but also their phenotypes and functional capacities distinguish immunologically "hottest" HPV-positive tumors with good prognosis from immunologically "colder," high-risk HNSCC. The individual features of the tumor-infiltrating immune cell populations are discussed below, and their prognostic impact is summarized in **Table 2**.

TUMOR-ASSOCIATED MACROPHAGES

Macrophages are monocyte-derived innate immune cells that, as sentinel and effector cells, play an essential role in the maintenance of tissue homeostasis, the control of pathogens, and the overall surveillance of tissue changes (65). According to their mechanisms of activation and subsequent roles in the polarization of the immune response, macrophages are divided into two main phenotypes. Inflammatory "fighting" M1 macrophages are activated by interferon (IFN)-γ and are involved in antitumoral helper T (Th)1 immune responses. Anti-inflammatory "healing" M2 macrophages, which are alternatively activated by IL-4, IL-10, IL-13, and/or prostaglandin E2, are associated with protumoral Th2 immune responses (66–69).

Macrophages are mainly recruited from the bone marrow *via* colony-stimulating factor 1 (CSF-1) and monocyte chemotactic protein 1 (MCP-1) signaling, which are particularly driven by the hypoxic conditions in the tumor tissue (70, 71). M1 macrophages express inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO), IL-12, IL-23, tumor necrosis factor (TNF), IL-1β and IL-6, whereas anti-inflammatory M2 tumor-associated macrophages (TAMs) secrete immunosuppressive cytokines and express arginase-1, which promotes the depletion of extracellular arginine and leads to the metabolic suppression of tumor-infiltrating T cells (65, 69, 71). Additionally, TAMs, as a major source of C-C motif chemokine ligand (CCL)22, help recruit Tregs into the tumor microenvironment *via* the CCL22/C-X-C motif ligand (CXCL)4 pathway (72, 73).

In HNSCC, TAMs generally show the tumor-promoting M2 phenotype that is associated with the production of the immunosuppressive cytokines IL-10 and TGF-β, and their presence in the tumor microenvironment is positively correlated with lymph node status and poor prognosis (71, 74–76). However, although the overall density of TAMs is comparable in HPV-positive and HPV-negative tumors (29, 37), Gameiro et al. (37) reported a significantly lower proportion of M2 macrophages in HPV-associated tumor tissues compared to that in HPV-negative tumor tissues. Similarly, Chen et al. (49) observed a higher M1/M2 macrophage ratio in HPV-positive tumors compared to that in HPV-negative tumors. Importantly, a high M1/M2 ratio correlated with better prognosis in both HPV-positive and HPV-negative HNSCC patients. Both analyses were performed at the mRNA level using publicly available databases.

MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs) form a heterogeneous population of immature myeloid cells, which under

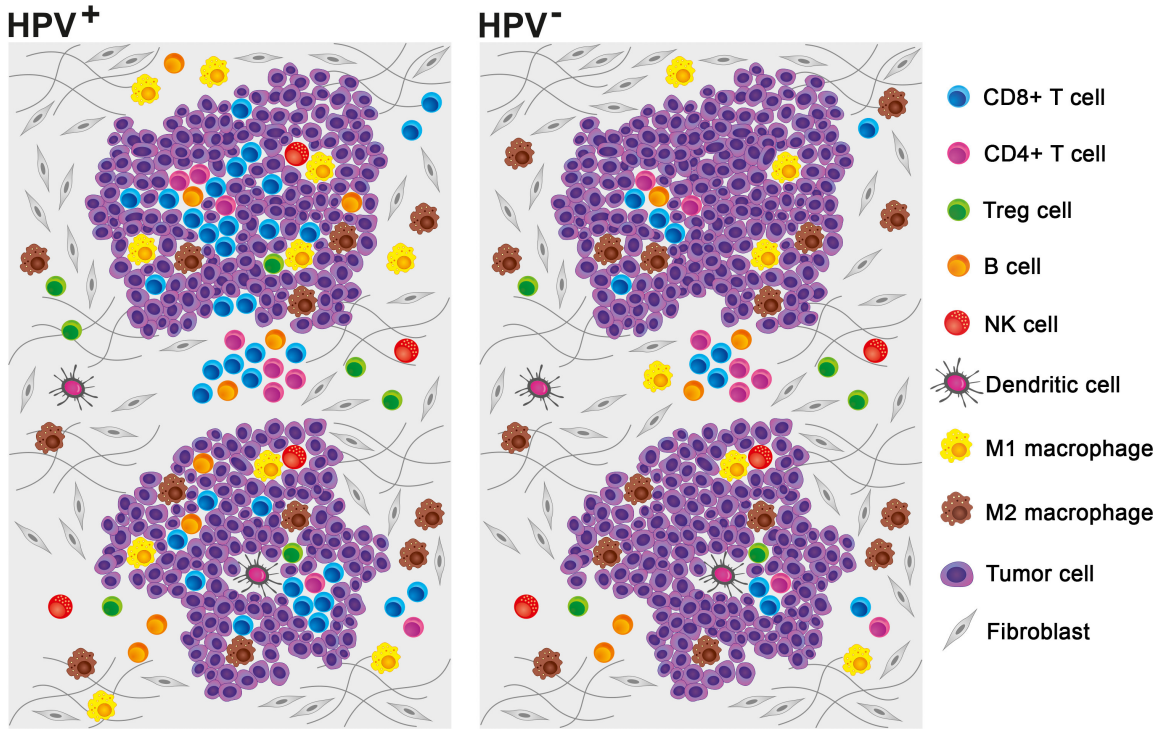

FIGURE 2 | Pattern of tumor-infiltrating immune cells in the human papillomavirus (HPV)-negative and HPV-associated head and neck squamous cell carcinoma (HNSCC) microenvironments. Although myeloid cells prevail in the HPV-negative tumor microenvironment, HPV-associated tumors are mostly characterized by high numbers of tumor-infiltrating lymphocytes. Neutrophils and myeloid-derived suppressor cells (MDSCs) are not included, as it was not possible to extrapolate the relative proportions of these cell populations from published data.

TABLE 2 | Prognostic impact of tumor-infiltrating immune cell populations in HNSCC.

Prognostic marker	Impact on prognosis HPV-	Impact on prognosis HPV+	References
High M1/M2 ratio	Positive	Positive	(49)
MDSC	NA	NA	
Neutrophils	None	Negative	(49)
NK cells	Positive	NA	(50)
mDC	Positive	None	(51–54)
pDC	Negative	NA	(55, 56)
CD8+ T cells	Positive	Positive	(36, 42, 43, 57)
CD4+ T cells	None	None	(49, 57, 58)
Tregs	Contradictory	Contradictory	(59–63)
B cells	NA	Positive	(43)
IL-10+ Bregs	Negative	NA	(64)

HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; M1/M2 ratio, ratio between inflammatory M1 and anti-inflammatory M2 macrophages; NK cells, natural killer cells; MDSC, myeloid-derived suppressor cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; Bregs, regulatory B cells; NA, not available.

physiological conditions represent only 0.5% of peripheral blood mononuclear cells (PBMCs) and consist of precursors of granulocytes, monocytes, and dendritic cells. There are two major subsets of MDSCs in humans, namely, $Lin^{-}HLA\text{-}DR^{-/lo}CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ granulocytic PMN-MDSCs and $Lin^{-}HLA\text{-}DR^{neg/lo}CD11b^{+}CD14^{+}CD15^{-}$ monocytic M-MDSCs (77, 78). Pathological MDSC accumulation is associated with chronic inflammation and cancer progression, and MDSCs are known to exhibit significant immunosuppressive and protumorigenic functions. These tumor-promoting activities include the production of immunosuppressive cytokines IL-10 and TGF-β, the secretion of angiogenic factors, NO and reactive oxygen species (ROS), the promotion and activation of Tregs, and the induction of arginine and cysteine deprivation, which result in the metabolic suppression of tumor-infiltrating T cells and the production of soluble factors that support tumor growth and invasion (71, 77, 79, 80).

MDSCs are mainly recruited to the tumor microenvironment *via* the prostaglandin E2-induced chemokines CCL2, IL-8, and CXCL12 (80, 81). Additionally, tumor cells are capable of producing mediators of chronic inflammation, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), TNF-α, IL-1β, and IL-6, which induce the generation and expansion of MDSCs *in situ* (80, 82). In HNSCC patients without a defined HPV status, the proportion of circulating PMN-MDSCs negatively correlated with overall survival. These peripheral blood-derived MDSCs were capable of suppressing T cell proliferation and cytokine production (83). Similarly, Chikamatsu et al. (84) reported

elevated levels and suppressive activities of MDSCs in the peripheral blood of HNSCC patients. In HPV-negative HNSCCs, tumor-derived MDSCs created a significant proportion of tumor-infiltrating immune cells and were capable of efficiently suppressing T cell (85) and natural killer (NK) cell functions (86). As all of these studies either did not specify the HPV status of the patients or included HNSCC patients with tumors localized outside the oropharynx, there is no report about the proportions and suppressive capacities of MDSCs in HPV-associated HNSCC to date.

NEUTROPHILS

Neutrophils represent the most abundant population of immune cells in humans and play an essential role in antimicrobial immune responses and wound healing (87). Depending on the signals from the tumor microenvironment, neutrophils can be either protumorigenic or antitumorigenic; however, most published studies describe neutrophils as tumor-promoting cells with a strong impact on the antitumor immune response (87, 88).

Similar to other malignancies, neutrophils were found at elevated levels in the peripheral blood of HNSCC patients, and their frequencies were inversely correlated with the frequencies of lymphocytes (89, 90). Patients with HPV-associated tumors had significantly lower levels of circulating neutrophils compared to patients with HPV-negative tumors, and the high absolute number of neutrophils correlated with poor prognosis in HPV-positive patients but not HPV-negative patients (91). However, if the abundance of neutrophils was related to the levels of circulating lymphocytes, a high neutrophil-to-lymphocyte ratio (NLR) was associated with poor prognosis in both groups of patients. As expected, patients with HPV-associated tumors showed lower NLR ratios compared to patients with HPV-negative tumors (89). Surprisingly, in patients with advanced oral squamous cell carcinoma (OSCC), both very high and very low NLRs were reported to be associated with increased risk of death (92). The authors suggest that compared to early-stage OSCC, where low NLR indicates unaffected immune system, in advanced-stage tumors, very low NLR may be a marker of immune system exhaustion.

The only publication that mentions the levels of neutrophils in the tumor microenvironment is an *in silico* study published by Chen et al. (49), which reported significantly lower levels of tumor-infiltrating neutrophils in HPV-associated samples compared to those in HPV-negative samples. Additionally, high infiltration of neutrophils was correlated with poor outcome in patients with HPV-associated HNSCC and was determined to be an independent prognostic marker based on the Cox proportional hazard model.

NATURAL KILLER CELLS

NK cells are generally considered to be effector lymphocytes of the innate immune system; however, they express a wide spectrum of activating and inhibitory receptors, which efficiently empower their cytotoxicity against virus-infected and tumor cells while concurrently ensuring self-tolerance (93). NK cells are known to recognize cells that escape detection by cytotoxic T cells due to the abnormal surface expression of HLA class I molecules. Indeed, a reduction in HLA class I expression is a very common mechanism used by viruses, such as HPV, and tumor cells to evade the host immune response (94). There are two major groups of NK cells, namely, cytokine-producing $CD56^{bright}CD16^{dim}$ immunoregulatory NK cells and $CD56^{dim}CD16^{bright}$ cytotoxic NK cells.

In HNSCC patients, peripheral $CD56^{dim}$ NK cells were shown to be functionally impaired and preferentially targeted for apoptosis (95). Subsequently, plasma TGFβ1 and soluble MHC class I chain-related peptide A (sMICA) were determined to be the main factors driving the loss of the functional capacities of peripheral NK cells in HNSCC (96). Although an *in silico* study published by Chen et al. (49) revealed no difference between the NK cell gene signatures in HPV-negative and HPV-positive HNSCC samples, Wagner et al. (50) found significantly higher numbers of tumor-infiltrating CD56+ NK cells in the microenvironment of HPV-positive OPSCC specimens compared to those in the microenvironment of HPV-negative OPSCC specimens. These cells mostly coexpressed granzyme B and CD16, suggesting their cytotoxic capacity and were correlated with increased overall survival independent of the HPV status of the patients.

MYELOID DENDRITIC CELLS

Myeloid dendritic cells (mDCs) are the most important antigen-presenting cells (APCs) with the highest capacity to initiate adaptive immune responses. Immature mDCs efficiently capture and process antigens, but due to the lack of co-stimulatory molecules, they are rather tolerogenic and may actually inhibit T cell responses (97, 98). Upon stimulation with microbial stimuli and inflammatory cytokines IL-1, TNFα, and IL-12, mDCs undergo maturation and migrate into T cell-rich areas of lymphoid organs. Mature mDCs produce substantial amounts of IL-12 and express high levels of HLA molecules and high levels of co-stimulatory molecules that are equally essential for T cell activation (99, 100).

Compared to healthy controls, HNSCC patients had significantly lower numbers of CD11c+ DCs in their peripheral blood. Interestingly, the decreased mDC levels normalized after tumor resection (101). In squamous cell carcinoma of the tongue, the presence of a high level of peritumoral CD1a+ DCs was shown to be associated with improved overall patient survival (52). High densities of stromal CD1a+ Langerhans cells were later confirmed to be a positive prognostic marker in HPV- HNSCC but not in HPV+ HNSCC (54). Similarly, in laryngeal (51) and oral (53) cancer patients, low densities of S-100+ DCs were associated with poor prognosis. To the best of our knowledge, compared to HPV-negative HNSCC, mDCs have not been considered a valid prognostic factor in HPV-associated oropharyngeal tumors to date.

In silico studies published by Chen et al. (49) and Gameiro et al. (37) did not reveal any statistically significant differences in the expression of mDC-related genes between HPV-positive and HPV-negative HNSCC samples (37, 49). In contrast, we observed significantly higher levels of CD45+LIN-HLA-DR+CD14-CD11c+ mDCs in HPV+ oropharyngeal tumors compared to those in HPV-negative HNSCC using flow cytometry (29). However, we did not show any differences between the densities of tumor-infiltrating DC-LAMP+ activated mDCs in HPV+ and HPV- oropharyngeal tumor samples using immunohistochemical staining, and we did not observe any associations between the DC-LAMP+ mDC densities and patient outcomes (43).

PLASMACYTOID DENDRITIC CELLS

Plasmacytoid dendritic cells (pDCs) play an essential role in the antiviral immune response and are characterized by their considerable production of IFNα in response to viral RNA or DNA, which are recognized by intracellular Toll-like receptors TLR7 and TLR9, respectively (102). Additionally, depending on the activation status of pDCs, these cells may act as efficient antigen-presenting cells or induce the differentiation and expansion of Tregs (103, 104).

Similar to other solid tumors, the pDCs infiltrating HNSCC were shown to be functionally impaired and were thought to be rather protumorigenic. Indeed, Hartmann et al. (105) reported a diminished capacity of HNSCC-infiltrating pDCs to produce IFNα upon TLR9 stimulation with CpG motif-containing oligonucleotides. Moreover, tumor-derived supernatants harvested from primary tumor cell cultures and HNSCC cell lines inhibited IFNα production in control peripheral pDCs. Bruchhage et al. (106) later suggested that IL-10 might be the major cytokine responsible for the impairment of pDC functional capacity in the HNSCC microenvironment. Consistent with these findings, high densities of pDCs were associated with poor prognosis in oral squamous cell carcinoma patients (55, 56).

T LYMPHOCYTES (TUMOR-INFILTRATING LYMPHOCYTES)

T lymphocytes are the pillars of adaptive immunity and are known to be essential in the control of tumor progression. Consequently, most of the immunotherapeutic protocols in cancer management, including highly successful immune checkpoint inhibitors, target T cell-related immune responses. Three major classes of T cells can be distinguished according to their primary function: cytotoxic CD8+ T cells, which are capable of killing infected or malignant cells; helper CD4+ T cells, which provide essential signals to B cells and polarize the immune response *via* cytokine production; and Tregs, which suppress the activity of other lymphocytes and help maintain peripheral tolerance.

Similar to the observations in other malignancies, the densities of CD8+ tumor infiltrating T cells were positively correlated with improved clinical outcome in both HPV-associated and HPV-negative HNSCC (36, 42, 43, 57, 107, 108). In general, tumors associated with HPV show significantly higher levels of T cell infiltration, especially CD8+ T cell infiltration (29, 36, 37, 49). Additionally, significantly higher proportions of CD8+ T cells infiltrating HPV-associated HNSCC were reported to be capable of producing pro-inflammatory cytokines, namely, IFNγ and IL-17 (29). However, a subgroup of cases with low proportions of infiltrating TILs and prognosis comparable to that of patients with HPV-negative tumors can be identified among HNSCC patients with HPV-positive tumors (26). These data suggest that the quantity and quality of the immune infiltrate is a valid prognostic tool that may markedly improve the stratification of HNSCC patients. Indeed, it has been shown that HPV-specific CD8+ T cells are detectable in 64–75% of HPV-positive HNSCC samples (109–111). These functional HPV-specific T cells were shown to be mostly PD-1+Tim-3- (111), and their presence was associated with improved overall survival (110). Thus, in addition to the density of CD8+ T cells, the presence of HPV-specific T cells seems to be a valid prognostic marker that can be used for better patient stratification.

In the case of CD4+ T cells, our study based on flow cytometry data showed significantly higher numbers of naive CD4+ T cells but not Th1 cells and Th17 cells in the tumor microenvironment of HPV-positive HNSCC samples compared to those in the tumor microenvironment of HPV-negative samples (29). A gene expression study published by Gameiro et al. (37) revealed higher numbers of follicular T helper (Tfh) cells and Tregs, but not memory CD4+ T cells, in HPV-associated tumor samples compared to those in HPV-negative tumor samples. Higher numbers of Tregs in HPV-positive HNSCC were also reported by several studies based on immunohistochemical staining of tumor sections (36, 58, 112). Unlike CD8+ T cells, the role of Tregs in HNSCC is not fully understood. Whereas, some studies suggest a negative impact of tumor-infiltrating Tregs on disease progression (60, 62), other publications reported a positive correlation between high densities of Tregs and patient outcome (59, 61, 63). The high numbers of tumor-infiltrating Tregs observed in immunologically "hot" HPV-associated tumors suggest that the proportions of Tregs or the CD8+ T cell/Treg ratio, rather than Treg numbers alone, might truly reflect the shape of the immune response within the tumor microenvironment. Indeed, we have observed that although the numbers of Tregs were slightly higher in HPV-associated HNSCC samples, the proportions of these cells were actually lower (29). Thus, the whole pattern of immune cells, which also reflects the relationships among various cell populations, provides the best information about the prevailing status of the immune response within the tumor microenvironment.

B LYMPHOCYTES

It is well-known that B lymphocytes play a central role in humoral immunity due to their capacity to produce antibodies. Different subsets of B cells are able to recognize either polysaccharides or lipid antigens, which leads to T cell-independent responses,

or protein antigens, which are presented to Tfh cells in the lymph nodes, Payer's patches, and spleen *via* HLA class II molecules. During T cell-dependent activation, Tfh cells stimulate B cell activation and differentiation into antibody-secreting plasmablasts *via* the CD40L-CD40 pathway and IL-21 and IL-4 production. Additionally, B cells can undergo further maturation in germinal centers and develop either into long-lived plasma cells that secrete high levels of antibodies or into memory B cells. Compared to the T cell-independent pathway of B cell activation, the T cell-dependent pathway of B cell activation leads to the production of high affinity class-switched antibodies (113, 114). In addition to antibody production, B cells are capable of producing immunomodulatory cytokines and chemokines, can play a role as antigen-presenting cells, and can efficiently stimulate both CD4+ T cells and CD8+ T cells (114–116).

Compared to T cells, the role of B cells in cancer immunology has been less extensively explored and generally underestimated. Thus, the role of B cells in tumor progression remains controversial. Whereas, B cells were shown to be rather protumorigenic in mice, high levels of tumor-infiltrating B cells in humans were mainly associated with good outcome and longer overall survival (114, 117). However, recent studies have shown that B cells play an essential role in the response to immune checkpoint inhibitors and thus might be much more important for successful immunotherapeutic approaches than expected (118).

In HNSCC, B cell signatures were able to distinguish between HPV-associated and HPV-negative carcinomas, with a significantly higher expression of B cell-related genes in HPV-associated tumors (37, 43, 49, 119). These data were confirmed at the cellular level, and significantly higher densities of tumor-infiltrating CD20+ B cells were observed in the microenvironment of HPV-associated tumor sections than in the microenvironment of HPV-negative samples (43, 112, 120). Compared to samples with low infiltrates of lymphocytes, B cells derived from TIL-rich tumors were shown to be activated and to express high levels of HLA and costimulatory molecules. Consistent with these findings, high B cell density was associated with good prognosis in OPSCC patients regardless of HPV status (43). Importantly, B cells were shown to create aggregates with CD8+ T cells, and the frequency of these B cell–CD8+ T cell interactions was positively associated with the proportions of HPV-specific CD8+ T cells infiltrating the tumor microenvironment, suggesting the importance of B cells for the T cell-related antitumor immune response (43). In contrast, the proportion of IL-10-producing regulatory B cells (Bregs) in HPV-associated tumor tissues was comparable to the levels of Bregs in control tonsils, indicating that Bregs do not accumulate in the tumor microenvironment of HPV-associated HNSCC (43). In HPV-negative tongue squamous cell carcinoma, the proportions of IL-10+CD19+ Bregs were also very low (below 1%); however, their levels were significantly enhanced compared to adjacent tissue and were significantly correlated with poor outcome in univariant, but not multivariant, survival analysis (64).

Besides the direct association between B cell densities in the tumor microenvironment and the disease outcome, the presence of antibodies against HPV16 E6 and E7 oncoproteins in patients' sera was positively correlated with the recurrence-free survival of HPV-positive OPSCC patients (121, 122). These findings support the importance of B cell-mediated immune responses in HPV-associated OPSCC.

CYTOKINE AND CHEMOKINE PROFILE

Similar to other malignancies, higher levels of pro-angiogenic cytokines IL-8 and VEGF were detected in HNSCC patients' sera compared to healthy controls (123). Expression of these cytokines by HNSCC cells was confirmed by immunohistochemistry (IHC), showing up to 90% of VEGF-positive tumors (123, 124). Together with pro-angiogenic effects, IL-8 and VEGF are known to promote tumor growth and metastasis (125). Comparing plasma levels of cytokines in HNSCC patients and healthy controls, Lathers et al. (126) showed that the cytokine profile of HNSCC patients is shifted toward Th2 bias. Indeed, HNSCC patients had significantly higher levels of IL-4, IL-6, and IL-10 in the plasma compared to controls. In agreement with this finding, lower levels of IFNγ were observed in HNSCC patients; however, the levels of IL-1, IL-2, and GM-CSF were increased, whereas Th1 cytokine IL-12 and immunosuppressive TGFβ remained unchanged (126). IL-6 and IL-10 were detected in HNSCC cell lines, primary HNSCC cells, as well as tumor-infiltrating immune cells (123, 127–129). Moreover, serum levels of IL-6 negatively correlated with HNSCC patients' prognosis (130). Despite exerting many pro-inflammatory properties, protumorigenic IL-6 is a pleiotropic cytokine, which affects cell growth, maturation, survival, and migration during immune responses (131, 132). In colorectal cancer, IL-6 was shown to stimulate IL-10 production by tumor cells (133). The role of IL-10 in cancer progression has been extensively studied. Mostly, IL-10 is regarded as an immunosuppressive, anti-inflammatory cytokine, which promotes tumor escape from immune surveillance. However, IL-10 was also shown to inhibit tumor-induced angiogenesis, enhance the production of NO, and increase tumor cell line immunogenicity in some preclinical models (134). Besides pro-angiogenic and Th2 cytokines, HNSCC tissues were reported to produce high levels of pro-inflammatory TNFα (29, 127). Immunohistochemical staining revealed that TNFα is mainly produced by tumor cells, TAMs, endothelial cells, stromal fibroblasts, and inflammatory tumor-infiltrating immune cells (127, 135, 136).

As most of the studies did not include HPV status, little is known about the differences in cytokine profile of HPV-positive and HPV-negative HNSCC. Partlová et al. (29) reported no statistically significant differences in cytokine production in cell culture supernatants derived from HPV-positive and HPV-negative HNSCC, although HPV-positive samples produced higher levels of IL-2, IL-17, IL-23, and IFNγ and slightly lower levels of IL-1β, IL-6, and TNFα compared to HPV-negative samples. However, HPV-positive samples produced markedly higher levels of pro-inflammatory chemokines CXCL9 and CXCL10, which characterize immunologically "hot" tumors (137). Additionally, HPV-positive samples produced significantly higher levels of CCL17 and CCL21. *Via* interaction with CCR4 and CCR8, CCL17 induces chemoattraction of T cells (mainly

Tregs and Th2 cells), macrophages, and activated NK cells (138–140). Surprisingly, in HNSCC, the levels of CCL17 positively correlated with the densities of Th17, Th1, and cytotoxic T cells, but not Tregs and macrophages (29). In secondary lymphoid organs, CCL21 attracts naive T cells facilitating their co-localization with antigen-stimulated DCs in T cell zones. In addition to chemoattraction, CCL21 favors expansion of $CD4^+$ and $CD8^+$ T cells and induces Th1 polarization, whereas Tregs are hyporesponsive to both CCL21-induced migration and CCL21 co-stimulation (141). In HNSCC, levels of CCL21 positively correlated with the frequency of Th17 cells (29).

CONCLUSIONS

Despite the markedly better prognosis of HNSCC patients with HPV-associated tumors and despite the recent segregation of HPV-associated and HPV-negative HNSCC into two different entities, the standard of care management remains the same in both groups of patients. Clinical trials focused on treatment deintensification strategies have not provided the necessary evidence to date to support deintensification protocols. The recently published multicenter DeESCAlaTE and RTOG 1016 clinical trials showed a significant decrease in tumor control in patients with HPV-associated OPSCC treated with radiotherapy plus cetuximab compared to those treated with radiotherapy plus cisplatin-based chemotherapy, and, moreover, there was no benefit in terms of reduced toxicity (142, 143). Indeed, the appropriate selection of patients who would profit from deintensified treatment is essential; however, a valid biomarker that is suitable for the precise stratification of patients with HPV-associated tumors has not yet been approved. As the density and pattern of the immune infiltrate in tumor tissues has been repeatedly associated with patient outcome in a wide range of malignancies, including HPV-associated HNSCC, high densities of CD8+ T cells and especially B cells or the presence of HPV-specific T cells within the tumor tissue might be considered possible biomarkers in treatment deintensification clinical trials. However, these markers would be applicable in surgically treated patients only, as tissue specimens are necessary for precise IHC or flow cytometry-based analyses. For non-surgically treated patients, IL-6 plasma levels and NLR might be candidates for stratification biomarkers. Nevertheless, to validate a biomarker, a large multicenter study needs to be performed to establish a proper cutoff. A precise and comprehensive immune monitoring of completed deintensification clinical trials would enable to preselect a biomarker worth validating.

The current knowledge about the HNSCC microenvironment might be also translated into novel immunotherapeutic approaches. Immune checkpoint inhibitors (ICIs) made a true breakthrough in cancer immunotherapy; nevertheless, primary or acquired resistance often accompanies this approach. Strategies combining multiple approaches thus achieve the highest response rate in cancer patients. In HNSCC, anti-PD-1 monoclonal antibodies nivolumab and pembrolizumab were recently approved as first-line treatment for patients with metastatic or unresectable, recurrent disease (144). Enhancement of Tim-3 expression on T cells following PD-1 blockade as a mechanism of acquired resistance (145) provides a rationale to combine anti-PD-1 therapy with anti-Tim-3 antibodies. High efficacy of simultaneously administered antigen and anti-PD-1 antibody (146) and the absence of Tim-3 overexpression in HPV E6/E7 peptide-stimulated T cells following PD-1 blockade (111) favors combining immune checkpoint inhibitors with HPV-specific vaccine. Indeed, the overall response rate of 33% was achieved with this approach in a phase 2 clinical trial enrolling incurable HPV16-positive OPSCC patients (147).

The importance of B cells in both patient stratification (43) and response to anti-PD-1 therapy (118, 148) suggests that B cells might be a useful target in future immunotherapy protocols. Thus, B cell-activating molecules, such as CD40 agonist antibodies, which are already tested in multiple clinical trials (149), might be interesting partners in novel combination approaches to immunotherapy.

Consequently, patient stratification as well as present immunotherapeutic approaches might be further refined based on the current knowledge of the HNSCC microenvironment, allowing beneficial changes in the standard of care for the treatment of HPV-associated HNSCC.

AUTHOR CONTRIBUTIONS

AF and VK wrote the manuscript. MH prepared the illustrations. KH searched for publications in public databases. RŠ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2018) 68:394–424. doi: 10.3322/caac.21492
2. Lajer CB, von Buchwald C. The role of human papillomavirus in head and neck cancer. *APMIS.* (2010) 118:510–9. doi: 10.1111/j.1600-0463.2010.02624.x
3. D'Souza G, Dempsey A. The role of HPV in head and neck cancer and review of the HPV vaccine. *Prev Med.* (2011) 53 (Suppl. 1):S5–S11. doi: 10.1016/j.ypmed.2011.08.001
4. Rautava J, Syrjanen S. Biology of human papillomavirus infections in head and neck carcinogenesis. *Head Neck Pathol.* (2012) 6 (Suppl. 1):S3–15. doi: 10.1007/s12105-012-0367-2
5. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer.* (2002) 2:342–50. doi: 10.1038/nrc798

6. Taberna M, Mena M, Pavon MA, Alemany L, Gillison ML, Mesia R. Human papillomavirus-related oropharyngeal cancer. *Ann Oncol.* (2017) 28:2386–98. doi: 10.1093/annonc/mdx304
7. Faraji F, Zaidi M, Fakhry C, Gaykalova DA. Molecular mechanisms of human papillomavirus-related carcinogenesis in head and neck cancer. *Microbes Infect.* (2017) 19:464–75. doi: 10.1016/j.micinf.2017.06.001
8. Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK, et al. Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. *Front Cell Dev Biol.* (2019) 7:52. doi: 10.3389/fcell.2019.00052
9. 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) 63:4417–21. doi: 10.1128/JVI.63.10.4417-4421.1989
10. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell.* (1990) 63:1129–36. doi: 10.1016/0092-8674(90)90409-8
11. Kim SH, Koo BS, Kang S, Park K, Kim H, Lee KR, et al. HPV integration begins in the tonsillar crypt and leads to the alteration of p16, EGFR and c-myc during tumor formation. *Int J Cancer.* (2007) 120:1418–25. doi: 10.1002/ijc.22464
12. Maleki Vareki S. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. *J Immunother Cancer.* (2018) 6:157. doi: 10.1186/s40425-018-0479-7
13. Eder T, Hess AK, Konschak R, Stromberger C, Jöhrens K, Fleischer V, et al. Interference of tumour mutational burden with outcome of patients with head and neck cancer treated with definitive chemoradiation: a multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group. *Eur J Cancer.* (2019) 116:67–76. doi: 10.1016/j.ejca.2019.04.015
14. Henderson S, Fenton T. APOBEC3 genes: retroviral restriction factors to cancer drivers. *Trends Mol Med.* (2015) 21:274–84. doi: 10.1016/j.molmed.2015.02.007
15. Faden DL, Ding F, Lin Y, Zhai S, Kuo F, Chan TA, et al. APOBEC mutagenesis is tightly linked to the immune landscape and immunotherapy biomarkers in head and neck squamous cell carcinoma. *Oral Oncol.* (2019) 96:140–7. doi: 10.1016/j.oraloncology.2019.07.020
16. Vogelmann R, Amieva MR. The role of bacterial pathogens in cancer. *Curr Opin Microbiol.* (2007) 10:76–81. doi: 10.1016/j.mib.2006.12.004
17. Whitmore SE, Lamont RJ. Oral bacteria and cancer. *PLoS Pathog.* (2014) 10:e1003933. doi: 10.1371/journal.ppat.1003933
18. Wolf A, Moissl-Eichinger C, Perras A, Koskinen K, Tomazic PV, Thurnher D. The salivary microbiome as an indicator of carcinogenesis in patients with oropharyngeal squamous cell carcinoma: a pilot study. *Sci Rep.* (2017) 7:5867. doi: 10.1038/s41598-017-06361-2
19. Licitra L, Perrone F, Bossi P, Suardi S, Mariani L, Artusi R, et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol.* (2006) 24:5630–6. doi: 10.1200/JCO.2005.04.6136
20. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, 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. doi: 10.1093/jnci/djn011
21. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol.* (2009) 27:1992–8. doi: 10.1200/JCO.2008.20.2853
22. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin.* (2017) 67:122–37. doi: 10.3322/caac.21389
23. Fouret P, Martin F, Flahault A, Saint-Guily JL. Human papillomavirus infection in the malignant and premalignant head and neck epithelium. *Diagn Mol Pathol.* (1995) 4:122–7. doi: 10.1097/00019606-199506000-00008
24. Benson E, Li R, Eisele D, Fakhry C. The clinical impact of HPV tumor status upon head and neck squamous cell carcinomas. *Oral Oncol.* (2014) 50:565–74. doi: 10.1016/j.oraloncology.2013.09.008
25. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med.* (2010) 363:24–35. doi: 10.1056/NEJMoa0912217
26. Ward MJ, Thirdborough SM, Mellows T, Riley C, Harris S, Suchak K, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer.* (2014) 110:489–500. doi: 10.1038/bjc.2013.639
27. Holmes BJ, Wenig BM. Virus-associated carcinomas of the head & neck: Update from the 2017 WHO classification. *Ann Diagn Pathol.* (2019) 38:29–42. doi: 10.1016/j.anndiagpath.2018.10.008
28. Seiwert TY, Zuo Z, Keck MK, Khattri A, Pedamallu CS, Stricker T, et al. Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. *Clin Cancer Res.* (2015) 21:632–41. doi: 10.1158/1078-0432.CCR-13-3310
29. Partlová S, Bouček J, Kloudová K, Lukešová E, Zábrodský M, Grega M, et al. Distinct patterns of intratumoral immune cell infiltrates in patients with HPV-associated compared to non-virally induced head and neck squamous cell carcinoma. *Oncoimmunology.* (2015) 4:e965570. doi: 10.4161/21624011.2014.965570
30. Chakravarthy A, Henderson S, Thirdborough SM, Ottensmeier CH, Su X, Lechner M, et al. Human papillomavirus drives tumor development throughout the head and neck: improved prognosis is associated with an immune response largely restricted to the oropharynx. *J Clin Oncol.* (2016) 34:4132–41. doi: 10.1200/JCO.2016.68.2955
31. Adelstein DJ, Ridge JA, Gillison ML, Chaturvedi AK, D'Souza G, Gravitt PE, et al. Head and neck squamous cell cancer and the human papillomavirus: summary of a National Cancer Institute State of the Science Meeting, November 9-10, 2008 Washington, D.C. *Head Neck.* (2009) 31:1393–422. doi: 10.1002/hed.21269
32. Kelly JR, Husain ZA, Burtness B. Treatment de-intensification strategies for head and neck cancer. *Eur J Cancer.* (2016) 68:125–33. doi: 10.1016/j.ejca.2016.09.006
33. Gillison ML, Zhang Q, Jordan R, Xiao W, Westra WH, Trotti A, et al. Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. *J Clin Oncol.* (2012) 30:2102–11. doi: 10.1200/JCO.2011.38.4099
34. Granata R, Miceli R, Orlandi E, Perrone F, Cortelazzi B, Franceschini M, et al. Tumor stage, human papillomavirus and smoking status affect the survival of patients with oropharyngeal cancer: an Italian validation study. *Ann Oncol.* (2012) 23:1832–7. doi: 10.1093/annonc/mdr544
35. Mandal R, Senbabaoglu Y, Desrichard A, Havel JJ, Dalin MG, Riaz N, Lee KW, et al. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight.* (2016) 1:e89829. doi: 10.1172/jci.insight.89829
36. Näsman A, Romanitan M, Nordfors C, Grün N, Johansson H, Hammarstedt L, et al. Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. *PLoS ONE.* (2012) 7:e38711. doi: 10.1371/journal.pone.0038711
37. Gameiro SF, Ghasemi F, Barrett JW, Koropatnick J, Nichols AC, Mymryk JS, et al. Treatment-naive HPV+ head and neck cancers display a T-cell-inflamed phenotype distinct from their HPV- counterparts that has implications for immunotherapy. *Oncoimmunology.* (2018) 7:e1498439. doi: 10.1080/2162402X.2018.1498439
38. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* (2006) 313:1960–4. doi: 10.1126/science.1129139
39. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol.* (2014) 232:199–209. doi: 10.1002/path.4287
40. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, et al. Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. *Cancer.* (2008) 113:1387–95. doi: 10.1002/cncr.23712
41. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA.* (2005) 102:18538–43. doi: 10.1073/pnas.0509182102

42. Solomon B, Young RJ, Bressel M, Urban D, Hendry S, Thai A, et al. Prognostic Significance of PD-L1(+) and CD8(+) Immune Cells in HPV(+) oropharyngeal squamous cell carcinoma. *Cancer Immunol Res.* (2018) 6:295–304. doi: 10.1158/2326-6066.CIR-17-0299
43. Hladíková K, Koucký V, Bouček J, Laco J, Grega M, Hodek M, et al. Tumor-infiltrating B cells affect the progression of oropharyngeal squamous cell carcinoma via cell-to-cell interactions with CD8(+) T cells. *J Immunother Cancer.* (2019) 7:261. doi: 10.1186/s40425-019-0726-6
44. Burnet FM. Cancer a biological approach. *Br Med J.* (1957) 1:841–7. doi: 10.1136/bmj.1.5023.841
45. Boshoff C, Weiss R. AIDS-related malignancies. *Nat Rev Cancer.* (2002) 2:373–82. doi: 10.1038/nrc797
46. Engels EA, Pfeiffer RM, Fraumeni JF Jr, Kasiske BL, Israni AK, Snyder JJ, et al. Spectrum of cancer risk among US solid organ transplant recipients. *JAMA.* (2011) 306:1891–901. doi: 10.1001/jama.2011.1592
47. Duray A, Demoulin S, Hubert P, Delvenne P, Saussez S. Immune suppression in head and neck cancers: a review. *Clin Dev Immunol.* (2010) 2010:701657. doi: 10.1155/2010/701657
48. Costello RT, Gastaut JA, Olive D. Tumor escape from immune surveillance. *Arch Immunol Ther Exp (Warsz).* (1999) 47:83–88.
49. Chen X, Yan B, Lou H, Shen Z, Tong F, Zhai A, et al. Immunological network analysis in HPV associated head and neck squamous cancer and implications for disease prognosis. *Mol Immunol.* (2018) 96:28–36. doi: 10.1016/j.molimm.2018.02.005
50. Wagner S, Wittekindt C, Reuschenbach M, Hennig B, Thevarajah M, Würdemann N, et al. CD56-positive lymphocyte infiltration in relation to human papillomavirus association and prognostic significance in oropharyngeal squamous cell carcinoma. *Int J Cancer.* (2016) 138:2263–73. doi: 10.1002/ijc.29962
51. Gallo O, Libonati GA, Gallina E, Fini-Storchi O, Giannini A, Urso C, et al. Langerhans cells related to prognosis in patients with laryngeal carcinoma. *Arch Otolaryngol Head Neck Surg.* (1991) 117:1007–10. doi: 10.1001/archotol.1991.01870210079015
52. Goldman SA, Baker E, Weyant RJ, Clarke MR, Myers JN, Lotze MT. Peritumoral CD1a-positive dendritic cells are associated with improved survival in patients with tongue carcinoma. *Arch Otolaryngol Head Neck Surg.* (1998) 124:641–6. doi: 10.1001/archotol.124.6.641
53. Reichert TE, Scheuer C, Day R, Wagner W, Whiteside TL. The number of intratumoral dendritic cells and zeta-chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma. *Cancer.* (2001) 91:2136–47. doi: 10.1002/1097-0142(20010601)91:11<2136::AID-CNCR1242>3.0.CO;2-Q
54. Kindt N, Descamps G, Seminerio I, Bellier J, Lechien JR, Pottier C, et al. Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas. *Oral Oncol.* (2016) 62:1–10. doi: 10.1016/j.oraloncology.2016.08.016
55. O'Donnell RK, Mick R, Feldman M, Hino S, Wang Y, Brose MS, et al. Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. *Cancer Lett.* (2007) 255:145–52. doi: 10.1016/j.canlet.2007.04.003
56. Han N, Zhang Z, Liu S, Ow A, Ruan M, Yang W, et al. Increased tumor-infiltrating plasmacytoid dendritic cells predicts poor prognosis in oral squamous cell carcinoma. *Arch Oral Biol.* (2017) 78:129–34. doi: 10.1016/j.archoralbio.2017.02.012
57. Nordfors C, Grün N, Tertipis N, Ährlund-Richter A, Haeggblom L, Sivars L, Du J, et al. CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. *Eur J Cancer.* (2013) 49:2522–30. doi: 10.1016/j.ejca.2013.03.019
58. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. *Cancer Res.* (2013) 73:128–38. doi: 10.1158/0008-5472.CAN-12-2606
59. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res.* (2006) 12:465–72. doi: 10.1158/1078-0432.CCR-05-1886
60. Sun J, Tang DN, Fu T, Sharma P. Identification of human regulatory T cells in the setting of T-cell activation and anti-CTLA-4 immunotherapy on the basis of expression of latency-associated peptide. *Cancer Discov.* (2012) 2:122–30. doi: 10.1158/2159-8290.CD-11-0236
61. Bron L, Jandus C, Andrejevic-Blant S, Speiser DE, Monnier P, Romero P, et al. Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. *Int J Cancer.* (2013) 132:E85–93. doi: 10.1002/ijc.27728
62. Hanakawa H, Orita Y, Sato Y, Takeuchi M, Ohno K, Gion Y, et al. Regulatory T-cell infiltration in tongue squamous cell carcinoma. *Acta Otolaryngol.* (2014) 134:859–64. doi: 10.3109/00016489.2014.918279
63. Santegoets SJ, Duurland CL, Jordanova ES, van Ham JJ, Ehsan I, van Egmond SL, et al. Tbet-positive regulatory T cells accumulate in oropharyngeal cancers with ongoing tumor-specific type 1 T cell responses. *J Immunother Cancer.* (2019) 7:14. doi: 10.1186/s40425-019-0497-0
64. Zhou X, Su YX, Lao XM, Liang YJ, Liao GQ. CD19(+)IL-10(+) regulatory B cells affect survival of tongue squamous cell carcinoma patients and induce resting CD4(+) T cells to CD4(+)Foxp3(+) regulatory T cells. *Oral Oncol.* (2016) 53:27–35. doi: 10.1016/j.oraloncology.2015.11.003
65. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: phenotypical vs. functional differentiation. *Front Immunol.* (2014) 5:514. doi: 10.3389/fimmu.2014.00514
66. Corraliza IM, Soler G, Eichmann K, Modolell M. Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages. *Biochem Biophys Res Commun.* (1995) 206:667–73. doi: 10.1006/bbrc.1995.1094
67. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* (2010) 141:39–51. doi: 10.1016/j.cell.2010.03.014
68. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* (2014) 6:13. doi: 10.12703/P6-13
69. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front Immunol.* (2014) 5:532. doi: 10.3389/fimmu.2014.00532
70. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel).* (2014) 6:1670–90. doi: 10.3390/cancers6031670
71. Davis RJ, Van Waes C, Allen CT. Overcoming barriers to effective immunotherapy: MDSCs, TAMs, and Tregs as mediators of the immunosuppressive microenvironment in head and neck cancer. *Oral Oncol.* (2016) 58:59–70. doi: 10.1016/j.oraloncology.2016.05.002
72. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* (2004) 10:942–9. doi: 10.1038/nm1093
73. Fialova A, Partlová S, Sojka L, Hromádková H, Brtnický T, Fučíková J, et al. Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells. *Int J Cancer.* (2013) 132:1070–9. doi: 10.1002/ijc.27759
74. Costa NL, Valadares MC, Souza PP, Mendonça EF, Oliveira JC, Silva TA, et al. Tumor-associated macrophages and the profile of inflammatory cytokines in oral squamous cell carcinoma. *Oral Oncol.* (2013) 49:216–23. doi: 10.1016/j.oraloncology.2012.09.012
75. Petruzzi MN, Cherubini K, Salum FG, de Figueiredo MA. Role of tumour-associated macrophages in oral squamous cells carcinoma progression: an update on current knowledge. *Diagn Pathol.* (2017) 12:32. doi: 10.1186/s13000-017-0623-6
76. He KF, Zhang L, Huang CF, Ma SR, Wang YF, Wang WM, et al. CD163+ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma. *Biomed Res Int.* (2014) 2014:838632. doi: 10.1155/2014/838632
77. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol.* (2012) 12:253–68. doi: 10.1038/nri3175
78. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor microenvironment. *Trends Immunol.* (2016) 37:208–20. doi: 10.1016/j.it.2016.01.004

79. Raber P, Ochoa AC, Rodriguez PC. Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives. *Immunol Invest.* (2012) 41:614–34. doi: 10.3109/08820139.2012.680634
80. Umansky V, Blattner C, Gebhardt C, Utikal J. The role of Myeloid-Derived Suppressor Cells (MDSC) in cancer progression. *Vaccines (Basel).* (2016) 4:36. doi: 10.3390/vaccines4040036
81. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res.* (2011) 71:7463–70. doi: 10.1158/0008-5472.CAN-11-2449
82. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J Immunol.* (2010) 185:2273–84. doi: 10.4049/jimmunol.1000901
83. Lang S, Bruderek K, Kaspar C, Höing B, Kanaan O, Dominas N, et al. Clinical relevance and suppressive capacity of human myeloid-derived suppressor cell subsets. *Clin Cancer Res.* (2018) 24:4834–44. doi: 10.1158/1078-0432.CCR-17-3726
84. Chikamatsu K, Sakakura K, Toyoda M, Takahashi K, Yamamoto T, Masuyama K. Immunosuppressive activity of CD14+ HLA-DR- cells in squamous cell carcinoma of the head and neck. *Cancer Sci.* (2012) 103:976–83. doi: 10.1111/j.1349-7006.2012.02248.x
85. Vasquez-Dunddel D, Pan F, Zeng Q, Gorbounov M, Albesiano E, Fu J, et al. STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. *J Clin Invest.* (2013) 123:1580–89. doi: 10.1172/JCI60083
86. Greene S, Robbins Y, Mydlarz WK, Huynh AP, Schmitt NC, Friedman J, et al. Inhibition of MDSC trafficking with SX-682, a CXCR1/2 inhibitor, enhances NK-Cell immunotherapy in head and neck cancer models. *Clin Cancer Res.* (2019) 6:1420–31. doi: 10.1158/1078-0432.CCR-19-2625
87. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer.* (2016) 16:431–46. doi: 10.1038/nrc.2016.52
88. Barros MR, Jr., de Melo CML, Barros M, de Cassia Pereira de Lima R, de Freitas AC, et al. Activities of stromal and immune cells in HPV-related cancers. *J Exp Clin Cancer Res.* (2018) 37:137. doi: 10.1186/s13046-018-0802-7
89. Rachidi S, Wallace K, Wrangle JM, Day TA, Alberg AJ, Li Z. Neutrophil-to-lymphocyte ratio and overall survival in all sites of head and neck squamous cell carcinoma. *Head Neck.* (2016) 38 (Suppl. 1):E1068–74. doi: 10.1002/hed.24159
90. Marchi F, Missale F, Incandela F, Filauro M, Mazzola F, Mora F, et al. Prognostic significance of peripheral T-cell subsets in laryngeal squamous cell carcinoma. *Laryngoscope Investig Otolaryngol.* (2019) 4:513–9. doi: 10.1002/lio2.304
91. Huang SH, Waldron JN, Milosevic M, Shen X, Ringash J, Su J, et al. Prognostic value of pretreatment circulating neutrophils, monocytes, and lymphocytes in oropharyngeal cancer stratified by human papillomavirus status. *Cancer.* (2015) 121:545–55. doi: 10.1002/cncr.29100
92. Mattavelli D, Lombardi D, Missale F, Calza S, Battocchio S, Paderno A, et al. Prognostic nomograms in oral squamous cell carcinoma: the negative impact of low neutrophil to lymphocyte ratio. *Front Oncol.* (2019) 9:339. doi: 10.3389/fonc.2019.00339
93. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science.* (2011) 331:44–9. doi: 10.1126/science.1198687
94. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* (2004) 23:255–9. doi: 10.1038/sj.emboj.7600019
95. Bauernhofer T, Kuss I, Henderson B, Baum AS, Whiteside TL. Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer. *Eur J Immunol.* (2003) 33:119–24. doi: 10.1002/immu.200390014
96. Kloss S, Chambron N, Gardlowski T, Arseniev L, Koch J, Esser R, et al. Increased sMICA and TGFbeta1 levels in HNSCC patients impair NKG2D-dependent functionality of activated NK cells. *Oncoimmunology.* (2015) 4:e1055993. doi: 10.1080/2162402X.2015.1055993
97. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. *Blood.* (2002) 100:174–7. doi: 10.1182/blood.V100.1.174
98. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, et al. A critical function for type I interferons in cancer immunoediting. *Nat Immunol.* (2005) 6:722–9. doi: 10.1038/ni1213
99. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol.* (2001) 31:3388–93. doi: 10.1002/1521-4141(200111)31:11<3388::AID-IMMU3388>3.0.CO;2-Q
100. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell.* (2001) 106:259–62. doi: 10.1016/S0092-8674(01)00456-1
101. Hoffmann TK, Muller-Berghaus J, Ferris RL, Johnson JT, Storkus WJ, Whiteside TL. Alterations in the frequency of dendritic cell subsets in the peripheral circulation of patients with squamous cell carcinomas of the head and neck. *Clin Cancer Res.* (2002) 8:1787–93.
102. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol.* (2011) 29:163–83. doi: 10.1146/annurev-immunol-031210-101345
103. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity.* (2008) 29:352–61. doi: 10.1016/j.immuni.2008.09.002
104. Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, et al. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med.* (2007) 204:105–15. doi: 10.1084/jem.20061660
105. Hartmann E, Wollenberg B, Rothenfusser S, Wagner M, Wellisch D, Mack B, et al. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res.* (2003) 63:6478–87.
106. Bruchhage KL, Heinrichs S, Wollenberg B, Pries R. IL-10 in the microenvironment of HNSCC inhibits the CpG ODN induced IFN-alpha secretion of pDCs. *Oncol Lett.* (2018) 15:3985–90. doi: 10.3892/ol.2018.7772
107. Balermpas P, Rödel F, Rödel C, Krause M, Linge A, Lohaus F, et al. CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: a multicentre study of the German cancer consortium radiation oncology group (DKTK-ROG). *Int J Cancer.* (2016) 138:171–81. doi: 10.1002/ijc.29683
108. Hanna GJ, Liu H, Jones RE, Bacay AF, Lizotte PH, Ivanova EV, et al. Defining an inflamed tumor immunophenotype in recurrent, metastatic squamous cell carcinoma of the head and neck. *Oral Oncol.* (2017) 67:61–9. doi: 10.1016/j.oraloncology.2017.02.005
109. Heusinkveld M, Goedemans R, Briet RJ, Gelderblom H, Nortier JW, Gorter A, et al. Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. *Int J Cancer.* (2012) 131:E74–85. doi: 10.1002/ijc.26497
110. Welters MJP, Ma W, Santegoets SJAM, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral HPV16-specific T cells constitute a type i-oriented tumor microenvironment to improve survival in HPV16-driven oropharyngeal cancer. *Clin Cancer Res.* (2018) 24:634–47. doi: 10.1158/1078-0432.CCR-17-2140
111. Hladíková K, Partlová S, Koucký V, Bouček J, Fonteneau JF, Zábrodský M, et al. Dysfunction of HPV16-specific CD8+ T cells derived from oropharyngeal tumors is related to the expression of Tim-3 but not PD-1. *Oral Oncol.* (2018) 82:75–82. doi: 10.1016/j.oraloncology.2018.05.010
112. Russell S, Angell T, Lechner M, Liebertz D, Correa A, Sinha U, et al. Immune cell infiltration patterns and survival in head and neck squamous cell carcinoma. *Head Neck Oncol.* (2013) 5:24.
113. Zubler RH. Naive and memory B cells in T-cell-dependent and T-independent responses. *Springer Semin Immunopathol.* (2001) 23:405–19. doi: 10.1007/s281-001-8167-7
114. Largeot A, Pagano G, Gonder S, Moussay E, Paggetti J. The B-side of cancer immunity: the underrated tune. *Cells.* (2019) 8:449. doi: 10.3390/cells8050449
115. Nelson BH. CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol.* (2010) 185:4977–82. doi: 10.4049/jimmunol.1001323
116. Nielsen JS, Sahota RA, Milne K, Kost SE, Nesslinger NJ, Watson PH, et al. CD20+ tumor-infiltrating lymphocytes have an atypical

CD27- memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clin Cancer Res.* (2012) 18:3281–92. doi: 10.1158/1078-0432.CCR-12-0234
117. Yuen GJ, Demissie E, Pillai S. B lymphocytes and cancer: a love-hate relationship. *Trends Cancer.* (2016) 2:747–57. doi: 10.1016/j.trecan.2016.10.010
118. Hollern DP, Xu N, Thennavan A, Glodowski C, Garcia-Recio S, Mott KR, et al. B cells and T follicular helper cells mediate response to checkpoint inhibitors in high mutation burden mouse models of breast cancer. *Cell.* (2019) 179:1191–206 e1121. doi: 10.1016/j.cell.2019.10.028
119. Wood O, Woo J, Seumois G, Savelyeva N, McCann KJ, Singh D, et al. Gene expression analysis of TIL rich HPV-driven head and neck tumors reveals a distinct B-cell signature when compared to HPV independent tumors. *Oncotarget.* (2016) 7:56781–97. doi: 10.18632/oncotarget.10788
120. Lechner A, Schlößer HA, Thelen M, Wennhold K, Rothschild SI, Gilles R, et al. Tumor-associated B cells and humoral immune response in head and neck squamous cell carcinoma. *Oncoimmunology.* (2019) 8:1535293. doi: 10.1080/2162402X.2018.1535293
121. Smith EM, Pawlita M, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer. *Int J Cancer.* (2010) 127:111–7. doi: 10.1002/ijc.25015
122. Lang Kuhs KA, Kreimer AR, Trivedi S, Holzinger D, Pawlita M, Pfeiffer RM, et al. Human papillomavirus 16 E6 antibodies are sensitive for human papillomavirus-driven oropharyngeal cancer and are associated with recurrence. *Cancer.* (2017) 123:4382–90. doi: 10.1002/cncr.30966
123. Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. *Clin Cancer Res.* (1999) 5:1369–79.
124. Seiwert TY, Cohen EE. Targeting angiogenesis in head and neck cancer. *Semin Oncol.* (2008) 35:274–85. doi: 10.1053/j.seminoncol.2008.03.005
125. Lapeyre-Prost A, Terme M, Pernot S, Pointet AL, Voron T, Tartour E, et al. Immunomodulatory activity of VEGF in cancer. *Int Rev Cell Mol Biol.* (2017) 330:295–342. doi: 10.1016/bs.ircmb.2016.09.007
126. Lathers DM, Achille NJ, Young MR. Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck. *Hum Immunol.* (2003) 64:1160–6. doi: 10.1016/j.humimm.2003.08.024
127. Nakano Y, Kobayashi W, Sugai S, Kimura H, Yagihashi S. Expression of tumor necrosis factor-alpha and interleukin-6 in oral squamous cell carcinoma. *Jpn J Cancer Res.* (1999) 90:858–66. doi: 10.1111/j.1349-7006.1999.tb00827.x
128. Chandler SW, Rassekh CH, Rodman SM, Ducatman BS. Immunohistochemical localization of interleukin-10 in human oral and pharyngeal carcinomas. *Laryngoscope.* (2002) 112:808–15. doi: 10.1097/00005537-200205000-00008
129. Hamzavi M, Tadbir AA, Rezvani G, Ashraf MJ, Fattahi MJ, Khademi B, et al. Tissue expression, serum and salivary levels of IL-10 in patients with head and neck squamous cell carcinoma. *Asian Pac J Cancer Prev.* (2013) 14:1681–5. doi: 10.7314/APJCP.2013.14.3.1681
130. Duffy SA, Taylor JM, Terrell JE, Islam M, Li Y, Fowler KE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. *Cancer.* (2008) 113:750–7. doi: 10.1002/cncr.23615
131. Hirano T. Interleukin 6 and its receptor: ten years later. *Int Rev Immunol.* (1998) 16:249–84. doi: 10.3109/08830189809042997
132. Park SJ, Nakagawa T, Kitamura H, Atsumi T, Kamon H, Sawa S, et al. IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J Immunol.* (2004) 173:3844–854. doi: 10.4049/jimmunol.173.6.3844
133. Suzuki S, Mita S, Kamohara H, Sakamoto K, Ishiko T, Ogawa M. IL-6 and IFN-gamma regulation of IL-10 production by human colon carcinoma cells. *Int J Oncol.* (2001) 18:581–6. doi: 10.3892/ijo.18.3.581
134. Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. *J Leukoc Biol.* (2005) 78:1043–51. doi: 10.1189/jlb.0705358
135. Piva MR, DE Souza LB, Martins-Filho PR, Nonaka CF, DE Santana Santos T, DE Souza Andrade ES, et al. Role of inflammation in oral carcinogenesis (Part II): CD8, FOXP3, TNF-alpha, TGF-beta and NF-kappaB expression. *Oncol Lett.* (2013) 5:1909–14. doi: 10.3892/ol.2013.1302
136. Parks RR, Yan SD, Huang CC. Tumor necrosis factor-alpha production in human head and neck squamous cell carcinoma. *Laryngoscope.* (1994) 104:860–4. doi: 10.1288/00005537-199407000-00015
137. De Guillebon E, Dardenne A, Saldmann A, Séguier S, Tran T, Paolini L, et al. Beyond the concept of cold and hot tumors for the development of novel predictive biomarkers and the rational design of immunotherapy combination. *Int J Cancer.* (2020) 147:1509–18. doi: 10.1002/ijc.32889
138. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today.* (1998) 19:568–74. doi: 10.1016/S0167-5699(98)01346-2
139. Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, et al. CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *Int J Cancer.* (2008) 122:2286–93. doi: 10.1002/ijc.23392
140. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, et al. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol.* (2010) 11:313–20. doi: 10.1038/ni.1848
141. Lin Y, Sharma S, John MS. CCL21 Cancer Immunotherapy. *Cancers (Basel).* (2014) 6:1098–110. doi: 10.3390/cancers6021098
142. Mehanna H, Robinson M, Hartley A, Kong A, Foran B, Fulton-Lieuw T, et al. Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. *Lancet.* (2019) 393:51–60. doi: 10.1016/S0140-6736(18)32752-1
143. Gillison ML, Trotti AM, Harris J, Eisbruch A, Harari PM, Adelstein DJ, et al. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. *Lancet.* (2019) 393:40–50. doi: 10.1016/S0140-6736(18)32779-X
144. Cohen EEW, Bell RB, Bifulco CB, Burtness B, Gillison ML, Harrington KJ, et al. The Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of squamous cell carcinoma of the head and neck (HNSCC). *J Immunother Cancer.* (2019) 7:184. doi: 10.1186/s40425-019-0662-5
145. Shayan G, Srivastava R, Li J, Schmitt N, Kane LP, Ferris RL. Adaptive resistance to anti-PD1 therapy by Tim-3 upregulation is mediated by the PI3K-Akt pathway in head and neck cancer. *Oncoimmunology.* (2017) 6:e1261779. doi: 10.1080/2162402X.2016.1261779
146. Verma V, Shrimali RK, Ahmad S, Dai W, Wang H, Lu S, et al. PD-1 blockade in subprimed CD8 cells induces dysfunctional PD-1(+)CD38(hi) cells and anti-PD-1 resistance. *Nat Immunol.* (2019) 20:1555. doi: 10.1038/s41590-019-0519-6
147. Massarelli E, William W, Johnson F, Kies M, Ferrarotto R, Guo M, et al. Combining immune checkpoint blockade and tumor-specific vaccine for patients with incurable human papillomavirus 16-related cancer: a phase 2 clinical trial. *JAMA Oncol.* (2019) 5:67–73. doi: 10.1001/jamaoncol.2018.4051
148. Petitprez F, de Reyniès A, Keung EZ, Chen TW, Sun CM, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. *Nature.* (2020) 577:556–60. doi: 10.1038/s41586-019-1906-8
149. Vonderheide RH. CD40 agonist antibodies in cancer immunotherapy. *Annu Rev Med.* (2020) 71:47–58. doi: 10.1146/annurev-med-062518-045435

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PERSPECTIVE
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Role of Tonsillectomy in the Management of Carcinomas of Unknown Primary of the Head and Neck: A Retrospective Study Based on p16 Analysis

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Purpose: To evaluate the impact of tonsillectomy on the detection of the primary tumor, based on p16 immunohistochemistry analysis, in patients with cervical unknown primary of squamous cell carcinoma (SCC-CUP).

Methods: This was a retrospective study of 63 patients, included from January 2008 to December 2017 in a single institution. All patients had an initial assessment with physical examination, CT scan of the neck and chest, whole body FDG-PET CT, and endoscopy under general anesthesia, which failed to determine the primary tumor.

Results: Forty-seven out of the 63 patients had an ipsi- or bilateral tonsillectomy which revealed 12 tonsil cancers (26%). The tonsil primary was ipsilateral to positive nodes in 10 cases, contralateral in 1 case and, in 1 case, the patient had bilateral neck involvement. The analysis of the p16 status was carried out in 41/63 patients (65%). Among the 32 patients who had a p16 analysis and tonsillectomy, the rate of primary detection was 59% (10/17) for p16-postives and 0% (0/15) for p16-negatives ($p < 0.001$).

Conclusion: These results suggest that an extended work-up should be systematically proposed including bilateral tonsillectomy (+/- mucosectomy of the base of tongue) in SCC-CUP p16-positive patients but not in p16-negatives.

Keywords: unknown primary, oropharyngeal cancer, human papillomavirus, head and neck cancer, squamous cell carcinoma

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PURPOSE

Carcinomas of unknown primary (CUP) of the head and neck are lymph node metastases with no primary tumor identified after a work-up including a physical examination and imaging tests (CT scan of the neck and chest, whole body FDG-PET CT). When no primary cancer is found, endoscopy under general anesthesia is performed, possibly associated with ipsi- or bilateral tonsillectomy.

Squamous cell carcinoma (SCC) is the most common histology of head and neck CUP. The incidence of head and neck CUP of squamous cell carcinoma (SCC-CUP) is rare, accounting for 1–4% of all head and neck cancers, and mortality is high, with mean 5-year survival rates that vary widely depending on the study, ranging from 24 to 79% of cases (1, 2). The detection of a primary tumor is an important goal to help improve both overall and disease-free survival. It is likely that this is related to the potential adaptation of treatment to the primary tumor by proposing a targeted treatment with curative surgery and a decrease in morbidity of an "over-treatment" (saving on adjuvant radiotherapy or modified radiation fields) (3).

It would appear that HPV or EBV status could point to a primary oropharyngeal or nasopharyngeal tumor. SCC-CUP represents a diagnostic challenge and, to date, there is no consensus on whether tonsillectomy should be performed as a single or bilateral procedure and on whether base of tongue mucosectomy should be conducted. Moreover, few studies have evaluated the impact of HPV status on the rate of discovery of the primary tumor (3, 4).

The aim of this study was to evaluate the impact of tonsillectomy on identification of the primary tumor, based on p16 immunohistochemistry analysis, in patients with SCC-CUP.

METHODS

Ethical Considerations

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study. Authorization to conduct this study was obtained from the Ethical Committee of our institution (Assistance Publique des Hôpitaux de Marseille, no. 2018-28).

Study Design

This was a retrospective, monocentric study analyzing the records of 63 patients managed from January 2008 to December 2017 with SCC-CUP (histologically confirmed by lymph node sampling) with no identified primary tumor after clinical examination, standardized imaging (CT scan of the neck and chest and whole body FDG-PET CT) and endoscopy under general anesthesia. Tonsillectomies were performed either during the initial endoscopy when no suspicious area was discovered or, in case of directed biopsy of any suspicious areas, during a second procedure when biopsies were finally negatives. Patients for whom a primary was visible during the endoscopy were not included in the study. Concerning the lymph node sampling, 33 patients (52%) had an adenectomy or lymph node open biopsy performed at another center and were then referred to our institution. Thirty-one patients (48%) had an initial Fine-needle aspiration cytology with, in all cases, confirmation of diagnosis (frozen section + histological analysis) after lymph node dissection. Patients with a history of head and neck cancer or radiotherapy of the neck were not included. Mean age of the patients was 63 years (range, 38 to 84). There were 51 males and 12 females. The rate of discovering a primary tonsillar tumor was noted as well as patient characteristics (sex, age, alcohol and tobacco consumption, lymph node location, TNM stage, histological criteria of aggressiveness, and HPV and EBV status).

Immunohistochemical Analysis

The presence of HPV in tumor cells was based on overexpression of the p16 protein using immunohistochemistry. The secondary antibody was clone E6H4 reference 6695248001, from the Roche laboratory, using Ventana BenchMark ULTRA automaton. The presence of EBV was tested using *in situ* hybridization with the EBER VENTANA-ROCHE probe (Epstein Barr virus Early RNA Probe; REF: 800.2842; GTIN: 04015630971923) using Ventana BenchMark ULTRA automaton.

Testing for the presence of EBV and HPV was performed in 41/63 patients (65%). These analyses had been performed at the time of initial assessment for 6 patients and were performed retrospectively for 35 patients. For 22 patients, the EBV or p16 status could not be determined as histological samples were not available.

Statistical Analysis

Categorical data were compared using Fisher's exact tests. Non-parametric Mann-Whitney tests were used to compare ordinal data. P values of less than 0.05 were taken to be statistically significant. All statistical analyses were two-sided and performed using IBM SPSS Statistics 20.0 (IBM Inc., New York, USA).

RESULTS

In the whole series, 12 primary tonsillar tumors were found (19%). Tonsillectomy was performed in 47 patients (bilateral in 36 and unilateral in 11) representing 75% of the patients in the series. Deep biopsies, without tonsillectomy, were performed in 10 patients (16%). Six other patients (9%) who had a history of bilateral tonsillectomy in childhood with no residual tonsil visible on endoscopy, had biopsies of the tonsil fossa. Three of the 47 patients with tonsillectomy (6%) had a postoperative hemorrhagic complication requiring hemostasis under general anesthesia: in two cases, the bleeding occurred in the tonsillar fossa ipsilateral to the lymphadenopathy. Among these two cases, we observed a primary carcinoma in one case. In one case, there was contralateral bleeding with no tumor.

For the 47 patients who had tonsillectomy, the procedure revealed 12 tonsil cancers (26%). The tonsil primary was ipsilateral to positive nodes in 10 cases (84%) and contralateral in 1 case (8%), and in 1 case (8%), the patient had bilateral neck involvement.

All the primary tumors were found on the tonsillectomy specimens. No primary tumors were found after deep biopsies without tonsillectomy. The median size of the primary tumors found after tonsillectomy was 6 mm (range, 2 to 18 mm). Among the 36 patients who had bilateral tonsillectomy, one primary contralateral to lymphadenopathy was found (3%).

For the 47 patients who had tonsillectomy, the procedure revealed 12 tonsil cancers (26%). The tonsil primary was ipsilateral to positive nodes in 10 cases (84%) and contralateral in 1 case (8%), and in 1 case (8%), the patient had bilateral neck involvement.

No statistically significant differences were found for age, sex, alcohol consumption, M stage, location of lymphadenopathies, extracapsular spread, and tumor differentiation (**Table 1**).

Of the 41 patients for which immunohistochemical analysis was performed, none was positive for EBV. Eighteen out of 41 patients (44%) were p16-positive, among which a primary tonsil

TABLE 1 | Comparison of patients with or without primary finding.

	Tonsil primary		No primary		Overall population	p (Fisher test)
	N	%	N	%	N (%)	
Population	12	19%	51	81%	63	
Sex						
Male	10	83%	41	80%	51 (81%)	0.99
Female	2	17%	10	20%	12 (19%)	
Tobacco consumption						
Yes	7	58%	44	86%	51 (81%)	**0.03**
No	5	42%	6	12%	11 (17%)	
Not available	0		1	2%	1 (2%)	
Alcohol consumption						
Yes	6	50%	27	53%	33 (52%)	0.75
No	6	50%	21	41%	27 (43%)	
Not available	0		3	6%	3 (5%)	
Stage N						
N1	7	58%	16	31%	23 (36%)	**0.04***
N2	4	33%	16	31%	20 (32%)	
N3	1	8%	19	37%	20 (32%)	
Stage M						
M0	12	100%	50	98%	62 (98%)	0.99
M1	0		1	2%	1 (2%)	
Tonsillectomy						
Unilateral	5	42%	6	12%	11 (17.5%)	0.17
Bilateral	7	58%	29	57%	36 (57%)	
No tonsillectomy	0	0%	16	31%	16 (25.5%)	
Lymph node levels involved						
I	1	8%	8	16%	9 (14%)	0.99
II	11	92%	42	82%	53 (84%)	0.67
III	3	25%	21	41%	24 (38%)	0.35
IV	1	8%	10	20%	11 (17%)	0.45
V	2	17%	8	16%	10 (16%)	0.99
Bilateral lymph node involvement						
Yes	1	8%	4	8%	5 (8%)	0.99
No	11	92%	47	92%	58 (92%)	
Extracapsular spread						
Yes	2	17%	19	37%	21 (34%)	0.30
No	9	75%	29	57%	38 (60%)	
Not available	1	8%	3	6%	4 (6%)	
p16 status						
Positive	10	83%	8	16%	18 (29%)	**<0.001**
Negative	0		23	45%	23 (36%)	
Not available	2	17%	20	39%	22 (35%)	
Tumor differentiation						
Well differentiated	6	50%	22	43%	28 (44%)	0.58*
Moderate differentiation	2	17%	7	14%	9 (14%)	
Poor/undifferentiated	4	33%	19	37%	23 (37%)	
Not available	0		3	6%	3 (5%)	

**Mann-Whitney test for ordinal data.*
Bold characters denote statistical significance.

tumor was found in 10 cases (56%). Twenty-three out of 41 patients (56%) were p16-negative, among which no primary tonsil tumor was found. Lastly, a primary tonsillar tumor was found in two patients whose p16 status was unknown.

Of the 32 patients who had a p16 analysis (all EBV-negative) and an ipsi- or bilateral tonsillectomy, 17 were p16-positive and 15 were p16-negative. Ten primary tonsil tumors were found, all in p16-positive patients. The primary detection rate in p16-positive patients with tonsillectomy was therefore 10/17 (59%) versus 0% for p16-negative patients ($p < 0.001$).

DISCUSSION

Benefits of Tonsillectomy

In our study, a tonsil primary tumor was found in 19% of cases (12 patients out of 63). However, this percentage is probably underestimated because 16 patients had only tonsil deep biopsies and no tonsillectomy.

In a cohort of 126 patients with SCC-CUP, Waltonen et al. reported a positive yield in 30% of patients who underwent tonsillectomy. In comparison, in the same study, deep biopsies identified the malignancy in only 3% of cases, reflecting the fact that some tumors are small and located within the tonsillar crypts and therefore cannot be identified by biopsy alone (5).

In our study, among the patients undergoing tonsillectomy, the latter was bilateral in 77% of cases and unilateral in 23% of cases. This distribution is similar to that found in 2016 by Farnebo et al. studying the management of patients with SCC-CUP in 22 main centers in five Nordic countries (Iceland, Norway, Sweden, Finland, and Denmark). Routine bilateral tonsillectomy was performed in about 80% of cases compared to 20% for unilateral tonsillectomy (6).

In our series, out of the 47 patients undergoing tonsillectomy, a primary tumor was found in 26% of cases, with a primary contralateral to the lymphadenopathy in 3% of the cases who underwent bilateral tonsillectomy. Our results are consistent with the literature reporting that ipsilateral tonsillectomy has a detection rate of 18 to 45%. However, they are lower for contralateral tonsillectomy with a likely detection rate ranging from 10 to 25% (7).

Di Maio et al. performed a systematic review and meta-analysis to evaluate the effectiveness of palatine tonsillectomy in patients with SCC-CUP. They analyzed 14 studies comprising 673 patients who underwent 416 palatine tonsillectomies, 338 performed during examination under anesthesia, and 78 managed with transoral robotic surgery (TORS). A total of 140 occult tonsillar malignancies were identified. Of these, 124 (89%) were ipsilateral, 2 were (1%) contralateral, and 14 were (10%) synchronous bilateral. A meta-analysis of 11 out of the 14 studies showed an overall detection by tonsillectomy rate of 0.34 (99% confidence interval, 0.23–0.46). The authors concluded that palatine tonsillectomy is a valuable diagnostic tool and that bilateral tonsillectomy should be considered mainly not only because of the non-negligible number of bilateral/contralateral occult tonsillar tumors reported in the literature but also because out of 204 bilateral tonsillectomies performed (from a total of 416), 2 bleeding episodes were reported in only one of the included articles (8). In our series, bleeding occurred in one (3%) contralateral tonsil fossa among the 36 patients undergoing bilateral tonsillectomy.

The American Society of Clinical Oncology has recently published evidence-based recommendations on the diagnosis and management of squamous cell carcinoma of unknown primary in the head and neck. They recommend that patients should undergo a complete operative upper aerodigestive tract evaluation of mucosal sites at risk (oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx), including directed biopsy of any suspicious areas. Random biopsies of nonsuspicious areas have a low yield and should not be performed. For patients with unilateral lymphadenopathy, if a primary site is not confirmed on initial evaluation, then the surgeon should perform ipsilateral palatine tonsillectomy. If palatine tonsillectomy fails to identify a primary, then ipsilateral lingual tonsillectomy may be performed. Bilateral palatine tonsillectomy may be considered according to clinical suspicion, at the discretion of the surgeon. For patients with bilateral lymphadenopathy, if a primary site is not confirmed on endoscopic examination, then the surgeon may perform unilateral lingual tonsillectomy on the side with the greater nodal burden and may perform contralateral lingual tonsillectomy if the ipsilateral procedure fails to identify a primary. Bilateral palatine tonsillectomy after bilateral lingual tonsillectomy should be avoided (9).

However, there is no international consensus on whether tonsillectomy should be a single or bilateral procedure and whether it should be combined with an ipsi- or bilateral base of tongue mucosectomy depending on p16 status (3, 10).

Impact of p16 Immunohistochemistry Analysis

It is widely accepted that the base of the tongue and the tonsils are the most common primary tumor sites found in the work-up for SCC-CUP (1, 3, 4).

The question is whether p16 status influences the detection rate of the primary tumor in the oropharynx. In our study, 10 of the 12 carcinomas found in the tonsils were p16-positive (83%), while for the other 2 patients, the p16 status was unknown. Also, among patients in whom a tonsillar primary was found, there were statistically fewer smokers than among those without a detected primary. In addition, the former had a lower lymph node stage. Most importantly, no tonsillar primary was found in p16-negative patients. Finally, among the patients in our series who underwent tonsillectomy, the detection rate of a tonsillar primary in p16-positive patients was 53% compared to 0% in p16-negative patients.

The role of p16 status on the rate of primary tumor detection in the oropharynx has been very little studied in the literature.

In the systematic review by Di Maio et al. the p16 status was available for 116 out of 673 patients. Of these, 104 (90%) were p16-positive and 12 (10%) p16-negative, but no information is given about the rate of primary findings in these patients (8).

Ryan et al. analyzed 80 p16-positive patients with SCC-CUP. After direct laryngoscopy with biopsies, 29/80 (35%) primary tumors were identified. Thirty-four patients with negative biopsies underwent palatine tonsillectomy. Fifteen of these 34 (44%) revealed the primary tumor, yielding a cumulative identification of 44/80 (55%) (11).

In our study, the presence of HPV in tumor cells was based on overexpression of the p16 protein using immunohistochemistry. According to the guideline the College of American Pathologists, the preferred method for initial high risk-HPV testing of tissue specimens (core biopsy or excisions) in high-prevalence settings is p16 immunohistochemistry, which is a sensitive surrogate marker (12).

Decision-Making Based on p16 Status

Our results showed that oropharyngeal primaries were found exclusively in p16-positive patients and never in p16-negative patients. The detection rate of a tonsil primary in p16-positive patients undergoing tonsillectomy was 53% in our series. This result is probably underestimated since not all our patients had bilateral tonsillectomy and none of them underwent base of tongue mucosectomy.

These findings highlight the need to intensify the search for the primary at oropharynx level in p16-positive patients. It is necessary, therefore, to ascertain the p16 status as early as possible by means of a cervical lymph node sample. In this way, p16-positive patients, in which there is the greatest likelihood of finding a primary tumor, could benefit from maximum sampling of the oropharynx. These patients could thus benefit from bilateral tonsillectomy possibly associated, at the same time or at a second stage, with a base of tongue mucosectomy to optimize the search for the primary tumor, as suggested by several authors (4, 13). Mehta et al. evaluated 10 patients with unknown primary tumors of the head and neck. All patients underwent a cervical biopsy, positron-emission tomography/computed tomography, formal endoscopy, and bilateral tonsillectomy. When the initial endoscopy and biopsies failed to locate a primary tumor, all patients underwent transoral robotic base of tongue resection. A primary was found in 9/10 (90%) patients, of which 8 out of the 9 (89%) were HPV-positive (13).

On the other hand, tonsillectomy and/or mucosectomy of the base of the tongue for p16-negative patients is much more debatable since, in our series, no primary was found in p16-negative patients who underwent tonsillectomy. This observation was already made by Kubic et al. (14), who analyzed the rate of primary detection in 23 p16-negative patients using TORS base of tongue mucosectomy. The primary tumor was identified in only 3 out of 23 cases (13%). In these three cases, the tumor was found in the ipsilateral base-of-tongue specimen in contrast with their previous series showing a tumor identification rate of 80% in the HPV-positive patients (4, 14).

CONCLUSION

Early determination of p16 status from a lymph node sample is important as it allows preferential referral to a primary oropharyngeal tumor and boosts the search for the primary tumor (bilateral tonsillectomy +/- base of tongue mucosectomy) in p16-positive patients. On the other hand, tonsillectomy and base of tongue mucosectomy for p16-negative patients are much more debatable, since, in our series, no primary was found in these patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by: Authorization to conduct this study was obtained from the Ethical Committee of our institution (Assistance Publique des Hôpitaux de Marseille, n° 2018-28). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concepts: NF and LS. Study design: NF and SW. Data acquisition: PP, JD, and LS. Quality control of data and algorithms: JMa. Data analysis and interpretation: PP and SS. Statistical analysis: JMa. Manuscript preparation: NF, PP, and TR. Manuscript editing: AG, PD, and JMi. Manuscript review: PD and NF. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Arosio AD, Pignataro L, Gaini RM, Garavello W. Neck lymph node metastases from unknown primary. *Cancer Treat Rev* (2017) 53:1–9. doi: 10.1016/j.ctrv.2016.11.014
2. Al Kadah B, Papaspyrou G, Linxweiler M, Schick B, Rübe C, Büchler BS, et al. Cancer of unknown primary (CUP) of the head and neck: retrospective analysis of 81 patients. *Eur Arch Otorhinolaryngol* (2017) 274:2557–66. doi: 10.1007/s00405-017-4525-8
3. Eskander A, Ghanem T, Agrawal A. Education Committee of American Head and Neck Society (AHNS). AHNS Series: Do you know your guidelines? Guideline recommendations for head and neck cancer of unknown primary site. *Head Neck* (2018) 40:614–21. doi: 10.1002/hed.25026
4. Geltzeiler M, Doerfler S, Turner M, Albergotti WG, Kubik M, Kim S, et al. Transoral robotic surgery for management of cervical unknown primary squamous cell carcinoma: updates on efficacy, surgical technique and margin status. *Oral Oncol* (2017) 66:9–13. doi: 10.1016/j.oraloncology.2016.12.033

5. Waltonen JD, Ozer E, Schuller DE, Agrawal A. Tonsillectomy vs. deep tonsil biopsies in detecting occult tonsil tumors. *Laryngoscope* (2009) 119:102–6. doi: 10.1002/lary.20017
6. Farnebo L, Laurell G, Mäkitie A. A Nordic survey on the management of head and neck CUP. *Acta Otolaryngol (Stockh)* (2016) 136:1159–63. doi: 10.1080/00016489.2016.1193894
7. Koch WM, Bhatti N, Williams MF, Eisele DW. Oncologic rationale for bilateral tonsillectomy in head and neck squamous cell carcinoma of unknown primary source. *Otolaryngol Head Neck Surg* (2001) 124:331–3. doi: 10.1067/mhn.2001.114309
8. Di Maio P, Iocca O, De Virgilio A, Ferreli F, Cristalli G, Pellini R, et al. Role of palatine tonsillectomy in the diagnostic workup of head and neck squamous cell carcinoma of unknown primary origin: A systematic review and meta-analysis. *Head Neck* (2019) 41:1112–21. doi: 10.1002/hed.25522
9. Maghami E, Ismaila N, Alvarez A, Chernock R, Duvvuri U, Geiger J, et al. Diagnosis and Management of Squamous Cell Carcinoma of Unknown Primary in the Head and Neck: ASCO Guideline. *J Clin Oncol* (2020) 38:2570–96. doi: 10.1200/JCO.20.00275
10. Martin JM, Galloway TJ. Evaluation and Management of Head and Neck Squamous Cell Carcinoma of Unknown Primary. *Surg Oncol Clin* (2015) 24:579–91. doi: 10.1016/j.soc.2015.03.007
11. Ryan JF, Motz KM, Rooper LM, Mydlarz WK, Quon H, Gourin CG, et al. The impact of a stepwise approach to primary tumor detection in squamous cell carcinoma of the neck with unknown primary. *Laryngoscope* (2019) 129:1610–16. doi: 10.1002/lary.27625
12. Fakhry C, Lacchetti C, Rooper LM, Jordan RC, Rischin D, Sturgis EM, et al. Human papillomavirus testing in head and neck carcinomas: ASCO clinical practice guideline endorsement of the College of American Pathologists guideline. *J Clin Oncol* (2018) 36:3152–61. doi: 10.1200/JCO.18.00684
13. Mehta V, Johnson P, Tassler A, Kim S, Ferris RL, Nance M, et al. A new paradigm for the diagnosis and management of unknown primary tumors of the head and neck: a role for transoral robotic surgery. *Laryngoscope* (2013) 123:146–51. doi: 10.1002/lary.23562
14. Kubik MW, Channir HI, Rubek N, Kim S, Ferris RL, von Buchwald C, et al. TORS base-of-tongue mucosectomy in human papilloma virus-negative carcinoma of unknown primary. *Laryngoscope* (2020). doi: 10.1002/lary.28617

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Pathology of HPV-Associated Head and Neck Carcinomas: Recent Data and Perspectives for the Development of Specific Tumor Markers

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A significant subset of carcinomas developed in the head and neck (H&NCs) are associated with specific human papillomaviruses (HPV) genotypes. In particular, 40–60% of oropharyngeal carcinoma cases are linked to HPV. Epidemiological studies have demonstrated that HPV oral infections are predominantly sexually transmitted and are more frequent among men (10–18%) than women (3.6–8.8%). Although there is a large diversity of HPV genotypes associated with H&NCs, HPV16 lineage represents 83% of the reported cases. The prognostic value of HPV as a biological parameter is well recognized. However, the use of HPV DNA as a diagnostic and/or predictive marker is not fully developed. Recent data reporting the physical state of the HPV genome in tumors have shown that HPV DNA integration into the tumor cell genome could lead to the alteration of cellular genes implicated in oncogenesis. Most importantly, HPV DNA corresponds to a tumor marker that can be detected in the blood of patients. Profile of the HPV DNA molecular patterns in tumor cells using New Genome Sequencing-based technologies, allows the identification of highly specific tumor markers valuable for the development of innovative diagnostic and therapeutic approaches. This review will summarize recent epidemiological data concerning HPV-associated H&NCs, the genomic characterization of these tumors, including the presence of HPV DNA in tumor cells, and will propose perspectives for developing improved care of patients with HPV-associated H&NCs, based on the use of viral sequences as personalized tumor markers and, over the longer term, as a therapeutic target.

Keywords: HPV—human papillomavirus, head and neck carcinoma, ctDNA, tumor markers, tumor microenvironment, viral integration, viral oncogenesis

INTRODUCTION

The first aim of this review is to summarize recent data concerning the pathology of head and neck carcinomas (H&NCs) associated with the human papillomaviruses (HPV), including prevalence and viral epidemiology which characterize these tumors as well as the specificities of HPV DNA as a tumor marker. In the second aim, we will explore specific perspectives focused on the use of HPV DNA as a prognostic tumor marker in the blood of patients with H&NCs and on the developments of new applications in clinical oncology related to the introduction of New Genome Sequencing (NGS) approaches as tools for the optimized characterization of viral DNA in the tumor cells of HPV-positive H&NCs.

EPIDEMIOLOGY

Epidemiology of HPV-Associated H&NCs

The reported worldwide prevalence of HPV-associated H&NCs varies between 25.9 (1) and 30% (2). The frequency of HPV association is different according to tumor localizations. The highest rate (35%) is observed for tumors located in the oropharynx (1, 3), particularly when developed in lympho-epithelial sites such as palatine tonsil (56 to 62%) (4, 5) and base of the tongue (40%) (4). Lower rates are observed in tumors developed in the oral cavity, (5.8 to 23.5%) (1, 3), in the larynx (3.3 to 24.0%) (1, 3), or in the soft palate (3.1%) (6).There is a striking geographic heterogeneity of HPV prevalence in oropharyngeal tumors: rates are higher in the United States (59.3%) and in Europe (31.1%) than in Brazil (4.1%) (3, 7). In the United States, an increase in the prevalence of H&NCs has been observed between 1984–1989 and 2000–2004, rising from 16.3 to 71.7% (8). A significant increase of HPV-related tumors developed in women has also been observed in France (9).

Viral Epidemiology of Asymptomatic Oral Infection and of H&NCs

In the general population, the prevalence of asymptomatic HPV infection was assessed by viral analyses of oral rinse specimens. The reported infection statistics ranged from 6.9% in the United States (10) to 13.1% in France (11). The prevalence is two- to three-fold higher in men (from 10.1 to 18%) (10–12) than in women (3.6 to 8.8%) (10, 11). Within non-tumor tonsils, HPV DNA was found in 3.6 to 4.9% from these specimens (11, 13). Paired analyses of tonsil brushing and oral rinse found a low agreement between the results (11) indicating that oral rinse, not able to reach the bottom of all of the tonsillar crypts, was a poor surrogate of HPV prevalence within tonsils. In a Japanese study concerning male patients, HPV DNA detected from mouth rinses was also found in the urine with a good agreement between the genotypes detected in these two specimens from the same patient (12).

The mode of contamination has been further analyzed from a large cohort study conducted with 5,500 subjects in the United States (10). This study showed that oral HPV infection was predominantly sexually transmitted: oral HPV prevalence was more than 8-fold higher among individuals who reported sexual relations *vs* no sexual relations (7.5 *vs* 0.9%). The prevalence increased with the number of partners but no evidence for increased risk related to particular sexual behavior was observed. However, one explanation for the observed higher prevalence among men could be attributed to the higher probability of HPV transmission through oral sex with women *vs* men. Indeed, oral HPV prevalence increased more sharply with the number of sexual partners for men than women (10). Low-risk HPV genotypes were two-fold more frequently detected than high-risk. Multivariate analyses showed that HPV prevalence was also related to cigarette smoking and to age, with a bimodal pattern characterized by peak prevalences among individuals aged between 30 to 34 and 60 to 64 years (10).

Patients with oropharyngeal cancer showed risk factors including a history of numerous sexual partners and oral sex (14–16). A study analyzing the risk of cancer among homosexual individuals showed an increased prevalence of oropharyngeal cancer in women but not in men (17). No significant increase in HPV infection was observed in partners of H&NCs patients (18).

When looking at H&NCs as well as cervical cancers, there is a large heterogeneity of the high-risk HPV genotypes encountered, but the prevalence of HPV16 in H&NCs (83.0%) (19) is significantly higher than in the cervix (55.5%) (20). In H&NCs, the other high-risk genotypes are found at lower rates: 3.3% for HPV33, 2.6% for HPV26, 2.2% for HPV35, 1.8% for HPV18, and below 1% for the other genotypes. In laryngeal tumors, the distribution of the respective genotypes is 50.8% for HPV16, 8.5% for HPV45, 6.6% for HPV6, 5.1% for HPV18, 3.4% each for HPV31 and HPV33, 1.7% for HPV35, and less than 1% for the other genotypes (19).

The improvement in sequencing technologies has revealed a high diversity of HPV16 DNA sequences. Four major variant lineages and up to 16 sublineages were identified (21), associated with different risks of persistence and of progression to invasive carcinoma (22) and with distinct histological types (23). Recently, among a series of 5,570 HPV16-infected case-control cervical samples, Mirabello et al. identified thousands of unique HPV16 viral isolates (24). In contrast to this variability, the HPV16 E7 gene showed extremely low variability in cervical cancers around the world, indicating that genetic conservation of this viral oncogene is critical for carcinogenesis. The role of HPV genome variants in the development of H&NCs merits updated and precise documentation in the HPV community (11).

GENETICS OF HPV POSITIVE AND HPV NEGATIVE H&NCS

Independently of their HPV status, H&NCs are characterized by recurrent mutations in the *TP53, CDKN2A, PI3KCA, HRAS, NOTCH1*, and *FBXW7* genes (25, 26) and in at least 30% of the cases, harbor mutations in genes regulating squamous cell differentiation, such as *NOTCH1, IRF6*, and *TP63* (27). Recurrent overexpression of sequences corresponding to relevant therapeutic targets, such as *PGF, PDL1, CDK6, MET*, and *EGFR*, were also observed (28).

Several studies aimed at determining the genetic alterations that distinguish HPV-positive from HPV-negative H&NCs (26, 27, 29, 30). In HPV-positive tumors, the mutation rate was two-fold lower (2.28 mutations per Mb) than in HPV-negative tumors (4.83 per Mb) (27). HPV-positive tumors harbor recurrent mutations of *PTEN*, *TRAF3* (TNF receptor associated factor 3), and *PIK3CA* and focal amplification of *E2F1* whereas HPV-negative cases are characterized by a high rate of *TP53* mutations and abrogation of the G1/S checkpoint *via CDKN2A/B* deletion and/or *CCND1* amplification. In line with an enhancement of cell proliferation induced by the E7 HPV protein (31), genome-wide expression profile of HPV-positive H&NCs revealed an up-regulation of a distinct and large subset of cell cycle genes as compared with HPV-negatives cases (32). In summary, these analyses show that HPV-positive H&NCs are mainly characterized by a low mutational load, a high proliferative index, integrity of p53, and a frequent alteration of the *PTEN/PIK3CA* pathway.

The HPV genome is a 7.8-kbp double-stranded DNA circular molecule and its presence in the nucleus of tumor cells represents *per se* a genetic alteration. In cervical neoplasias, the analyses of the interactions between viral and cell genomes have shown that the physical state of HPV genomes in tumor cells is different according to the types of lesions. In benign or in intraepithelial lesions, viral genomes are present as free episomal molecules in the nucleus of infected cells whereas, in most invasive cancers, part of the viral DNA is integrated into the cell genome (33). HPV DNA integration is clonal, stable over time and, homogeneously distributed throughout different tumor regions, does not depend upon intra-tumor heterogeneity (34). Host chromosomal structural alterations at the HPV integration locus are frequently observed (35) and the nature of these changes is related to the recombination mechanisms of viral insertions (36). These chromosomal alterations, as well as the introduction of illegitimate viral DNA enhancer sequences into the cell genome, have consequences on the expression of cellular genes located near integrated viral sequences (37). Tumor cells may also contain non-integrated episomal molecules in various quantities, ranging from a few to several thousand per nucleus (38).

For H&NCs, few analyses have been performed concerning the physical state of HPV genomes, but the reported pattern is similar to that observed in genital carcinomas. Viral integration was detected in 60.7% (51/84) (39) to 71.4% (25/35) (40) of the cases, although a low prevalence of 15.4% (2/13) was observed in another series (41). As in genital tumors, integration was frequently found within or in close vicinity (<20 Kb) to cellular genes and tumors without viral integrants displayed distinct gene expression profile (40). Reported target genes for HPV insertion were *RAD51B*, *ETS2*, *NR4A2*, *KLF5*, *KLF12*, *p63*, *CD274*, *FLJ3745*, and *TTC6* (39, 40).

HPV AS A TUMOR MARKER IN H&NCS

Prognostic and Predictive Value of HPV DNA in H&NCs

Many strategies for HPV characterization in H&NCs have been used (42, 43) including Polymerase Chain Reaction (PCR) or Quantitative Reverse Transcription-PCR (qRT-PCR) for the direct detection of HPV DNA or RNA, and immunohistochemistry (IHC) for the detection of P16INK4A (p16) cell protein. Viral E7 oncoprotein expressed from high-risk HPVs inactivates cellular Rb protein leading to the up-regulation of various cell cycle associated protein, including p16, which is commonly used as an indirect surrogate marker of HPV infection. Consistent discrepancies in the respective positivity of p16 and HPV DNA/RNA have been reported (44). Due to the high sensitivity and specificity of PCR and qRT-PCR, these methods are widely accepted for the clinically significant detection of HPV infection in H&NCs but p16 IHC is commonly used as a complementary procedure due to its low cost and sensitivity (43). Guidelines suggest to perform p16 IHC as the initial test for HPV characterization in tumor tissue, followed by additional molecular test at the discretion of pathologists (45). It was shown that combined p16 and HPV DNA testing discriminates subgroup of tumors with significantly distinct outcome (44, 46).

The outcome of HPV-associated H&NCs is better than that of HPV-negative cases (47). In a meta-analysis including 42 studies, both progression-free survival and disease-free survival were significantly improved in HPV-positive tumors (48). However, the prognostic value of the HPV status should be appreciated differently according to tumor histology. Most HPV-positive H&NCs correspond to SCC, but undifferentiated carcinomas may also be HPV-associated and present a favorable outcome (49) whereas large cell neuro-endocrine carcinoma (50) and high-grade neuro-endocrine carcinomas (51) correspond to diseases of poor outcome whatever their HPV status may be. Neuro-endocrine tumors frequently express p16 and this marker is a poor surrogate for HPV-association in these tumors (52). In addition, H&NCs associated with HPV genotypes other than HPV16 were found to have unfavorable outcomes as compared with HPV16-positive cases (53), an inversed association to that observed in cervical cancers (20). Integration pattern might also be clinically predictive: H&N squamous cell carcinoma with presence of episomal form of the viral genome without integration were associated with a better outcome than HPV integration-positive or HPV-negative cases (39) and tumor relapse was more frequently observed when HPV-DNA was found inserted in cancer-related genes rather than in intergenic loci (54).

In patients with carcinomas of the oral cavity or of the oropharynx, the oral rinse sampling for prognostic purpose may represent a convenient approach for sequential viral analyses. A diagnostic rate of 81 and 100% of sensitivity and specificity for HPV-16-positive cases was observed (55). After completion of primary therapies, the presence of persisting viral DNA in the oral cavity was associated with an increased risk of recurrence: the disease-free survival at 2 years was 55% in patients with persistent HPV detection *versus* 88% for viral-negative cases (55).

It is likely that the HPV status has also a predictive value. A specific HPV-related tumor immune microenvironment (56) may be implicated in improving sensitivity to treatments. The analysis of the immune infiltrate in H&NCs has shown that HPV-positive cases exhibited greater CD8+ T cell infiltrate and PD-L1 expression than HPV-negative tumors (57–60). This immune

pattern was associated with favorable outcome after chemo-radiotherapy (58). Moreover, clinical trials provide data indicating that anti-PD-1/PD-L1 therapy results in anti-tumor activity in H&NCs (61) and report a higher response rate in HPV-positive than in HPV-negative patients (57). Immunological analyses found that an HPV-positive status contributes to T-cell infiltration and enhanced cytolytic activity which result in a better response to anti PD-1/PD-L1 therapy (57). Nevertheless, significant PD-L1 expression can also be observed in HPV-negative H&NCs and thus, the HPV status is not a prerequisite for immunotherapy in these tumors (62).

HPV Integration Signatures and Viral DNA Used as Specific Tumor Markers

HPV DNA integration pattern can be used as specific tumor markers helpful for personalized patient follow-up (34, 63). This pattern encompasses several parameters, (I) locus at the molecular level, (II) break locus on the viral genome, (III) deletion of part of the viral genome, and (IV) characteristic patterns of the viral/cell genome junctions. The high specificity of this signature is essential for diagnostic purposes. For instance, in a patient previously treated for an HPV-associated tumor and developing a second tumor, the differential diagnosis between a metastasis versus the *de novo* development of an independent second tumor associated with the same viral genotype may be difficult. In this situation, the presence of the same insertional signature in the two lesions confers a very high level of specificity for a diagnosis of metastasis. As an example, we have demonstrated recently that a carcinoma developed on the base of the tongue in a male patient previously treated for a carcinoma of the anal canal shared the same HPV16 specific insertion molecular signature as that characterizing the anal tumor and we could conclude that the tongue tumor corresponded unambiguously to the metastasis of the primary anal tumor (64). The distinction can be important since the therapeutic approach is different in case of metastasis vs a second primary tumor. A limitation of this approach for diagnostic purpose relies on the fact that 30–40% of H&NCs harbor only episomal HPV DNA and thus HPV-chromosome insertional signatures are lacking. However, the identification of the precise lineage and sub-lineage of the HPV viral genotype should nevertheless provide a valuable tumor marker, particularly when different HPV strains/genotypes are detected in the respective heterogeneous tumors.

PERSPECTIVES

Three major bio-clinical perspectives may be considered, (a) the development of the detection of circulating tumor DNA (ctDNA) using HPV sequences as a tumor marker, (b) the introduction of NGS methods for the optimal molecular characterization of viral DNA in clinical oncology, and (c) the analysis of the tumor immune microenvironment in the frame of immunotherapeutic protocols.

HPV Genome: A Tumor Marker for the Detection of ctDNA

Circulating HPV DNA: A specific Form of ctDNA

Numerous applications of ctDNA as diagnostic and predictive/prognostic marker in oncology are under development (65–67). In most models, ctDNA is detected *via* the presence of somatic mutations, but the low rate of target molecules, especially in early stage tumors, implies that their detection can be affected by stochastic sampling leading to a lack of target molecules in some specimens (68), a limitation for clinical applications, (69). In this context, HPV-associated tumors represent a privileged model for the detection of ctDNA. As mentioned above, the HPV genome is a 7.8 kbp-long circular double-strand DNA molecule present in the nucleus of tumor cells, as free episomes and/or as an integrated form, in copy numbers varying from a few to thousands per cell. The viral DNA fragments shed in the blood corresponds thus to a large target of foreign DNA that can be more easily detected than ctDNA fragments harboring point mutations dispersed among germline circulating DNA. Indeed, circulating HPV DNA can be detected in patients with various types of HPV-associated carcinoma (70, 71). Studies found no circulating viral DNA in non-tumor patients or in patients with intra-epithelial neoplasia and circulating HPV DNA, referred to as ctHPV DNA, can thus be considered as a specific form of ctDNA (71) and serve as a tumor marker for improved diagnosis, prognosis, and treatment monitoring (72).

ctHPV DNA: A Diagnostic Marker

At the time of diagnosis of HPV-positive H&NCs, the rates of ctHPV DNA positivity ranged from 60.5 to 95.9% (57, 70, 71, 73, 74). In a study focused on early stage H&NCs and using an NGS-based approach for the detection of HPV16, all cases (55/55) were ctHPV DNA positive (75). Although a positive correlation between ctHPV DNA levels and tumor stages has been observed (71, 76, 77), a striking and recurrent fact is that small tumors may be associated with high ctHPV DNA levels whereas low levels are found in more advanced cases (70, 71, 74). The reasons for this discrepancy are unclear. It could be related to variations in tumor viral load and/or integration signatures, but the comparison between tumor viral loads and ctHPV DNA levels revealed only a weak correlation for H&NCs (70). Other factors such as tumor differentiation, necrosis, proliferative rate, or immune response might be involved in the release of viral DNA. Like in other tumor models, the data collected suggest that ctHPV DNA levels are not simply associated with tumor burden or the number of dying cells but they correspond to a complex combination of tumor biology factors, potentially playing an active role in immunomodulation or in other processes regulating cell homeostasis (68).

From these works, three major conclusions can be drawn. (I) High rates of ctHPV DNA positivity are observed at diagnosis in patients with HPV-associated H&NCs; (II) The ctHPV DNA level is poorly related to tumor volume; (III) ctHPV DNA is already detectable in patients with subclinical disease. In clinical oncology, the first potential application is the possibility of using ctHPV DNA detection as an alternative approach to histology for

the diagnosis of HPV-associated invasive carcinoma. This new approach could notably be used for the diagnosis of relapse in patients previously treated for an HPV-associated tumor and presenting abnormal imaging. In this situation, the detection of ctHPV DNA should be a sufficient criterion to confirm the diagnosis of relapse, avoiding unnecessary biopsy procedure that may cause morbidity. However, the question of the specificity of this approach has to be considered since some cases may be difficult to interpret. We previously provided a proof of concept showing that the highly specific viral insertional signature could be detected in the blood of patients (78) and that this approach provides a valuable tool to ascertain the specificity of the result of ctHPV DNA analysis when necessary.

Dynamic of ctHPV DNA Load: A Prognostic/ Predictive Marker

The comparison between virological and clinical data showed that ctHPV DNA load at diagnosis is poorly indicative of disease outcome (76, 77). This is in contrast with the Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma model in which patients with a high plasma EBV load at diagnosis present a higher tumor stage and metastatic status (79). This discrepancy may be related to a poor correlation between tumor size and HPV ctDNA load in H&NCs. In contrast, longitudinal studies report that the dynamics of ctHPV DNA load under treatment can be a surrogate for the quality of tumor response and represent a valuable prognostic factor (70, 75, 77). Response kinetics to chemoradiation showed a high degree of heterogeneity among patients, but a drop in ctHPV DNA load was observed in most cases, preceding tumor regression at imaging (70). In particular, the post-therapeutic ctHPV DNA load demonstrated a major surrogate marker for the quality of the tumor response (75). At the end of treatment, early relapse in patients with positive ctHPV DNA was observed whereas, in patients showing abnormal fixation at imaging and ctHPV DNA negative detection, no residual disease was detected (75). Altogether, these observations suggest that post treatment ctHPV DNA status has positive as well as negative predictive values.

The high level of sensitivity and specificity of ctHPV DNA as a tumor marker in patients with HPV-associated carcinomas advocates for use of this marker to improve the biological follow-up of patients. The clinical relevance of this approach has been documented in colon carcinomas (80). However, there is no current evidence that, in all H&NCs cases, a biological relapse characterized by ctHPV DNA positivity would be followed by a clinical relapse at short term. Prospective studies are necessary to document and provide the clinical validation of this approach. Other pending questions are to address the specificity of the test, as well as the clinical utility of this approach. Concerning the specificity, the criteria necessary to affirm a biological relapse should be determined according to the clinical situation. In case of doubt, the specificity of the insertional signature would be a formal argument for a diagnosis of relapse and discard the possibility of the subclinical development of a second HPV-associated tumor. The clinical utility of the detection of subclinical relapse has also to be discussed. What should be the attitude in case of ctHPV DNA positivity in patients with no clinical or radiological evidence of a tumor? The design of innovative therapeutic protocols targeting subclinical diseases will be necessary to address this question and, in this perspective, ctHPV DNA will be a valuable surrogate marker to measure the efficiency of these treatments.

NGS for the Improved Characterization of HPV-Associated H&NCs

The detection of HPV DNA in biological specimens is commonly performed using q-PCR technique. However, this technology is not sufficient to allow the identification of the specific molecular markers useful for the diagnostic or follow-up purposes described above. In contrast, NGS is a powerful tool able to provide, in a single experiment, the extensive molecular characterization of HPV DNA in tumors: the complete nucleotide sequence of the viral genome (genotyping), its host chromosomal modifications (deletion, amplification), physical status, integration signature, chromosomal integration site(s), and identification of the gene(s) located in the vicinity (36, 81–84) (**Figure 1**). For instance, the CaptHPV method that we have developed (36) includes a double capture of HPV DNA fragments using single-stranded biotinylated probes recognizing 235 unique HPV types and variants. DNA sequences captured are sequenced using MiSeq instruments and raw sequencing data are aligned on the 235 HPV reference strains. A second alignment on human genome is performed to select hybrid reads that align on both HPV and human genomes and a map of the HPV pattern is deduced. This global approach has been validated technically and may be used in clinical oncology. In H&NCs, most of the data described above concerning integration targets were obtained using NGS methods (39–41, 75). These data have identified recurrent integration sites (39) and have shown that, as in genital tumors, viral insertion could impact the host genome by amplification of oncogenes and disruption of tumor suppressors as well as by driving inter- and intrachromosomal rearrangements (40). The clinical relevancy of the HPV integration status has been suggested (39) and, if confirmed, represents an important biological parameter to take into account when defining therapeutic strategies. Moreover, NGS approaches provide precise identifications of the viral sequences associated with the tumor, corresponding either to a specific HPV16 strain (75) or to any other genotypes (20% of the cases) (39, 40), genotypes that constitute valuable tumor markers.

Importantly, the NGS-based method developed for the molecular characterization of HPV DNA in cervical tumors can be successfully applied to the detection and characterization of ctHPV DNA (36). The NGS approach applied to the analysis of a standard blood sample allows the identification of any HPV genotype as well as the characterization of the insertional signature. Using this methodology, a diagnosis of HPV-associated invasive carcinoma can be obtained from a blood sample whatever the viral genotype involved. A prospective study is in progress to provide the clinical validation of this

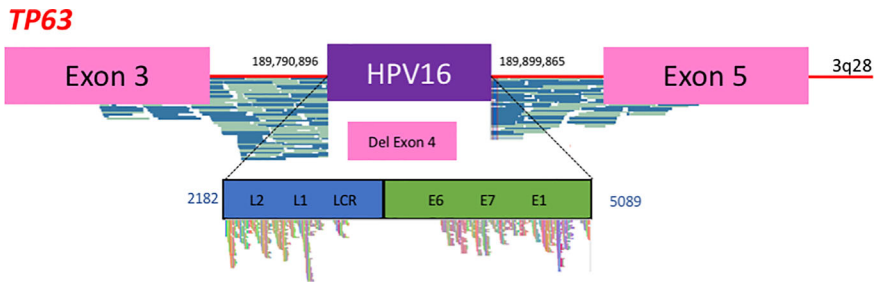

FIGURE 1 | Next-generation sequencing data using the CaptHPV assay. The results presented here are sequences obtained from tumor tissue analyze in a patient with a HPV16-induced carcinoma. DNA sequences show viral DNA inserted within the *TP63* gene (3q28 chromosomal band), between exon 3 and exon 5 with gene disruption and loss of the exon 4. All genomics coordinates are presented in *Hg38* reference genome.

approach as an alternative to histology for a diagnosis of HPV-associated carcinoma, and to determine its limitation in terms of sensitivity and specificity (Sastre-Garau et al., in preparation). Furthermore, an NGS approach can be designed to combine the characterization of HPV DNA with the identification of somatic mutations frequent in HPV negative tumors. Using such a combined approach, Wang et al. detected ctDNA in 86 to 100% of HPV-positive or -negative H&NCs cases (57). Therefore, the design of NGS approaches combining extensive HPV DNA analysis and the detection of recurrent mutations can allow the determination of molecular markers and targets associated with in H&NCs independently of their HPV status (29). For the moment, NGS-based approaches remain relatively expensive and are not used routinely for sequential analyses during patient' follow-up care in most hospitals or clinics worldwide. Their implementation requires specific facilities, including dedicated bioinformatics pipeline and trained team for technical processing and data interpretation. However, once we have a full picture of the HPV integration pattern that characterizes each tumor using NGS, the relevant markers determined can be extrapolated and sequentially analyzed using specific q-PCR method, allowing optimized long-term follow-up at reduced costs.

Analysis of the Tumor Immune Microenvironment

The important role of the immune microenvironment as a major feature for tumor response to therapies of subsequent disease outcome has been underlined. Among the various parameters that can be analyzed, includes the density of the effector T cells (CD8+) and of PD-L1 in both immune cells and tumor cells that represent key prognostic and predictive parameters recurrently found in several studies (58–60). The development of immunotherapy protocols will require the evaluation of these markers using standardized parameters.

CONCLUSION

Over the last 10 years, a number of viro-clinical studies have permitted us to obtain a better knowledge of the prevalence of HPV association in H&NCs, including the viral epidemiology and the oncogenesis of these tumors, allowing us to evolve from the concept of HPV-positive towards that of HPV-driven H&NCs (85). However, the use of HPV DNA as a diagnostic and/or predictive marker is not yet fully developed, in large part due to a lack of clinical validation. In the process of validation of tumor biomarkers, three main steps should be distinguished: analytical validity, clinical validity, and clinical utility (86). In the model of HPV-associated H&NCs, two major steps have already been reached: (I) the analytical validity of the HPV status using different methods (immunohistochemistry, PCR, or NGS) and (II) the clinical validity of the prognostic value of the HPV status when considered as a binary parameter (positive *versus* negative) (45). However, the clinical validity of other potential prognostic viral-related markers, such as the exact genotype or the physical state of viral DNA, needs to be further documented.

HPV-associated tumors represent a privileged model for the analysis of ctDNA and viral sequences constitute a very convenient biological role model to assess the course of the disease. ctHPV DNA is a sensitive tumor marker and this should facilitate the clinical validation of the "liquid biopsy approach" for the diagnosis of invasive carcinomas, for instance in case of suspicion of relapse. This validation will require the analysis of various tumors differing in size, localization, and viral genotype before the ctHPV DNA approach could be implemented as a recognized diagnostic tool in clinical oncology, avoiding more invasive procedures such as biopsies or fine needle aspirations. The ctDNA load at diagnosis is a poor prognostic marker, but its dynamic during the treatment is a promising predictive surrogate of tumor response. During the follow-up procedures, the clinical validation of ctHPV DNA as a predictive marker of relapse will solicit prospective studies demonstrating that all of the biological relapses precede clinical relapses. Difficulties need to be overcome. For instance, current studies report that advanced cases of HPV-positive tumors remain ctHPV DNA negative and the identification of the parameters accounting for this discrepancy is a prerequisite for a large use of ctDNA as a surrogate marker of the course of H&NCS.

Once these clinical validations are obtained, a major challenge remains to document the clinical utility of the diagnosis of sub-

clinical disease, which depends mainly on the possibility of treatment. New tools will be necessary for the care of subclinical relapses and viral-associated tumors represent an attractive model for the development of immunotherapy. As an example, the treatment of high grade cervical intra-epithelial neoplasia using therapeutic HPV16/18 vaccine is currently in evaluation and provides encouraging results (87). Such an approach might be extended to sub-clinical diseases. The design of these innovative treatments as well as the assessment of their efficiency will require extensive molecular characterization of the viral sequences, and tools allowing this characterization are available.

The analysis of a large series of H&NCs using NGS approaches should enhance our knowledge about the biology of these tumors and favor further developments in diagnosis, follow-up, and treatments. The implementation of large data bases collecting the biological and clinical data obtained will be a powerful common tool favoring these advances.

AUTHOR CONTRIBUTIONS

XS-G wrote the manuscript and AH reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prevent: Publ Am Assoc Cancer Res Cosponsored Am Soc Prevent Oncol* (2005) 14(2):467–75. doi: 10.1158/1055-9965.EPI-04-0551
2. de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *Int J Cancer* (2017) 141 (4):664–70. doi: 10.1002/ijc.30716
3. Anantharaman D, Abedi-Ardekani B, Beachler DC, Gheit T, Olshan AF, Wisniewski K, et al. Geographic heterogeneity in the prevalence of human papillomavirus in head and neck cancer. *Int J Cancer* (2017) 140(9):1968–75. doi: 10.1002/ijc.30608
4. Haeggblom L, Ramqvist T, Tommasino M, Dalianis T, Nasman A. Time to change perspectives on HPV in oropharyngeal cancer. A systematic review of HPV prevalence per oropharyngeal sub-site the last 3 years. *Papillomavirus Res* (2017) 4:1–11. doi: 10.1016/j.pvr.2017.05.002
5. Charfi L, Jouffroy T, de Cremoux P, Le Peltier N, Thioux M, Freneaux P, et al. Two types of squamous cell carcinoma of the palatine tonsil characterized by distinct etiology, molecular features and outcome. *Cancer Lett* (2008) 260 (1–2):72–8. doi: 10.1016/j.canlet.2007.10.028
6. Gelwan E, Malm IJ, Khararjian A, Fakhry C, Bishop JA, Westra WH. Nonuniform Distribution of High-risk Human Papillomavirus in Squamous Cell Carcinomas of the Oropharynx: Rethinking the Anatomic Boundaries of Oral and Oropharyngeal Carcinoma From an Oncologic HPV Perspective. *Am J Surg Pathol* (2017) 41(12):1722–8. doi: 10.1097/PAS.0000000000000929
7. Abogunrin S, Di Tanna GL, Keeping S, Carroll S, Iheanacho I. Prevalence of human papillomavirus in head and neck cancers in European populations: a meta-analysis. *BMC Cancer* (2014) 14(1):968. doi: 10.1186/1471-2407-14-968
8. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol: Off J Am Soc Clin Oncol* (2011) 29(32):4294–301. doi: 10.1200/JCO.2011.36.4596
9. Jehannin-Ligier K, Belot A, Guizard AV, Bossard N, Launoy G, Uhry Z, et al. Incidence trends for potentially human papillomavirus-related and -unrelated head and neck cancers in France using population-based cancer registries data: 1980-2012. *Int J Cancer* (2017) 140(9):2032–9. doi: 10.1002/ijc.30631
10. Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L, et al. Prevalence of oral HPV infection in the United States, 2009-2010. *Jama* (2012) 307(7):693–703. doi: 10.1001/jama.2012.101
11. Combes JD, Franceschi S. Human papillomavirus genome variants and head and neck cancers: a perspective. *Infect Agents Cancer* (2018) 13:13. doi: 10.1186/s13027-018-0185-6
12. Nakashima K, Shigehara K, Kawaguchi S, Wakatsuki A, Kobori Y, Nakashima K, et al. Prevalence of human papillomavirus infection in the oropharynx and urine among sexually active men: a comparative study of infection by papillomavirus and other organisms, including Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma spp., and Ureaplasma spp. *BMC Infect Dis* (2014) 14:43. doi: 10.1186/1471-2334-14-43
13. Rieth KKS, Gill SR, Lott-Limbach AA, Merkley MA, Botero N, Allen PD, et al. Prevalence of High-Risk Human Papillomavirus in Tonsil Tissue in Healthy Adults and Colocalization in Biofilm of Tonsillar Crypts. *JAMA Otolaryngol Head Neck Surg* (2018) 144(3):231–7. doi: 10.1001/jamaoto.2017.2916
14. Heck JE, Berthiller J, Vaccarella S, Winn DM, Smith EM, Shan'gina O, et al. Sexual behaviours and the risk of head and neck cancers: a pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. *Int J Epidemiol* (2010) 39(1):166–81. doi: 10.1093/ije/dyp350
15. Chancellor JA, Ioannides SJ, Elwood JM. Oral and oropharyngeal cancer and the role of sexual behaviour: a systematic review. *Community Dentistry Oral Epidemiol* (2017) 45(1):20–34. doi: 10.1111/cdoe.12255
16. Schnelle C, Whiteman DC, Porceddu SV, Panizza BJ, Antonsson A. Past sexual behaviors and risks of oropharyngeal squamous cell carcinoma: a case-case comparison. *Int J Cancer* (2017) 140(5):1027–34. doi: 10.1002/ijc.30519
17. Saunders CL, Meads C, Abel GA, Lyratzopoulos G. Associations Between Sexual Orientation and Overall and Site-Specific Diagnosis of Cancer: Evidence From Two National Patient Surveys in England. *J Clin Oncol: Off J Am Soc Clin Oncol* (2017) 35(32):3654–61. doi: 10.1200/JCO.2017.72.5465
18. D'Souza G, Gross ND, Pai SI, Haddad R, Anderson KS, Rajan S, et al. Oral human papillomavirus (HPV) infection in HPV-positive patients with oropharyngeal cancer and their partners. *J Clin Oncol: Off J Am Soc Clin Oncol* (2014) 32(23):2408–15. doi: 10.1200/JCO.2014.55.1341
19. Castellsague X, Alemany L, Quer M, Halec G, Quiros B, Tous S, et al. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients. *J Natl Cancer Institute* (2016) 108(6):djv403. doi: 10.1093/jnci/djv403
20. de Cremoux P, de la Rochefordiere A, Savignoni A, Kirova Y, Alran S, Fourchotte V, et al. Different outcome of invasive cervical cancer associated with high-risk versus intermediate-risk HPV genotype. *Int J Cancer* (2009) 124(4):778–82. doi: 10.1002/ijc.24075
21. Mirabello L, Clarke MA, Nelson CW, Dean M, Wentzensen N, Yeager M, et al. The Intersection of HPV Epidemiology, Genomics and Mechanistic Studies of HPV-Mediated Carcinogenesis. *Viruses* (2018) 10(2):80. doi: 10.3390/v10020080
22. Burk RD, Harari A, Chen Z. Human papillomavirus genome variants. *Virology* (2013) 445(1-2):232–43. doi: 10.1016/j.virol.2013.07.018
23. Nicolas-Parraga S, Alemany L, de Sanjose S, Bosch FX, Bravo IG, Ris Hpv TT, et al. Differential HPV16 variant distribution in squamous cell carcinoma, adenocarcinoma and adenosquamous cell carcinoma. *Int J Cancer* (2017) 140 (9):2092–100. doi: 10.1002/ijc.30636
24. Mirabello L, Yeager M, Yu K, Clifford GM, Xiao Y, Zhu B, et al. HPV16 E7 Genetic Conservation Is Critical to Carcinogenesis. *Cell* (2017) 170(6):1164–74 e6. doi: 10.1016/j.cell.2017.08.001

25. Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol Rev* (2003) 67(4):657–85. doi: 10.1128/MMBR.67.4.657-685.2003
26. Cancer Genome Atlas N Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* (2015) 517(7536):576–82. doi: 10.1038/nature14129
27. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science* (2011) 333(6046):1157–60. doi: 10.1126/science.1208130
28. Sablin MP, Dubot C, Klijanienko J, Vacher S, Ouafi L, Chemlali W, et al. Identification of new candidate therapeutic target genes in head and neck squamous cell carcinomas. *Oncotarget* (2016) 7(30):47418–30. doi: 10.18632/oncotarget.10163
29. Tuna M, Amos CI. Next generation sequencing and its applications in HPV-associated cancers. *Oncotarget* (2017) 8(5):8877–89. doi: 10.18632/oncotarget.12830
30. Lechner M, Frampton GM, Fenton T, Feber A, Palmer G, Jay A, et al. Targeted next-generation sequencing of head and neck squamous cell carcinoma identifies novel genetic alterations in HPV+ and HPV- tumors. *Genome Med* (2013) 5(5):49. doi: 10.1186/gm453
31. Rosty C, Sheffer M, Tsafrir D, Stransky N, Tsafrir I, Peter M, et al. Identification of a proliferation gene cluster associated with HPV E6/E7 expression level and viral DNA load in invasive cervical carcinoma. *Oncogene* (2005) 24(47):7094–104. doi: 10.1038/sj.onc.1208854
32. Pyeon D, Newton MA, Lambert PF, den Boon JA, Sengupta S, Marsit CJ, et al. Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. *Cancer Res* (2007) 67(10):4605–19. doi: 10.1158/0008-5472.CAN-06-3619
33. Hopman AH, Smedts F, Dignef W, Ummelen M, Sonke G, Mravunac M, et al. Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *J Pathol* (2004) 202(1):23–33. doi: 10.1002/path.1490
34. Carow K, Golitz M, Wolf M, Hafner N, Jansen L, Hoyer H, et al. Viral-Cellular DNA Junctions as Molecular Markers for Assessing Intra-Tumor Heterogeneity in Cervical Cancer and for the Detection of Circulating Tumor DNA. *Int J Mol Sci* (2017) 18(10):2032. doi: 10.3390/ijms18102032
35. Peter M, Stransky N, Couturier J, Hupe P, Barillot E, de Cremoux P, et al. Frequent genomic structural alterations at HPV insertion sites in cervical carcinoma. *J Pathol* (2010) 221(3):320–30. doi: 10.1002/path.2713
36. Holmes A, Lameiras S, Jeannot E, Marie Y, Castera L, Sastre-Garau X, et al. Mechanistic signatures of HPV insertions in cervical carcinomas. *NPJ Genom Med* (2016) 1:16004. doi: 10.1038/npjgenmed.2016.4
37. Peter M, Rosty C, Couturier J, Radvanyi F, Teshima H, Sastre-Garau X. MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors. *Oncogene* (2006) 25(44):5985–93. doi: 10.1038/sj.onc.1209625
38. Saunier M, Monnier-Benoit S, Mauny F, Dalstein V, Briolat J, Riethmuller D, et al. Analysis of human papillomavirus type 16 (HPV16) DNA load and physical state for identification of HPV16-infected women with high-grade lesions or cervical carcinoma. *J Clin Microbiol* (2008) 46(11):3678–85. doi: 10.1128/JCM.01212-08
39. Koneva LA, Zhang Y, Virani S, Hall PB, McHugh JB, Chepeha DB, et al. HPV Integration in HNSCC Correlates with Survival Outcomes, Immune Response Signatures, and Candidate Drivers. *Mol Cancer Res: MCR* (2018) 16(1):90–102. doi: 10.1158/1541-7786.MCR-17-0153
40. Parfenov M, Pedamallu CS, Gehlenborg N, Freeman SS, Danilova L, Bristow CA, et al. Characterization of HPV and host genome interactions in primary head and neck cancers. *Proc Natl Acad Sci U S A* (2014) 111(43):15544–9.
41. Gao G, Johnson SH, Kasperbauer JL, Eckloff BW, Tombers NM, Vasmatzis G, et al. Mate pair sequencing of oropharyngeal squamous cell carcinomas reveals that HPV integration occurs much less frequently than in cervical cancer. *J Clin Virol: Off Publ Pan Am Soc Clin Virol* (2014) 59(3):195–200. doi: 10.1016/j.jcv.2013.12.006
42. Westra WH. The pathology of HPV-related head and neck cancer: implications for the diagnostic pathologist. *Semin Diagn Pathol* (2015) 32(1):42–53. doi: 10.1053/j.semdp.2015.02.023
43. Economopoulou P, de Bree R, Kotsantis I, Psyrri A. Diagnostic Tumor Markers in Head and Neck Squamous Cell Carcinoma (HNSCC) in the Clinical Setting. *Front Oncol* (2019) 9:827. doi: 10.3389/fonc.2019.00827
44. Albers AE, Qian X, Kaufmann AM, Coordes A. Meta analysis: HPV and p16 pattern determines survival in patients with HNSCC and identifies potential new biologic subtype. *Sci Rep* (2017) 7(1):16715. doi: 10.1038/s41598-017-16918-w
45. Fakhry C, Lacchetti C, Rooper LM, Jordan RC, Rischin D, Sturgis EM, et al. Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. *J Clin Oncol: Off J Am Soc Clin Oncol* (2018) 871: JCO1800684. doi: 10.1200/JCO.18.00684
46. Salazar CR, Anayannis N, Smith RV, Wang Y, Haigentz MJr., Garg M, et al. Combined P16 and human papillomavirus testing predicts head and neck cancer survival. *Int J Cancer* (2014) 135(10):2404–12. doi: 10.1002/ijc.28876
47. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *New Engl J Med* (2010) 363(1):24–35. doi: 10.1056/NEJMoa0912217
48. O'Rorke MA, Ellison MV, Murray LJ, Moran M, James J, Anderson LA. Human papillomavirus related head and neck cancer survival: a systematic review and meta-analysis. *Oral Oncol* (2012) 48(12):1191–201. doi: 10.1016/j.oraloncology.2012.06.019
49. Carpenter DH, El-Mofty SK, Lewis JSJr. Undifferentiated carcinoma of the oropharynx: a human papillomavirus-associated tumor with a favorable prognosis. *Modern Pathol: Off J U States Can Acad Pathol Inc* (2011) 24(10):1306–12. doi: 10.1038/modpathol.2011.87
50. Thompson ED, Stelow EB, Mills SE, Westra WH, Bishop JA. Large Cell Neuroendocrine Carcinoma of the Head and Neck: A Clinicopathologic Series of 10 Cases With an Emphasis on HPV Status. *Am J Surg Pathol* (2016) 40(4):471–8. doi: 10.1097/PAS.0000000000000580
51. Westra WH. Human Papillomavirus-Related Neuroendocrine Carcinomas of the Head and Neck. *Head Neck Pathol* (2018) 12(1):9–12. doi: 10.1007/s12105-018-0886-6
52. Alos L, Hakim S, Larque AB, de la Oliva J, Rodriguez-Carunchio L, Caballero M, et al. p16 overexpression in high-grade neuroendocrine carcinomas of the head and neck: potential diagnostic pitfall with HPV-related carcinomas. *Virchows Archiv: Int J Pathol* (2016) 469(3):277–84. doi: 10.1007/s00428-016-1982-1
53. Mazul AL, Rodriguez-Ormaza N, Taylor JM, Desai DD, Brennan P, Anantharaman D, et al. Prognostic significance of non-HPV16 genotypes in oropharyngeal squamous cell carcinoma. *Oral Oncol* (2016) 61:98–103. doi: 10.1016/j.oraloncology.2016.08.019
54. Pinatti LM, Walline HM, Carey TE. Human Papillomavirus Genome Integration and Head and Neck Cancer. *J Dental Res* (2018) 97(6):691–700. doi: 10.1177/0022034517744213
55. Fakhry C, Blackford AL, Neuner G, Xiao W, Jiang B, Agrawal A, et al. Association of Oral Human Papillomavirus DNA Persistence With Cancer Progression After Primary Treatment for Oral Cavity and Oropharyngeal Squamous Cell Carcinoma. *JAMA Oncol* (2019) 5(7):985–92. doi: 10.1001/jamaoncol.2019.0439
56. Lechien JR, Seminerio I, Descamps G, Mat Q, Mouawad F, Hans S, et al. Impact of HPV Infection on the Immune System in Oropharyngeal and Non-Oropharyngeal Squamous Cell Carcinoma: A Systematic Review. *Cells* (2019) 8(9):1061. doi: 10.3390/cells8091061
57. Wang J, Sun H, Zeng Q, Guo XJ, Wang H, Liu HH, et al. HPV-positive status associated with inflamed immune microenvironment and improved response to anti-PD-1 therapy in head and neck squamous cell carcinoma. *Sci Rep* (2019) 9(1):13404. doi: 10.1038/s41598-019-49771-0
58. Balermpas P, Rodel F, Krause M, Linge A, Lohaus F, Baumann M, et al. The PD-1/PD-L1 axis and human papilloma virus in patients with head and neck cancer after adjuvant chemoradiotherapy: A multicentre study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). *Int J Cancer* (2017) 141(3):594–603. doi: 10.1002/ijc.30770
59. Gameiro SF, Ghasemi F, Barrett JW, Koropatnick J, Nichols AC, Mymryk JS, et al. Treatment-naive HPV+ head and neck cancers display a T-cell-inflamed phenotype distinct from their HPV- counterparts that has implications for immunotherapy. *Oncoimmunology* (2018) 7(10):e1498439. doi: 10.1080/2162402X.2018.1498439

60. Ock CY, Keam B, Kim S, Lee JS, Kim M, Kim TM, et al. Pan-Cancer Immunogenomic Perspective on the Tumor Microenvironment Based on PD-L1 and CD8 T-Cell Infiltration. *Clin Cancer Res: Off J Am Assoc Cancer Res* (2016) 22(9):2261–70. doi: 10.1158/1078-0432.CCR-15-2834
61. Ferris RL, Blumenschein GJr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *New Engl J Med* (2016) 375(19):1856–67. doi: 10.1056/NEJMoa 1602252
62. Cohen EEW, Bell RB, Bifulco CB, Burtness B, Gillison ML, Harrington KJ, et al. The Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of squamous cell carcinoma of the head and neck (HNSCC). *J Immunother Cancer* (2019) 7(1):184. doi: 10.1186/s40425-019-0662-5
63. Arfi A, Hequet D, Bataillon G, Tran-Perennou C, Farkhondeh F, Sastre-Garau X, et al. HPV DNA integration site as proof of the origin of ovarian metastasis from endocervical adenocarcinoma: three case reports. *BMC Cancer* (2019) 19(1):375. doi: 10.1186/s12885-019-5582-8
64. Harle A, Guillet J, Thomas J, Demange J, Dolivet G, Peiffert D, et al. HPV insertional pattern as a personalized tumor marker for the optimized tumor diagnosis and follow-up of patients with HPV-associated carcinomas: a case report. *BMC Cancer* (2019) 19(1):277. doi: 10.1186/s12885-019-5447-1
65. Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, et al. Direct detection of early-stage cancers using circulating tumor DNA. *Sci Trans Med* (2017) 9(403). doi: 10.1126/scitranslmed.aan2415
66. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Trans Med* (2014) 6(224):224ra24. doi: 10.1158/1538-7445.AM2014-5606
67. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet* (2019) 20(2):71–88. doi: 10.1038/s41576-018-0071-5
68. Kustanovich A, Schwartz R, Peretz T, Grinshpun A. Life and death of circulating cell-free DNA. *Cancer Biol Ther* (2019) 20(8):1057–67. doi: 10.1080/15384047.2019.1598759
69. Cabel L, Decraene C, Bieche I, Pierga JY, Bennamoun M, Fuks D, et al. Limited Sensitivity of Circulating Tumor DNA Detection by Droplet Digital PCR in Non-Metastatic Operable Gastric Cancer Patients. *Cancers* (2019) 11 (3):PP396. doi: 10.3390/cancers11030396
70. Damerla RR, Lee NY, You D, Soni R, Shah R, Reyngold M, et al. Detection of Early Human Papillomavirus-Associated Cancers by Liquid Biopsy. *JCO Precis Oncol* (2019) 3:1–17. doi: 10.1200/PO.18.00276
71. Jeannot E, Becette V, Campitelli M, Calmejane MA, Lappartient E, Ruff E, et al. Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma. *J Pathol Clin Res* (2016) 2 (4):201–9. doi: 10.1002/cjp2.47
72. Lauritano D, Oberti L, Gabrione F, Lucchese A, Petruzzi M, Carinci F, et al. Liquid biopsy in head and neck squamous cell carcinoma: Prognostic significance of circulating tumor cells and circulating tumor DNA. A systematic review. *Oral Oncol* (2019) 97:7–17. doi: 10.1016/j.oraloncology.2019.07.003
73. Dahlstrom KR, Anderson KS, Cheng JN, Chowell D, Li G, Posner M, et al. HPV Serum Antibodies as Predictors of Survival and Disease Progression in Patients with HPV-Positive Squamous Cell Carcinoma of the Oropharynx. *Clin Cancer Res: Off J Am Assoc Cancer Res* (2015) 21(12):2861–9. doi: 10.1158/1078-0432.CCR-14-3323
74. Veyer D, Wack M, Mandavit M, Garrigou S, Hans S, Bonfils P, et al. HPV circulating tumoral DNA quantification by droplet-based digital PCR: A promising predictive and prognostic biomarker for HPV-associated oropharyngeal cancers. *Int J Cancer* (2019) 147(4):1222–7. doi: 10.1002/ijc.32804
75. Lee JY, Garcia-Murillas I, Cutts RJ, De Castro DG, Grove L, Hurley T, et al. Predicting response to radical (chemo)radiotherapy with circulating HPV DNA in locally advanced head and neck squamous carcinoma. *Br J Cancer* (2017) 117(6):876–83. doi: 10.1038/bjc.2017.258
76. Dahlstrom KR, Li G, Hussey CS, Vo JT, Wei Q, Zhao C, et al. Circulating human papillomavirus DNA as a marker for disease extent and recurrence among patients with oropharyngeal cancer. *Cancer* (2015) 121(19):3455–64. doi: 10.1002/cncr.29538
77. Hanna GJ, Supplee JG, Kuang Y, Mahmood U, Lau CJ, Haddad RI, et al. Plasma HPV cell-free DNA monitoring in advanced HPV-associated oropharyngeal cancer. *Ann Oncol: Off J Eur Soc Med Oncol* (2018) 29 (9):1980–6. doi: 10.1093/annonc/mdy251
78. Campitelli M, Jeannot E, Peter M, Lappartient E, Saada S, de la Rochefordiere A, et al. Human papillomavirus mutational insertion: specific marker of circulating tumor DNA in cervical cancer patients. *PloS One* (2012) 7(8): e43393. doi: 10.1371/journal.pone.0043393
79. Zhao FP, Liu X, Chen XM, Lu J, Yu BL, Tian WD, et al. Levels of plasma Epstein-Barr virus DNA prior and subsequent to treatment predicts the prognosis of nasopharyngeal carcinoma. *Oncol Lett* (2015) 10(5):2888–94. doi: 10.3892/ol.2015.3628
80. Tie J, Cohen JD, Wang Y, Christie M, Simons K, Lee M, et al. Circulating Tumor DNA Analyses as Markers of Recurrence Risk and Benefit of Adjuvant Therapy for Stage III Colon Cancer. *JAMA Oncol* (2019) 5(12):1710–7. doi: 10.1001/jamaoncol.2019.3616
81. Xu B, Chotewutmontri S, Wolf S, Klos U, Schmitz M, Durst M, et al. Multiplex Identification of Human Papillomavirus 16 DNA Integration Sites in Cervical Carcinomas. *PloS One* (2013) 8(6):e66693. doi: 10.1371/journal.pone.0066693
82. Hu Z, Zhu D, Wang W, Li W, Jia W, Zeng X, et al. Genome-wide profiling of HPV integration in cervical cancer identifies clustered genomic hot spots and a potential microhomology-mediated integration mechanism. *Nat Genet* (2015) 47(2):158–63. doi: 10.1038/ng.3178
83. Akagi K, Li J, Broutian TR, Padilla-Nash H, Xiao W, Jiang B, et al. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome Res* (2014) 24(2):185–99. doi: 10.1101/gr.164806.113
84. Lagstrom S, Umu SU, Lepisto M, Ellonen P, Meisal R, Christiansen IK, et al. TaME-seq: An efficient sequencing approach for characterisation of HPV genomic variability and chromosomal integration. *Sci Rep* (2019) 9(1):524. doi: 10.1038/s41598-018-36669-6
85. Boscolo-Rizzo P, Pawlita M, Holzinger D. From HPV-positive towards HPV-driven oropharyngeal squamous cell carcinomas. *Cancer Treat Rev* (2016) 42:24–9. doi: 10.1016/j.ctrv.2015.10.009
86. Hayes DF. Biomarker validation and testing. *Mol Oncol* (2015) 9(5):960–6. doi: 10.1016/j.molonc.2014.10.004
87. Das M. A promising therapeutic vaccine for cervical precancer. *Lancet Oncol* (2019) 20(12):e671. doi: 10.1016/S1470-2045(19)30752-1

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REVIEW
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The Tumor Microenvironment and Immunotherapy of Oropharyngeal Squamous Cell Carcinoma

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Oropharyngeal squamous cell carcinoma (OPSCC) develops as a consequence of several mutations in the tumor suppressor pathways or after a progressive infection with high risk human papillomavirus (HPV). The dismal side effects of the current standard of care and the clear involvement of the immune system has led to a surge in clinical trials that aim to reinforce the tumor-specific immune response as a new treatment option. In this review, we have focused on the most recent literature to discuss the new findings and insights on the role of different immune cells in the context of OPSCC and its etiology. We then applied this knowledge to describe potential biomarkers and analyzed the rationale and outcomes of earlier and ongoing immunotherapy trials. Finally, we describe new developments that are still at the preclinical phase and provide an outlook on what the near future may bring, now that several new and exciting techniques to study the immune system at the single cell level are being exploited.

Keywords: tumor microenvironment, immunotherapy, oropharyngeal cancer, T cells, myeloid cells, clinical outcome, survival

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INTRODUCTION

Head and neck cancer is the sixth most prevalent cancer type and mainly consists of squamous cell carcinoma [90%; HNSCC (1, 2)]. The tumor can develop in the oral cavity, the larynx, pharynx (hypopharynx, nasopharynx, or oropharynx) and in the sinonasal tract. While the incidence of head and neck cancer located at most oral sites is marginally decreasing due to the knowledge of tobacco and alcohol as risk factors for its development, the incidence of oropharyngeal carcinomas is increasing, especially in the developed world (3). Oropharyngeal squamous cell carcinomas (OPSCC) include oropharyngeal, tonsillar and base-of-tongue tumors. A high percentage (60–80%) of the OPSCC are induced by high risk human papillomavirus type 16 (HPV16) (4–6). Patients with HPV+ OPSCC often tend to be younger, approximately 75% is male, a minority smokes and they often have lymph node metastasis when first visiting the clinic (7, 8). HPV is a double stranded DNA virus encoding for early and late (envelop) proteins (9, 10). The early proteins E6 and E7 are oncoproteins and responsible for the malignant transformation of HPV infected epithelial cells and maintenance hereof (11, 12). Patients with HPV16+ OPSCC display a longer overall survival (OS) and a lower recurrence rate after standard of care treatment than patients with HPV-negative OPSCC (13, 14).

Only recently the difference between these two OPSCC entities has been acknowledged and a debate on treatment has been started (15, 16). To understand the differences between the

HPV+ and HPV-negative tumors, in-dept analysis have been performed on various levels including genetics (DNA), epigenetics, (micro)RNA and the immune system (7). De-intensified treatment, by lowering the dose of the (chemotherapeutic) drug or radiotherapy or by replacement of the drug, have been suggested in order to decrease side effects particularly in patients with HPV+ OPSCC (15, 16). This led to inferior survival and new treatment designs are required (17). Immunotherapy may form a new effective treatment in OPSCC and is currently under investigation. While it is clear that during HPV infection and subsequent transformation several ways are exploited to escape from the immune system (18–22), HPV16 E6/E7-specific T-cells are often detected in OPSCC tumors and their presence is associated with improved clinical outcome (6, 23, 24). In addition, a whole series of articles exists on the association between intratumoral T-cell infiltration and better clinical outcome after standard of care therapy (25–28). These type of data paved the way for immunotherapeutic strategies to harness the immune response to OPSCC. Indeed, blockade of CTLA-4 and/or PD-1 has been studied and showed a survival benefit in a subset of patients with different types of HNSCC when compared to the standard of care treatment arm (29). The vast majority of patients did not benefit from this treatment, illustrating the requirement for in-depth studies on systemic and local host-tumor interactions. In particular, studies on the tumor immune microenvironment (TME) are a prerequisite to understand what hurdles are at play and need to be overcome in order for the immune system to effectively control tumor growth.

A search in PubMed using the key terms "Oropharyngeal cancer immunity", "Oropharyngeal cancer myeloid cells", "Oropharyngeal cancer immunotherapy trial", and "Head and Neck squamous cell carcinoma immunotherapy trial" was performed for articles published in the last 10 years (until august 2020) describing cohorts of patients with head and neck cancer, of which at least 25% had OPSCC and the results were not typical for one type of HNSCC, or with <25% of OPSCC patients but with results specific for the OPSCC group. Also, studies comparing the results between HPV-negative and HPV-positive HNSCC were included. In addition, a search in Clinicaltrials.gov for registered and ongoing trials in patients with HNSCC, including OPSCC, was performed. From these articles a selection was made to discuss the insights and developments in OPSCC immunity, biomarkers, and immunotherapy within the last decade. Last but not least, these studies were supplemented with general literature to explain concepts.

THE TUMOR IMMUNE MICROENVIRONMENT

The TME plays a pivotal role in the clinical behavior and response to different sorts of therapy of various cancer types (30). In-depth studies on the type, balance and interaction of immune cells in the TME and how this may affect clinical behavior could open the door to rationally designed strategies to treat patients with OPSCC (23).

Lymphocytes

The influx of high number of T cells was positively associated with clinical outcome of OPSCC after standard of care therapy (23, 31–33). In particular, strong infiltration by CD8+ and CD4+ T cells in pretreatment OPSCC tissues was associated with lower T stage, improved disease specific survival (DSS) and prolonged overall survival (OS). The clinical benefit of tumor infiltration with T cells was irrespective of HPV status of the tumor, albeit that HPV-induced tumors were more often strongly infiltrated by these T cells (31, 34–38). The functional activity of these tumor-infiltrating T cells is also important. Higher interferon gamma (IFNγ) and lower interleukin-4 (IL-4) and transforming growth factor-beta (TGF-β) cytokine expression levels were observed in HPV+ than HPV-negative OPSCC (39). In line with this, HPV-induced OPSCC contain high numbers of IFNγ or IL-17 producing CD8+ T cells (40) and the presence of IFNγ-producing CD4+ and CD8+ T cells in OPSCC, as inferred by the expression of the transcription factor T-box (Tbet) expressed in T cells, was related to better OS (6). Interestingly, the presence of IL-17-producing non-T cells in HPV+ OPSCC was associated with worse clinical benefit (38).

The analysis of freshly dissociated OPSCC tissues by mass cytometry revealed that HPV16+ OPSCC, comprising HPV16-specific T cells, also contained high numbers of CD161+ classical T cells, CD103+ tissue resident CD8+ T cells, dendritic cells (DCs) and DC-like macrophages (6). CD161+ T cells were highly activated, as shown by the high expression levels of PD-1, CD38 and HLA-DR, and were shown to be superior effector cells as they produced more IFNγ per cell (24). Notably, a higher frequency of CD161+CD4+ T cells was found to be associated with prolonged survival in OPSCC (24). The abundant presence of CD103+CD8+ T cells in HPV16+ OPSCC and its correlation with better prognosis was also demonstrated by others (41, 42). These CD103+CD8+ T cells represent non-circulating memory T cells that play a key role in local immunosurveillance (43) and are enriched for a number of genes associated with tissue resident cells (44). Single cell RNA analysis of 13 OPSCC revealed that these cells also expressed genes associated with cytotoxic potential, exhaustion/co-inhibition programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG3), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3)) as well as activation/co-stimulation [CD27, inducible T cell costimulator (ICOS) and tumor necrosis factor receptor superfamily member 14 (TNFRSF14)] (45).

An important aspect in tumor control by T cells is the interaction with human leukocyte antigen (HLA) molecules presenting tumor-derived antigens at the cell surface of tumor cells, yet HLA expression is often lost or decreased in OPSCC (46, 47). Antigen presentation to CD8+ T cells may also be impaired by alterations in antigen processing pathway components such as the endoplasmic reticulum aminopeptidase 1 (ERAP1), an enzyme involved in trimming the N-terminus of peptide until it fits in the HLA class I molecule. Some polymorphisms in ERAP-1 were associated with high or low T cell infiltration of OPSCC. Interestingly, only the ERAP1 allotypes present in highly infiltrated HPV16+ OPSCC were capable of trimming model

antigens, including HPV16 E7, to the proper HLA class I binding epitope (48). In contrast to HPV-negative OPSCC, there is no correlation between HLA class I expression and survival for HPV+ OPSCC (49, 50). The expression of HLA class II, which was more often found on tumor cells in HPV+ OPSCC, is associated with longer OS, DFS and disease specific survival (DSS) in OPSCC (46), supporting a role for CD4+ T cells in the control of OPSCC.

The Curious Relationship Between Regulatory T Cells and OPSCC Survival

Regulatory T cells (Tregs) act as gatekeepers of immunological tolerance, and dysfunction in Treg-mediated control plays an important role in autoimmune and allergic disorders and cancer. Tregs play a dismal role in tumor immunity, with numerous studies revealing their role in suppressing anti-tumor immune responses and promoting tumor progression in various types of cancer. In HNSCC, including OPSCC, there is conflicting evidence regarding their role in suppressing tumor immunity and survival (51). In two small scale studies, a negative impact on clinical outcome and disease progression was observed for high frequencies of Tregs in the peripheral blood of patients with OPSCC (52, 53), whereas other studies including OPSCC demonstrated no impact of tumor-infiltrating Tregs (54) or a positive impact of circulating or tumor-infiltrating Tregs (38, 55, 56) on clinical outcome and/or survival. A similar debate has been described for colorectal cancers (57, 58), resulting in the definition of three populations of CD4+Foxp3+ T cells, namely highly suppressive CD45RA-negative $Foxp3^{hi}$ effector Tregs ($Foxp3^{hi}$ eTregs), suppressive CD45RA+$Foxp3^{int}$ naïve Tregs ($Foxp3^{int}$ nTregs), and non-suppressive cytokine-producing CD45RA-negative $Foxp3^{int}$ T cells (59–61). Patients with colorectal tumors that were predominantly infiltrated with non-suppressive and cytokine-producing CD45RA-negative $Foxp3^{int}$ T cells displayed a much better survival than patients with tumors mainly infiltrated with suppressive $Foxp3^{hi}$ eTregs (60). This phenomenon may also play a role in studies that evaluated the clinical significance of intratumoral Tregs in OPSCC by means of immunohistochemistry (IHC), since discrimination between $Foxp3^{int}$ and $Foxp3^{hi}$ is difficult using this technique.

Part of the controversy on the prognostic value of Tregs may also be attributed to differences in the type of samples assessed, i.e. peripheral blood versus tumor tissues, and the absence of knowledge about the HPV status of the assessed tumors (51). The latter may be important as HPV-associated OPSCC is a distinct clinical entity with a different intratumoral immune cell make-up and a much better prognosis after (chemo)radiotherapy than HPV-negative OPSCC, particularly in patients with a concomitant HPV-specific and type 1-oriented intratumoral T cell response (6, 14, 32, 62).

Alternatively, differences in the effects of Tregs on clinical outcome may also come from their functional adaptability. Tregs can mirror effector cells by adopting the transcriptional profile of the cells they aim to suppress. In mice, Foxp3+ Tregs have been described to upregulate signal transducer and activator of transcription 3 (STAT3), GATA binding protein 3 (GATA-3)/interferon regulatory factor 4 (IRF4), or Tbet to control T helper type 17 (Th17), Th2 or Th1 inflammatory responses, respectively, in persistent infection and autoimmunity (63–66). We recently described that Tbet+ Tregs accumulate in tumors of HPV16+ OPSCC patients, and that their presence was associated with prolonged survival following standard of care therapy (67). Albeit detected at lower levels, the number of infiltrating Tbet+ Tregs strongly correlated with the number of tumor-infiltrating Tbet-positive CD4+ and CD8+ effector T cells as well as with the detection of HPV16-specific T cells, both of which we previously demonstrated to be associated with better tumor control on standard of care therapy. These data suggested that Tbet expression within the intratumoral Treg population and the association with prolonged survival is merely the reflection of an ongoing beneficial Th1-oriented T cell response, and that the balance between pro- and anti-tumor T cells eventually determines clinical outcome.

Myeloid Cells

The myeloid cell compartment constitutes another major player in the TME. One of the most abundant types of myeloid cells in the TME are macrophages. High numbers of tumor associated macrophages (TAMs) in the TME is most often associated with poor prognosis (68, 69). Macrophages are highly plastic and display a functional status ranging between classically activated anti-tumor type 1 macrophages (M1) and the alternatively activated tumor promoting type 2 macrophages (M2) (70). Incoming monocytes from the blood into the tumor can differentiate into macrophages dependent on the different cytokines they sense and the interactions they have with other cells present in the TME. While an inflamed TME with IFNγ results in M1 differentiation, high levels of interleukin 4 (IL-4) and IL-13 turn them into M2 (71). M2 can produce cytokines, such as TGF-β, vascular endothelial growth factor (VEGF), IL-6, IL-10, and prostaglandin E2 (PGE2), and chemokines that promote angiogenesis or destruct the extracellular matrix, thereby helping tumor cells to invade and metastasize and they can stimulate Treg development and expansion while suppressing CD8+ T cell function (72, 73). Moreover, in response to inflammation (IFNγ production) or hypoxia TAMs can upregulate inhibitory receptor ligands for T cells like PDL-1 and PDL-2 (69, 72).

CD68+CD163+ M2 macrophages are often detected in OPSCC (46, 74). A higher infiltration with these macrophages was associated with poor clinical outcome in two studies on HNSCC tumors with the majority or all being OPSCC (46, 75). The strong stromal presence of CD68+CD163+ macrophages was associated with shorter DFS, DSS and OS, independent of HPV status in OPSCC (46), but this does not mean that all macrophages are bad as high infiltration with macrophages, the minority being M2, was associated with better PFS in p16+ OPSCC (47). This suggests that other macrophages may benefit tumor control. Indeed, in various tumor types high infiltration with M1 (CD14+CD163-negative) macrophages is associated with prolonged survival (76, 77). It also implies that simply depleting all macrophages in the TME may have detrimental

effects and even accelerate tumor growth or increase recurrences (72, 78, 79). Therefore, reprogramming TAMs is currently under investigation in solid tumors, to move the balance from pro-tumorigenic towards tumor fighting macrophages (72, 80) and it is highly likely that also a strong Th1 cell response may aid in this process (73).

Similar to TAMs also myeloid derived suppressor cells (MDSCs) can be recruited from the blood or generated locally by arresting monocytes in their immature state. The phenotype of these MDSC is often described as CD14+ HLA-DR-CD33+ CD11b+ (81, 82), hence monocytic MDSC (mMDSC). High circulating numbers of these cells are associated with metastasis and recurrences in OPSCC patients (83). Interestingly, treatment with tadalafil, a phospohodiesterase 5 inhibitor to block nitric oxide and arginase 1 production by myeloid cells, resulted in the activation of a stronger tumor-specific T-cell response with no difference in responsiveness between HPV16+ OPSCC and HPV-negative (non)oropharyngeal cancer patients (84). Several other studies to deplete mMDSCs, prevent their recruitment or block their suppressive function have been undertaken (85). Next to these mMDSC also granulocytic MDSC (gMDSC) and neutrophils may enter the tumor to suppress effector T cells in the TME. However, no studies on the role of these cells in the TME of OPSCC have been reported.

Finally, recruited monocytes can also differentiate into DCs. High numbers of CD14+CD11b+CD11c+ DC-like macrophages and CD14-negative CD11b^{dim}CD11c+ DCs have been detected in the TME of OPSCC patients with a good clinical outcome (6). In another study, high numbers of stromal CD11c+ DC was an independent prognostic factor for better survival in HNSCC, including OPSCC (55). Current studies have analyzed DCs by only a few markers. Recently, two in-depth studies on blood derived DCs have divided these in several phenotypic and functional subtypes (86, 87). We applied high-dimensional flow cytometry and multispectral imaging to reveal a new subset of DCs, called CD163+ cDC2 or DC3, the presence of which was directly related to survival in HPV16+ OPSCC (74). Moreover, these DCs displayed the capacity to specifically trigger type 1 T cell responses by their production of IL-12 and IL-18 (74) and may be involved in conferring a tissue-resident signature to stimulated T cells (88).

Expression of Immune Checkpoints

Intratumoral T cell reactivity is regulated *via* so-called co-stimulatory and co-inhibitory (or checkpoint) molecules. Well-known is the suppression of T cells expressing PD-1 *via* PD-1 ligand (PD-L1) and blockade of this axis has resulted in spectacular clinical responses for a number of tumor types. Analyses of checkpoint expression in OPSCC revealed that the expression of PD-1 and/or PD-L1 was related to a stronger immune infiltration and good prognosis after standard therapy (89–92), most likely as it reflects an ongoing immune response in which type I and II interferons are produced. The presence of intratumoral PD-L1 expressing CD68+ macrophages and CD8+ T cells was found to be associated with improved OS (93). In addition, rich immune infiltration, comprising PD1+CD8+ T cells and CD68+ macrophages, was found to be associated with a better clinical response to checkpoint therapy (94). While the numbers of infiltrating total CD8+ T cells and CD68+ macrophages were higher in HPV+ OPSCC, the percentage of CD8+PD-1+ T cells was similar, and the percentage of CD68+ PD-L1+ macrophages lower in HPV+ OPSCC compared to HPV-negative OPSCC (95).

Another actionable co-inhibitory molecule is natural killer group 2 member A (NKG2A) (96, 97), which together with its co-receptor CD94 is expressed by many of the tumor-infiltrating CD8+ T cells and only by a minority of the CD4+ T cells in OPSCC (45). Remarkably, NKG2A expression on CD8+ in OPSCC is independent from PD-1 and often found on CD103+ early effector tissue resident CD8+ T cells (45, 97). The frequency of intratumoral NKG2A/CD94+ CD8+ T cells was higher in in HPV16+ OPSCC patients with a demonstrable ongoing HPV16-specific T cell response when compared to HPV16+ OPSCC lacking such an anti-tumor response or to HPV-negative OPSCC patients (6, 97). NKG2A interacts with HLA-E, which is a non-classical highly-conserved HLA class I molecule that is expressed by many cancers (96, 98, 99), including OPSCC (50). The interaction between NKG2A and HLA-E is thought to block the cytotoxic activity of CD8+ T cells and NK cells (100) and a couple of studies have shown that expression of HLA-E by tumor cells restrained the prognostic impact of tumor-infiltrating CD8+ T cells (98, 99), including that of HPV16+ OPSCC (97).

Other inhibitory receptors found to be upregulated on activated T cells in the TME of OPSCC include TIM3, LAG3 and T cell immunoreceptor with Ig and ITIM domains (TIGIT) and others (45). All expressed on higher numbers of T cells in HPV+ when compared to virus-negative head and neck tumors, but only in HPV+ tumors each of these markers was associated with prolonged survival (101).

Overall, the expression of inhibitory receptors are more indicative for an inflamed TME with ongoing antitumor immunity than for an exhausted T cell response in OPSCC. Nevertheless, the interaction between inhibitory receptors and their ligands will inhibit the activation and effector functions of T cells impairing their capacity to control OPSCC growth.

THE BLOOD COMPARTMENT FOR BIOMARKER ANALYSIS

An important question is whether the TME biomarkers associated with clinical outcome are also detectable and prognostic when analyzed on immune cells present in blood, as this compartment is easily accessible and allows for kinetic studies. The easiest approach is to determine differential leukocyte counts on blood samples, which is used in all hospitals as a normal diagnostic routine. High neutrophil counts in OPSCC, and more specifically high neutrophil-to-lymphocyte ratio (NLR) in the blood sample prior, during and after radiotherapy correlated with poor OS, recurrence free survival (RFS) and/or DSS as well as distant metastasis (102–105). Also, in HPV16+ OPSCC patients, a high NLR in the blood sample obtained prior to concurrent chemoradiation correlated with decreased OS. Neutrophils appear to have an unique

phenotype of immature granulocytes CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}, which are absent in blood of healthy donors (106).

High circulating monocyte counts prior to therapy also correlated to reduced OS and RFS in HPV16+ OPSCC patients (102, 107). While monocyte counts in HPV-negative OPSCC patients were even higher, the absolute monocyte counts were not related to clinical outcome in this patient group (102), suggesting that these monocytes might have a negative impact only in highly immunogenic tumors. Potentially, the composition of the monocyte subtypes may be of importance as some of them may constitute CD14+ MDSC, which have shown to exhibit immune suppressive effects (6, 108). Furthermore, the percentage of classical (CD14^{high}CD16-negative) monocytes were shown to be increased in OPSCC patients as well as expressed PD-L1 (107). Also a high monocyte-to-lymphocyte ratio was associated with lower OS in OPSCC (107, 109).

So far, no comparative studies have been performed with respect to neutrophils and MDSC in order to analyze if the blood compartment reflects the TME.

The relation between higher numbers of tumor-infiltrating T cells and clinical outcome prompts the question if this can also be detected in the blood. High pre-treatment numbers of CD8+ T cells in the blood correlated with improved OS in HPV16+ OPSCC patients (14). However, absolute CD4 counts as well as frequencies of CD4+ T cells measured in blood of OPSCC was not correlating to clinical outcome (14). This seems logical as the population of CD4+ T cells comprises naïve T cells, type 1 and type 2 effector T cells, memory T cells and also Tregs which dilutes the real impact of one of these subpopulations. Moreover, the peripheral blood levels of CD4+ and CD8+ T cells were not related to the type and degree of specific T cell subset infiltration in OPSCC (34). Interestingly, it was found that HPV+ OPSCC with elevated Treg frequencies in the blood displayed a better OS than patients with HPV-negative tumors or patients with HPV+ OPSCC patients displaying relatively low numbers of circulating Tregs (56).

Last but not least, HPV16-specific T cells have been detected in the circulation of patients with OPSCC using functional assays in which PBMC were stimulated with the HPV-encoded antigens (110–112). The detection of such an HPV16-specific T cell response in the peripheral blood did not always coincide with the presence of these HPV16-specific T cells in the TME, this (110). HPV16-specific T cells could also be detected in a large percentage of healthy individuals (113–115) indicating that their presence is not enough to function as a biomarker.

All together there are a couple of opportunities (neutrophils, monocytes, Tregs) that may be exploited as blood biomarkers reflecting the TME, but more sophisticated measurements of subpopulations and validation of the used techniques are warranted.

THE ROLE OF TUMOR-SPECIFIC T CELLS IN THE TME OF OPSCC

There is ample evidence that tumors highly infiltrated with CD4+ and/or CD8+ T cells (also referred to as immunologically "hot" tumors) respond better to (immuno-)therapy (27, 116). In OPSCC, HPV infection may play a very important role in this phenomenon. Superior prognosis of HPV+ OPSCC over HPV-negative OPSCC following chemoradiotherapy has been reported (13, 117). Interestingly, for the majority of HPV+ OPSCC this was related with a more dense and activated T cell infiltrate, suggesting a role for HPV-specific immunity in tumor control (14, 62, 118).

HPV16+ tumors express the virally derived oncoproteins E6 and E7, and it has been suggested that these "non-self" HPV antigens can evoke immune responses. Indeed, cellular and humoral immunity against these antigens can be detected in the peripheral blood and TME of HPV16+ OPSCC patients (110, 119–123). Notably, there is a direct link between the presence of an intratumoral HPV-specific T-cell response and the good prognosis of HPV16-driven OPSCC (6). Patients with an HPV16-driven tumor and a concomitant intratumoral HPV16-specific T cell response have a much better survival after (chemo) radiotherapy than patients with an HPV16-driven tumor without such an HPV16-specific T cell response or patients with HPV-negative OPSCC. Moreover, the presence of these HPV-specific T cells was associated with a type 1 oriented TME and a much higher level of activated CD161-expressing effector memory CD4+ T cells,CD103+ tissue-resident CD8+ T cells, and tissue-resident memory T cell-stimulating CD163+ cDC2 (6, 74).

Although a clear relation could be found between the dense, type 1 oriented HPV-specific immune infiltrate and disease outcome in HPV16-driven OPSCC, there is also evidence for improved survival of HPV-negative OPSCC patients with strongly T-cell infiltrated tumors (37, 124). Unfortunately, knowledge on the tumor-specificity is lacking in these studies. A promising and emerging field in studies of the anti-tumor response is the recognition of tumor neoantigens. Tumors with high numbers of nonsynonymous mutations have a greater likelihood of presenting mutation-derived neoepitopes and consequently mounting a T-cell response against these epitopes thereby improving the clinical response (125). Tumor mutational burden (TMB) in HNSCC is relatively high and comparable to other smoking related tumor types. Viral HNSCC display only half of the mutations rate observed in non-viral HNSCC (125, 126). CD8+ T cells responding to such mutations have been detected in a few patients with non-OPSCC either spontaneously induced (127) or following a complete response after pembrolizumab treatment (128). Notably, such neoantigen specific T cells can also be detected in low TMB tumors (129), suggesting that they may also be present in OPSCC.

IMMUNOTHERAPY IN OPSCC

During the last two decades, several strategies aiming to boost the immune response to cancer have been developed and tested in patients with cancer, ranging from immune modulators to checkpoint therapy, adoptive cell transfer, and vaccines. **Table 1** summarizes active/recruiting immunotherapy trials in patients with OPSCC and HNSCC, including OPSCC.

Immune Modulators

One of the earliest immune stimulators tested is recombinant IL-2 (rIL-2), used to directly stimulate the activity of tumor-specific T cells. In a phase III trial with 200 patients rIL-2 was injected close to the ipsilateral lymph node, each day for 10 days before surgery and radiotherapy and then each month at the contralateral LN site for 1 year. The treatment had no significant side effects but improved the DFS (from 51 to 64%) and OS (from 55 to 73%) at 5 years (130). At a later stage, IRX-2 was developed to stimulate T cell activity. IRX-2 is a mix of purified cytokines obtained after stimulating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA). In a phase IIa trial treatment naïve patients with HNSCC received subcutaneous IRX-2 injections near the TDLN for 10 days prior to surgical resection, which with the knowledge of today is an interesting choice as our studies showed that the great majority of TDLN comprise tumor-specific T cells even when they are hardly detectable in the tumor itself (24, 131). Injection of IRX-2 was associated with small radiological reductions in tumor size, including patients with OPSCC (132). In a first analysis, pre-and-post IRX-2 samples were analyzed and showed marked increases in CD3+ T cells and CD68+ macrophages (133). In a subgroup of 7 patients pre-and post-samples could be compared, showing potential increases in CD4+ T cells (6/7) and CD68 macrophages (4/7) as well as decreases in CD8+ T cells (5/7) patients after IRX-2 treatment. Unfortunately, HPV status was not determined but one could envision that most of the responders would have a HPV-positive tumor. Nanostring analyses using the PanCancer IO360 immune profiling panel confirmed the increases in CD4+ T cells and most markedly in DC as well as in the immune modulatory cytokines IL-6 and IL-10 (134). Another compound that indirectly could stimulate the tumor-specific T cell response is the toll-like receptor 8 (TLR8) agonist motolomid, which is known to activate monocytes, DCs and NK cells (135). Treatment of recurrent or metastatic HNSCC patients after the first 6 cycles of chemotherapy with weekly infusions of cetuximab with or without motolomid did not result in improved OS in a trial with n=195 patients. However, in a prespecified subgroup of n=83 OPSCC patients the motolomid arm displayed longer PFS and OS when the tumor was induced by HPV (136), suggesting that motolomid may have boosted the existing but probably weak HPV-specific T cell response in these patients.

Other compounds target immune suppressive cells that are active in the TME. For instance, metformin, which is widely used as a drug to manage diabetes type 2, has been described to have anti-cancer effects (137). In mice, metformin increased the number of tumor infiltrating CD8+ T cells, and the combination of metformin and a tumor vaccine improved the multifunctionality of the vaccine-induced CD8+ tumor infiltrating lymphocytes (TIL) (138). Another potential mechanism is the effect of metformin on Tregs, as it also inhibits mammalian target of rapamycin (mTOR). Metformin treatment for a minimum of 9 days before surgery of HNSCC, more than half being OPSCC, resulted in a strong decrease of intratumoral Foxp3+ Tregs and increase of stromal CD8+ T cells between pre-and post-treatment samples (n=16 HPV+ and n=20 HPV-negative), independent of HPV status (139). Potentially, metformin treatment may be interesting for HPV+ OPSCC patients with ongoing tumor immunity. As high numbers of Tregs have infiltrated those tumors (67), the use of metformin may tip the balance in favor of spontaneous anti-tumor responses in these patients, resulting in tumor shrinkage. Similarly, it may provide benefit in combination with other types of immunotherapy. Several new immune modulators are currently tested in the clinic (**Table 1**).

Checkpoint Blockade Therapy

The use of antibodies that block the interaction between PD-1 and PD-L1 are successfully used in several types of immunogenic cancers with a high mutation rate (e.g. melanoma, lung cancer) or induced by the Merkel cell virus (140–142). Several studies have been reported on the efficacy of such antibodies for the treatment of HNSCC, including patients with OPSCC (143–147).

In CheckMate 141, a phase III trial, platinum-resistant patients with recurrent or metastatic tumors were treated with the anti-PD-1 antibody nivolumab. The response rate was 13.3% in the nivolumab group versus 5.8% in patients treated with standard of care. The objective response rate (ORR) to nivolumab was 15.9% in HPV+ patients while it was 8.0% in HPV-negative patients (143). The 2-year OS in this study was 16.9% in patients with PD-L1 expression, regardless of the HPV status (148). Nivolumab also induced clinical benefit in patients lacking PD-L1 expression, albeit in a lower proportion of patients (148). Interestingly, continued treatment of patients with nivolumab with slow progressing disease still resulted in clinical benefit, irrespective of the HPV status of the tumor. This was associated with a decrease in the percentage of PD-1+ Tregs in PBMC (149), suggesting that an ongoing immune response was present in these patients but which was simply too weak to be effective. Importantly, nivolumab treatment improved OS irrespective of prior treatment with cetuximab, an antibody known for its immune modulating side effects (150–153), albeit that the reduction in risk of death was lower in pre-treated patients (154). A similar response rate with PD-1 blockade using pembrolizumab was found in the KEYNOTE-012 phase Ib study of 60 patients with recurrent or metastatic tumors with PD-L1 expression. In total 18% of the patients displayed a clinical response, divided as 25% (4/16) in the HPV+ HNSCC patient group and 14% (4/29) in HPV-negative group (144).

The PD-L1 antibody durvalumab was tested in the expansion phase of a phase I/II study for treatment of patients with recurrent or metastatic tumors, 40% of which were HPV+. The ORR was 6.5% (4/62) in the total group with 15% in the PD-L1 expressing subgroup and 2.6% in the group with low PD-L1 expression. None of the HPV+ patients showed a response (145).

Another important checkpoint molecule is NKG2A. Preclinical experiments revealed that anti-NKG2A therapy promoted tumor immunity and synergized with PD-1 blockade. In addition, it was shown that NKG2A blockade

TABLE 1 | Immunotherapy in patients with OPSCC.

NCT number	Phase	Number of arms	Randomized	Treatment	Target	Setting	Previous therapy	Number of patients	Endpoint	Trial Status
OPSCC	Immune modulators									
2002182	2	2	No (open label)	ADXS11-0001 vs control (SoC)	ADXS11-0001: HPV16 E7	Neoadjuvant (pre-surgery)	None	30 HPV+	Safety + IR	Active (Not recruiting)
4106362	2	2	Yes (open label)	RT-CT + Cetuxi vs RT-CT	Cetuxi: anti EGFR	First line	None	70 HPV+ KRA variant+ stage III–IV	Efficacy + toxicity	Recruiting
4508829	2	1	No (open label)	IMRT + CT + anti EGFR moAb	Anti EGFR	Concurrent	SoC (no RT)	52 (advanced stage)	Efficacy	Recruiting
OPSCC	Checkpoints (+ combinations)									
3144778	1	2	Yes (open label)	Durva vs Durva + Treme	Durva: anti PD-L1 Treme: anti CTLA-4	Durva + Treme prior to surgery	None	28 stage II–IVA	Safety + IR	Active (not recruiting)
3618134	1/2	1	No (open label)	RT + Durva vs RT + Durva + Treme	Durva: anti PD-L1 Treme: anti CTLA-4	Prior to surgery	None	82 HPV+ stage I–III	Safety + efficacy	Recruiting
3715946	2	1	No (open label)	RT (reduced) + Nivo	Nivo: anti PD-1	Adjuvant	Surgery	135 (primary tumor)	Efficacy	Recruiting
3799445	2	1	No (open label)	Ipi + Nivo + RT	Ipi: anti CTLA-4 Nivo: anti PD-1	First line	None	180 HPV+ stage I–IVA	Safety + efficacy	Recruiting
3838263	2	2	Yes (2:1; open label)	Nivo vs CRT (control)	Nivo: anti PD-1	Nivo before RT-CT	None	61 HPV+	Safety + efficacy	Recruiting
3811015	2/3	3 (incl. cross over)	Yes (open label)	IMRT + CT + Nivo vs IMRT + CT (control)	Nivo: anti PD-1	Nivo post RT-CT	None	744 HPV+	Efficacy + Prognostic biomarker	Recruiting
3952585	2/3	3	Yes (open label)	IMRT + CT vs IMRT (reduced) + CT vs Nivo + IMRT (reduced) + CT	Nivo: anti PD-1	Nivo prior to IMRT (reduced) + CT	None	711 p16+	Efficacy	Recruiting
OPSCC	ACT									
4015336	2	1	No (open label)	E7 TCR T cells	E7 TCR T cells: HPV16 E7	HLA-A0201	None	180 HPV16+ stage II–III	Safety	Recruiting
OPSCC	Vaccines (+ combinations)									
3258008	2	1	No (open label)	Utomi + ISA101b	Utomi: agonistic CD137 ISA101b: HPV16 E6E7	Adjuvant	SoC	27 HPV+	Efficacy + toxicity	Active (not recruiting)
3669718	2	2	Yes (Double blinded)	ISA101b + Cemip vs Cemip	ISA101b: HPV16 E6E7 Cemip: anti PD-1	Adjuvant	SoC	194 HPV16+ R/M	Efficacy + toxicity	Recruiting
4001413	2	2	Yes (open label)	Durva vs Durva + MEDI0457	Durva: anti PD-L1 MEDI0457: HPV16/18 E6E7	Adjuvant	SoC	66 HPV+	Efficacy + toxicity	Recruiting
HNSCC	Immune modulators									
3088059	2	Multiple	No (open label)	Afatinib, Palbociclib, Niraparib, Rogaratinib (BAY1163877)	Afatinib: kinase inhibitor Palbociclib: kinase inhibitor Niraparib: PARP inhibitor Rogaratinib: FGFR tyrosine kinase inhibitor	Adjuvant	CT	340 R/M	Efficacy + Biomarker	Recruiting
3795610	2	1	No (open label)	IPI-549	IPI-549: PI3Ky inhibitor	Neoadjuvant (pre-surgery)	None	15 advanced	PI3Ky changes + IR + toxicity	Recruiting
HNSCC	Checkpoints (+ combinations): Ipilimumab									
1935921	1	1	No (open label)	Ipi + cetuxi +RT	Ipi: anti CTLA-4 Cetuxi: anti EGFR	Concurrent	None	19 stage III–IV	Dose finding + efficacy + biomarkers	Active (not recruiting)

(Continued)

TABLE 1 | Continued

NCT number	Phase	Number of arms	Randomized	Treatment	Target	Setting	Previous therapy	Number of patients	Endpoint	Trial Status
HNSCC	Checkpoints (+ combinations): Nivolumab									
2741570	3	2	Yes (open label)	Nivo + ipi vs SoC	Nivo: anti PD-1 Ipi: anti CTLA-4	First line	None	947 R/M	Efficacy	Active (not recruiting)
2764593	1	4	No (open label)	Nivo + CT + IMRT, Nivo + high CT + IMRT, Nivo + Cetuxi + RT, Nivo + IMRT	Nivo: anti PD-1 Cetuxi: anti EGFR	Neoadjuvant (pre-RT)	None	40 stage I–IV	Toxicity	Active (not recruiting)
2823574	2	2	Yes (double blinded)	Nivo + ipi vs nivo (control)	Nivo: anti PD-1 Ipi: anti CTLA-4	Adjuvant	None	675 R/M	Efficacy	Active (not recruiting)
2834013	2		No (open label)	Nivo + ipi vs nivo (control)	Nivo: anti PD-1 Ipi: anti CTLA-4	Adjuvant	SoC	818 PD (rare tumors)	Efficacy + toxicity	Recruiting
3247712	1/2	4	No (open label)	Nivo + RT	Nivo: anti PD-1	Neoadjuvant (pre-surgery)	None	28 (eligible for surgery)	Safety + feasibility + efficacy	Recruiting
3317327	1/2	1	No (open label)	Nivo	Nivo: anti PD-1	Neoadjuvant (pre-RT)	SoC	20 R	Safety + efficacy	Recruiting
3341936	2	1	No (open label)	Nivo + Liri	Nivo: anti PD-1 Liri: anti KIR2DL1/2L3	Neoadjuvant (pre-salvage surgery)	SoC	58 R	Safety + efficacy	Recruiting
3406247	2	2	No (open label)	Nivo alone vs nivo + ipi	Nivo: anti PD-1 Ipi: anti CTLA-4	Adjuvant	Salvage surgery after RT	140 P/R	Safety + efficacy	Not yet recruiting
3620123	2	2	Yes (open label)	Nivo + ipi vs docetaxel	Nivo: anti PD-1 Ipi: anti CTLA-4	Palliative	SoC	280 R/M	Efficacy	Recruiting
3854032	2	2	Yes (open label)	Nivo + BMS-986205 vs Nivo	Nivo: anti PD-1 BMS-986205: IDO1 inhibitor	Neoadjuvant (pre-surgery)	None	48 stage II–IV (non R)	Efficacy + IR + toxicity	Recruiting
4080804	2	3	Yes (open label)	Nivo + ipi vs nivo + Relatlimab vs nivo	Nivo: anti PD-1 Ipi: anti CTLA4 Relatlimab: anti LAG3	Neoadjuvant (pre-surgery)	None	60 advanced	Safety + IR + efficacy	Recruiting
HNSCC	Checkpoints (+ combinations): Pembrolizumab									
2296684	2	1	No (open label)	Pembro	Pembro: anti PD-1	Neoadjuvant (pre-surgery)	None	66 stage III–IV	Efficacy + toxicity	Recruiting
2586207	1	1	No (open label)	Pembro + CRT	Pembro: anti PD-1	Concomitant	SoC	57 stage III–IV	Safety + QoL	Active (not recruiting)
2707588	2	2	Yes (open label)	Pembro + RT vs Cetuxi + RT	Pembro: anti PD-1 Cetuxi: anti EGFR p16/HPV	Concomitant	SoC	133 advanced	Efficacy + toxicity + QoL +impact	Active (not recruiting)
2718820	1/2	1	No (open label)	Pembro + docetaxel	Pembro: anti PD-1	Adjuvant	SoC	22 R/M	Efficacy + QoL + toxicity	Active (not recruiting)
2769520	2	1	No (open label)	Pembro	Pembro: anti PD-1	Adjuvant	Surgery	45 R	Efficacy + toxicity	Recruiting
2775812	1	1	No (open label)	Pembro + CT + IMRT	Pembro: anti PD-1 CT: cisplatin	Concomitant	SoC	37 stage III–IV (high risk)	RP2D + efficacy + toxicity + biomarkers	Active (not recruiting)
2777385	2	2	Yes (open label)	Pembro + CT + IMRT	Pembro: anti PD-1 CT: cisplatin	Adjuvant	SoC	90 (non M)	Efficacy + toxicity	Recruiting
2819752	1	2	No (open label)	Pembro + CRT	Pembro: anti PD-1	Concomitant	SoC	36 stage IV (HPV+ vs HPV-)	MTD + toxicity + efficacy	Active (not recruiting)
2841748	2	2	Yes (double-blinded)	Pembro vs placebo	Pembro: anti PD-1	Adjuvant	SoC	100 stage III–IV (high risk)	Efficacy	Recruiting
3082534	2	4	No (open label)	Pembro + Cetuxi	Pembro: anti PD-1 Cetuxi: anti EGFR	Concurrent	SoC	83 R/M	Efficacy	Recruiting

(Continued)

TABLE 1 | Continued

NCT number	Phase	Number of arms	Randomized	Treatment	Target	Setting	Previous therapy	Number of patients	Endpoint	Trial Status
3383094	2	2	Yes (open label)	Pembro + IMRT vs IMRT + CT (control)	Pembro: anti PD-1	Concurrent + adjuvant	None	114 p16+ stage III–IV	Efficacy + toxicity	Recruiting
3546582	2	2	Yes (open label)	Pembro + RT vs RT	Pembro: anti PD-1	Adjuvant	RT	102 R or second primary	Safety + efficacy	Recruiting
3695510	2	1	No (open label)	Afatinib + Pembro	Afatinib: kinase inhibitor Pembro: anti PD-1	Adjuvant	SoC	29 R/M	Efficacy + toxicity	Recruiting
HNSCC	Checkpoints (+ combinations): Pembro									
4193293	1/2	1	No (open label)	Duvelisib + pembro	Duvelisib: PI3K inhibitor Pembro: anti PD-1	Adjuvant	SoC	30 R/M	DLT + efficacy + safety	Recruiting
HNSCC	Checkpoints (+ combinations): Durvalumab									
2551159	3	3	Yes (open label)	Durva vs durva + treme vs SoC	Durva: anti PD-L1 Treme: anti CTLA-4	First line	None	823 R/M	Efficacy + PK + IR + QoL	Active (not recruiting)
2827838	2	1	No (open label)	Durva	Durva: anti PD-L1	Neoadjuvant (pre-surgery)	None	20 stage I–IV	IR vs HPV status	Recruiting
2997332	1	1	No (open label)	Durva + CT	Durva: anti PD-L1	Adjuvant	SoC	36 advanced	Safety + RP2D	Recruiting
3051906	1/2	1	No (open label)	Durva + cetuxi + RT	Durva: anti PD-L1 Cetuxi: anti EGFR	Adjuvant	SoC	69 advanced	Efficacy + toxicity	Not yet recruiting
3088059	2	Multiple	No (open label)	SoC, IPH2201 or durva	IPH2201: anti NKG2A Durva: anti PD-L1	Adjuvant	CT	340 R/M	Efficacy + Biomarker	Recruiting
3258554	2/3	2	Yes (open label)	RT + durva vs RT + cetuxi	Durva: anti PD-L1 Cetuxi: anti EGFR	Adjuvant	SoC	523 stage III–IV	DLT + efficacy +QoL	Recruiting
3426657	2	1	No (open label)	Durva + treme + + CT + RT	Durva: anti PD-L1 Treme: anti CTLA-4	Adjuvant	CT	120 advanced	Feasibility + IR + toxicity + efficacy	Recruiting
3509012	1	8	No (open label)	Durva + treme + CRT	Durva: anti PD-L1 Treme: anti CTLA-4	Adjuvant	SoC	360 advanced	Toxicity + efficacy	Active (not recruiting)
3529422	2	1	No (open label)	Durva + Treme + RT	Durva: anti PD-L1 Treme: anti CTLA-4	Adjuvant	Surgery	24 stage III–IV (non M)	Safety + efficacy	Recruiting
HNSCC	Checkpoints (+ combinations): others									
2999087	3	4	Yes (open label)	Avelu + cetuxi + RT vs cetuxi + RT vs CRT	Avelu: anti PD-L1 Cetuxi: anti EGFR		None	688 advanced	Efficacy	Recruiting
4129320	2/3	4	Yes (open label)	Enoblitu + MGA012 vs Enoblitu + MGA012 + CT vs MGA012 + CT vs Pembro + CT (control)	Enoblituzumab: anti B7H3 (MGA271) MGA012: anti PD-1 Pembro: anti PD-1		None	750 R/M	Efficacy + toxicity + IR + QoL	Not yet recruiting
2274155	1	3	No (open label)	MEDI6469	MEDI6469: OX40 agonist	Neoadjuvant (pre-surgery)	None	17 advanced	Safety + feasibility + IR + efficacy	Active (not recruiting)
HNSCC	ACT									
3083873	2	5	No (open label)	LN-145 non-cryopreserved vs cryopreserved	autologous TIL	Adjuvant	SoC	55 R/M	Efficacy + safety	Recruiting
3578406	1	2	Yes (open label)	TCR-T with or without anti PD-1 secreting element	HPV16 E6-specific T cells	Adjuvant	SoC	20 HPV16 + R/M	MTD	Recruiting
HNSCC	Vaccine (+ combinations)									
1998542	2	1	No (open label)	AlloVax + CRCL + AlloStim	AlloVax CRCL: chaperone rich cell lysate AlloStim: adjuvant	Adjuvant	SoC	12 R/M	Safety + efficacy	Completed

(Continued)

TABLE 1 | Continued

NCT number	Phase	Number of arms	Randomized	Treatment	Target	Setting	Previous therapy	Number of patients	Endpoint	Trial Status
2865135	1b/2	1	No (open label)	DPX-E7	DPX-E7:HPV16 $E7_{1-19}$	Adjuvant	SoC	44 HPV+ HLA-A*02 +	Safety + efficacy	Active (not recruiting)
3162224	1b/2a	1	No (open label)	MEDI0457 + Durva	MEDI0457: HPV16/18 E6E7 Durva: anti PD-L1	Adjuvant	SoC	35 HPV+ R/M	Safety + efficacy	Active (not recruiting)
3260023	1b/2	1	No (open label)	TG4001 + Avelu	TG4001: HPV16 E6E7 Avelu: anti PD-L1	Adjuvant	SoC	52 HPV+ R/M	Safety + DLT (+ efficacy in phase II)	Recruiting
3633110	1/2	2	No (open label)	GEN-009 vs GEN-009 + anti PD-1	GEN-009: neoepitope SLP vaccine Anti PD-1: nivo or pembro	Adjuvant	SoC	99 NED	Safety + IR + efficacy	Active (not recruiting)
3946358	2	1	No (open label)	UCPVax + Atezo	UCP: telomerase derived	Adjuvant	SoC	47 HPV+ advanced/M	Efficacy + QoL	Recruiting
4180215	1/2	Multiple	No (open label)	HB-201 (IV or IT) vs HB-201 + CI	HB-201: HPV16 E7E6	Adjuvant	SoC	100 HPV+	Dose finding + toxicity	Recruiting
4287868	1/2	2	No (open label)	PDS0101 + M7824 + NHS-IL12	PDS0101: HPV M7824: anti PD-L1/TGFβ NHS-IL12: IL12	Adjuvant	SoC	40 HPV+ advanced/M	Safety + efficacy	Recruiting
4432597	1/2	4	No (open label)	PRGN-2009 vs PRGN-2009 + M7824	PRGN-2009: HPV M7824: anti PD-L1/TGFβ	Neoadjuvant or induction	SoC	70 HPV+ R/M	Safety + efficacy	Recruiting

Clinical trials studying immunotherapy in patients with OPSCC and HNSCC (including OPSCC). Afatinib, protein kinase inhibitor, highly selective irreversible ErbB family blocker (including EGFR); AlloStim, living cell product, consisting of activated allogeneic type 1 $CD4^+$ memory T cells containing cytolytic activity and have the following phenotype $CD45RO^{hi}$, $CD62L^{lo}$, $CD40L^{hi}$, $CD25^+$, IFN-gamma$^+$, IL-4$^-$, granzyme$^+$, and perforin$^+$, and is used as adjuvant; Avelu, avelumab (anti PD-L1 antibody); Cemip, Cemiplimab REGN2810 (anti PD-1 antibody); Cetuxi, cetuximab (anti EGFR antibody); CRCL, chaperone rich cell lysate as source of tumor antigens prepared from autologous tumor; CRT, chemoradiation therapy; CT, chemotherapy; CTLA-4, cytotoxic T lymphocyte antigen 4; DLT, dose limiting toxicity; Durva, Durvalumab (anti PD-L1 antibody); EGFR, epidermal growth factor receptor; Enoblitu, Enoblituzumab (anti B7H3/CD276 antibody); FGFR, fibroblast growth factor receptor; HB-201, arenavirus vector-based vaccine expressing inactivated fusion protein HPV16 E7E6; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma (including OPSCC); HPV, human papillomavirus; IDO-1, indoleamine 2,3-dioxygenase-1; IMRT, intensity modulated radiotherapy; IPH2201, Monalizumab (anti NKG2A antibody); Ipi, ipilimumab (anti CTLA-4); IR, immune response; KIR, killer cell immunoglobulin-like receptor; LAG-3, lymphocyte activation gene 3; Liri, Lirilumab (anti KIR2DL1/2L3 antibody); LN-145, Lifileucel, autologous TIL adoptively transferred with addition of IL-2 in patients receiving a nonmyeloablative lymphocyte depletion; M, metastatic cancer patients; M7824, bintrafusp alfa, fusion protein, bispecific antibody directed to TGF-beta and PD-L1; MEDI0457, INO-3112 HPV DNA vaccine; MoAb, monoclonal antibody; MTD, maximum tolerated dose; NED, no evidence of disease anymore after treatment; Nivo, Nivolumab (anti PD-1 antibody); NKG2A, natural killer group 2A inhibitory receptor, ligand of HLA-E; OPSCC, oropharyngeal squamous cell carcinoma; P, primary tumor; PARP, poly ADP ribose polymerase enzyme; PD, progressive disease; PD-1, programmed cell death protein 1; Pembro, Pembrolizumab (anti PD-1 antibody); PD-L1, programmed cell death ligand 1; PI3K, Phosphatidylinositol-3-kinase; PK, pharmacokinetics; R, recurrent cancer patients; RT, radiotherapy; QoL, quality of life; RP2D, recommended phase 2 dose; R/M, recurrent/metastasis patients; RT-CT, radiotherapy-chemotherapy (standard therapy); SLP, synthetic long peptide; SoC, standard of care therapy; TCR, T cell receptor; TCR-T, engineered T cells bearing HPV E6 specific TCR and armed with PD-1 antagonist that will be secreted; TG-4001, Vaccinia vector (MVA)-HPV16E6/E7-IL2 vaccine; TIL, tumor infiltrating lymphocytes; Treme, Tremelimumab (anti CTLA-4); UCPVax, Universal cancer peptides (UCP2 and UCP4) derived from telomerase, a CD4 T helper type 1 inducer cancer vaccine; Utomi, utomilumab (agonistic anti CD137 antibody).

improved the efficacy of tumor vaccines and adoptive cell therapy (ACT) (96, 97). The humanized NKG2A antibody monalizumab was tested together with cetuximab in previously treated recurrent or metastatic tumors, including 32% OPSCC. A preliminary report on the outcome of the trial revealed a confirmed clinical partial response (PR) in 8 out of 26 patients (31%) and stable disease (SD) in 14 out of 26 patients, which was regarded as promising when compared to historical cetuximab data (96).

In comparison to the high ORR in virus-driven Merkel cell carcinoma after checkpoint therapy (56% (142)), the response in HPV+ patients to checkpoint therapy is disappointingly low. However, in view of the fact that patients with both an HPV+ tumor and a strong immune response to HPV display an excellent response to standard of care therapy (6, 24), this result was to be expected. The majority of HPV+ patients with recurrent or metastatic tumors most likely are those in whom there was no or a very weak intratumoral T-cell response to HPV during first line treatment and it is known that checkpoint blockade works best in patients with a pre-existing tumor-specific immune response (155). Hence, in OPSCC these therapies should be combined with modalities that enhance the tumor-reactive T cell response in patients.

Adoptive Cell Therapy

The infusion of large numbers of tumor-reactive T cells, being either ex-vivo expanded TILs, T-cell receptor (TCR) transduced T cells or chimeric antigen receptor (CAR)-T cells is one way to enhance the number of tumor-reactive T cells and has proven to be clinically effective in patients with advanced cancer (156–158). The presence of two strong tumor-specific antigens E6 and E7 in

HPV-driven cancers provides the opportunity to specifically stimulate and expand tumor-specific T cells for ACT. Stimulation of peripheral blood lymphocytes with overlapping peptide pools resulted in the expansion of HPV16-specific T cells in 33 of 52 HPV16+ OPSCC patients. Most of the cell cultures comprised HPV16-specific CD4+ T cells and infrequently CD8+ T cells with the capacity to kill target tumor cells (122). A highly reproducible method to stimulate these cells under good manufacturing practice (GMP)-conditions resulted in the expansion of IFNγ-producing HPV-specific CD4+ T cells (11 of 11 cases) and CD8+ T cells (3 of 11) cases (131). In a phase II ACT trial, one out of 5 OPSCC patients resolved most of the metastatic lesions (complete response, CR) except for one brain metastasis, which was resected. Responding patients received higher numbers of IFNγ-producing HPV-specific TILs and the frequency of HPV-specific T cells in the blood one month after ACT correlated with clinical outcome. The question is how HPV-specific ACT will be used as most patients of whom their primary tumors display HPV-specific TIL reactivity respond well to chemoradiation while the tumors of most patients with recurrent disease are expected to contain no or few HPV-specific TILs (6). If present in the tumor, ex-vivo expansion and reinfusion of HPV-specific TIL may help to prevent or to reduce chemoradiation as primary treatment.

The absence of HPV-specific T cells mandates alternative approaches such as the infusion of ex-vivo TCR-transduced autologous T cells. Draper *et al.* reported the isolation of an HLA-A*0201 restricted HPV16 $E6_{29-38}$-specific TCR from a patient with HPV16-induced anal cancer. The use of this TCR is expected to contribute to tumor immunity as the tumor site was highly enriched for this TCR clonotype when compared to blood and T cells transduced with this TCR were shown to recognize several HPV16+ cell lines of different origins (159). Another study reported the isolation of an HLA-DRB1*04 restricted TCR reactive to the HPV16 $E7_{70-89}$ epitope. TCR-transduced cells were shown to specifically produce IFNγ when stimulated with HPV16 E6 and E7 containing tumor lysate (160). The isolation of TCR responsive to HPV epitopes restricted by a series of HLA class I and II molecules will lead to a warehouse of HPV-specific TCR required for personalized treatment of patients with recurrent or metastatic HPV-induced OPSCC. Currently, the use of T cells transgenic for an E7-specific TCR is tested in the clinic (**Table 1**). The fact that most mutations (neoantigens) are patient specific makes a similar approach for HPV-negative OPSCC complicated but not impossible (161).

Vaccines

Therapeutic vaccination is another option to increase the number of tumor-reactive T cells in OPSCC, preferentially against tumor-specific epitopes created by DNA mutations or oncogenic viruses. Based on a whole series of different trials it was concluded that the induction of tumor reactivity correlated with clinical outcome after vaccination in patients with pre-cancers or in settings of low tumor burden, and with tumor regressions if broad type 1 T cell reactivity was established (162). The treatment of most established cancers will require the combination of therapeutic vaccination with other modalities to overcome immune suppression and escape (163).

Two types of vaccines, one aiming to induce responses to MAGE-A3 and HPV and the other aiming to induce T-cell reactivity against p53 (for HPV-negative tumors) were tested in groups of HNSCC patients, including 30–40% OPSCC patients. These vaccines displayed a weak immunogenicity and did not alter clinical outcome (164, 165).

However, in a single arm phase II trial, designed based on the observation that HPV16+ OPSCC patients lacking tumor infiltration with HPV16-specific T cells are most likely the ones that are diagnosed with incurable HPV16+ cancer (6, 24) and that PD-1 checkpoint therapy has most impact in patients with pre-existing tumor-specific T cell responses (155), HPV16+ OPSCC patients received a combined treatment with ISA101 and nivolumab (146). ISA101 is an HPV16 -E6 and -E7-specific synthetic long peptide vaccine, which previously was shown to induce CR and PR in patients with HPV16-induced high-grade but premalignant disease (166, 167). The ORR of 33% and median OS of 17.5 months was regarded promising (146) when compared to PD-1 inhibition only in a similar patient group (143) or when compared to the study with durvalumab in which none of the HPV+ patients responded (145). A randomized trial comparing the efficacy of PD-1 blockade alone versus vaccination + PD-1 blockade is underway (**Table 1**). The outcome of the combination ISA101 and nivolumab trial argue for tumor-specific vaccination strategies. Approaches to stimulate the neoantigen repertoire are also warranted for the treatment of in particular HPV-negative OPSCC, similar to what has been done in other types of cancers (168–170).

Another option to stimulate tumor-reactive T cells might be through irradiation, as this has been shown to result in the release of antigens from tumor cells as well as in the induction of proinflammatory signals that trigger the innate immune system (171). Upon radiation, the tumor may serve as an *in situ* vaccine with the capacity to activate tumor-specific T cells. Spurred by the results with checkpoint blockade after radiation therapy in lung cancer (172), new trials are initiated in patients with HNSCC, including OPSCC (**Table 1**), using radiotherapy and checkpoint inhibition together or in combination with chemotherapy (173–175). The feasibility of using PD-L1 antibody avelumab with conventional cetuximab-radiotherapy as an alternative for advanced stage OPSCC patients unfit for cisplatin treatment was tested in a phase I study with 8 patients with OPSCC, 4 of which were HPV+. Immune toxicity was transient and manageable, and objective responses were observed in 4 of 6 evaluable OPSCC patients (3 complete responses and one partial response), including 3 HPV+ patients (176).

PRECLINICAL DEVELOPMENTS

There are several mouse models for HPV+ HNSCC. Study of cell surface proteins expressed by MTEC tumors, made from mouse tonsillar epithelial cells that are transformed by HPV16 E6, E7, and hRAS, showed the expression of CD47 (177). By binding to signal-regulatory protein alpha (SIRP-alpha) expressed on antigen processing cells, this transmembrane molecule blocks the phagocytosis and clearance of cells expressing CD47, resulting in

the suppression of innate and adaptive anti-tumor responses. Targeting of CD47 using antibodies in rituximab-refractory non-Hodgkin's lymphoma re-sensitized about 50% of these patients (178). The CD47-SIRP-alpha axis can also be targeted pharmacologically (179). The efficacy of radiation, which is a key component of the standard of care treatment for OPSCC, was shown to depend on intact T cell responses and was improved after CD47 knock-down in MTEC tumors (177), suggesting that CD47 may also play a key role in regulating immunity and as such the efficacy of standard of care treatment in OPSCC. Another study with mEER tumor cells, derived from the metastases of an HPV+ oropharyngeal murine cancer, injected into the flank of mice showed that the response to standard cisplatin-radiation therapy could be improved by adding cyclophosphamide and an inducible nitric oxide synthase (iNOS) inhibitor. Standard therapy did not alter the tumor microenvironment, which remained cold as indicated by high numbers of immune suppressive immune cells (MDSC, Tregs) and low numbers of T cells, M1 macrophages, and DCs (180). The addition of cyclophosphamide to the standard treatment converted treated tumors to hot but the combination with the iNOS inhibitor resulted in a strong influx of CD8+ and CD4+ Th1 cells as well as lead to durable control of established tumors (180). The use of cisplatin chemotherapy has been shown to increase tumor immune infiltration (181) and tumor cell killing by tumor necrosis factor alpha (TNFα)-producing T cells (182). The use of a low dose cisplatin was also shown to augment the effects of an adenovirus-based oncolytic virus therapy of subcutaneously injected mEER tumors. The effects of the oncolytic virus were clearly CD8+ T cell dependent and cisplatin was shown to augment the infiltration of the mEER tumors with HPV16 E7-specific CD8+ T cells (183). Interestingly, the anatomical location of the mEER tumors had a profound impact on the composition of the tumor infiltrating immune cells (184), quite reminiscent of what was observed in patients with HPV16-induced OPSCC and cervical cancer (24). Orthotopic injection of mEER tumor cells resulted in a much more inflamed tumor immune microenvironment, reflected by higher numbers of tumor-infiltrating CD8+ T cells and a stronger type 1 and 2 gene signature, than when these tumor cells were injected in the flank. Moreover, whereas the orthotopic growing mEER tumors were directly responsive to treatment with anti-PD-1 (> 50% survival) and even better to the combination of anti-PD-1 and anti-CTLA-4 (> 90% survival), flank injected tumors did not show any response to PD-1 blockade alone whereas only 40% responded to the combination treatment. Intratumoral injections of STING agonist, to increase IFN signaling, resulted in a strong decrease of Tregs and MDSC and an almost complete eradication of flank tumors (184). The decrease of immune suppressive immune cells in flank-injected mEER tumors seems essential for a better outcome after both standard chemoradiation (180) and immunotherapy (184).

PERSPECTIVES

The use of 15 parameter flow cytometry, the development of over 30 marker panels by mass cytometry and the introduction of unbiased bioinformatical approaches to cluster cells based on the expression of all proteins assessed have led to a much better definition and quantification of the immune cell phenotypes that are present in OPSCC, solved debates on the prognostic role of certain subtypes and led to the identification of subtypes strongly associated with clinical outcome. The use of single cell RNA sequencing to study the transcriptome of intratumoral cells has provided new means to identify subtypes of immune cells and understand their functional properties as well as defined gene expression signatures with clinical outcome. In a first study of 26 patients with all types of HNSCC, including 7 OPSCC, the transcriptional signatures in helper CD4+ T cells and B cells were quite divergent between HPV-negative and HPV+ tumors, whereas that of CD8+ T cells and Tregs was quite similar (185). These results may not necessarily reflect a difference caused by HPV but can be caused by the different types of tumor and their location (6, 24, 184). In a similar, but purely OPSCC and T cell focused transcriptome study, highly active tumor resident and tumor-reactive populations of CD4+ and CD8+ T cells that displayed actionable checkpoints were found in OPSCC (45). Whilst these techniques give an unprecedented insight in the complexity of the immune infiltrate of tumors, they lack the spatial information on each cell. The fact that a fairly simple classification based on the distribution of T cells has a major impact on prognosis and response to immunotherapy stresses the importance of such analyses (27). Multispectral imaging using the Vectra not only allows for a more in-depth analyses of different types of immune cells within the same tumor section but also to study their interaction. The use of mass cytometry based imaging allows for many more markers to be studied in a spatial context (186) and one can foresee that this will bring a much deeper understanding on the types of cells in the TME as well as their interaction and how this affect clinical outcome.

The much better outcome of HPV+ OPSCC compared to their HPV-negative counterparts after standard of care therapy prompted discussions on de-intensified treatments, specifically with respect to the dose of cisplatin chemotherapy (15), but this led to inferior survival (17). It should be realized that the reason why patients with HPV+ tumors do better on this standard therapy is because of their extensive immune infiltration and this is enhanced by cisplatin (67, 180). Thus, rather than downscaling the use of chemotherapy one should consider to make optimal use of it. For instance, by taking advantage of the fact that chemotherapy may remove some of the immunosuppressive mechanisms that are at play in HPV-induced cancers (187, 188). Rational combination of the immunomodulatory properties of chemotherapy and radiation that turns up the heat in tumors are highly warranted. Based on a recent publication on therapeutic vaccination (189), it is unlikely that checkpoint therapy or ACT approaches will manage to do this. Potentially, oncolytic virus therapy may aid in this (NCT02626000).

Finally, checkpoint therapy has only limited effect in patients with head and neck cancer. One of the problems may be that these patients failed to or mounted only a weak response to tumor antigens. Whereas this can be achieved with therapeutic vaccines in patients with HPV+ tumors, this is more difficult when the tumor antigens are unknown, as is the case in HPV-negative OPSCC. To increase the number of tumor-reactive T

cells, approaches are undertaken to either transfer TCR transgenic T cells into patients or by isolation and expansion of T cells which are most likely to be enriched for tumor-reactive T cells using the expression of the activation markers CD137, PD-1, or CD39 to select the T cells (190–193). These approaches have mostly targeted tumor-reactive CD8+ T cells, but in view of the important role (194–196) and efficacy of tumor-reactive CD4+ T cells (197–199) strategies to rapidly isolate that specific T cell fraction are needed. This may be achieved using the expression of CD39 on tumor-infiltrating CD4+ T cells (45).

AUTHOR CONTRIBUTIONS

The authors contributed equally to the review. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. *CA Cancer J Clin* (2017) 67(1):51–64. doi: 10.3322/caac.21384
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* (2015) 65(2):87–108. doi: 10.3322/caac.21262
3. Mehanna H, Jones TM, Gregoire V, Ang KK. Oropharyngeal carcinoma related to human papillomavirus. *BMJ* (2010) 340:c1439. doi: 10.1136/bmj.c1439
4. Brown LM, Check DP, Devesa SS. Oropharyngeal cancer incidence trends: diminishing racial disparities. *Cancer Causes Control* (2011) 22(5):753–63. doi: 10.1007/s10552-011-9748-1
5. van Monsjou HS, van Velthuysen ML, van den Brekel MW, Jordanova ES, Melief CJ, Balm AJ. Human papillomavirus status in young patients with head and neck squamous cell carcinoma. *Int J Cancer* (2012) 130(8):1806–12. doi: 10.1002/ijc.26195
6. Welters MJP, Ma W, Santegoets S, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral HPV16-Specific T Cells Constitute a Type I-Oriented Tumor Microenvironment to Improve Survival in HPV16-Driven Oropharyngeal Cancer. *Clin Cancer Res* (2018) 24(3):634–47. doi: 10.1158/1078-0432.CCR-17-2140
7. Wang HF, Wang SS, Tang YJ, Chen Y, Zheng M, Tang YL, et al. The Double-Edged Sword-How Human Papillomaviruses Interact With Immunity in Head and Neck Cancer. *Front Immunol* (2019) 10:653. doi: 10.3389/fimmu.2019.00653
8. Syrjanen S, Syrjanen K. HPV in Head and Neck Carcinomas: Different HPV Profiles in Oropharyngeal Carcinomas - Why? *Acta Cytol* (2019) 63(2):124–42. doi: 10.1159/000495727
9. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. *Vaccine* (2012) 30 Suppl 5:F55–70. doi: 10.1016/j.vaccine.2012.06.083
10. Stanley M. Immunobiology of HPV and HPV vaccines. *Gynecol Oncol* (2008) 109(2 Suppl):S15–21. doi: 10.1016/j.ygyno.2008.02.003
11. Gupta S, Kumar P, Das BC. HPV: Molecular pathways and targets. *Curr Probl Cancer* (2018) 42(2):161–74. doi: 10.1016/j.currproblcancer.2018.03.003
12. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* (2002) 2(5):342–50. doi: 10.1038/nrc798
13. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med* (2010) 363(1):24–35. doi: 10.1056/NEJMoa0912217
14. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. *Arch Otolaryngol Head Neck Surg* (2010) 136(12):1267–73. doi: 10.1001/archoto.2010.211
15. Gillison ML, Trotti AM, Harris J, Eisbruch A, Harari PM, Adelstein DJ, et al. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. *Lancet* (2019) 393(10166):40–50. doi: 10.1016/S0140-6736(18)32779-X
16. Patel RR, Ludmir EB, Augustyn A, Zaorsky NG, Lehrer EJ, Ryali R, et al. De-intensification of therapy in human papillomavirus associated oropharyngeal cancer: A systematic review of prospective trials. *Oral Oncol* (2020) 103:104608. doi: 10.1016/j.oraloncology.2020.104608
17. Ventz S, Trippa L, Schoenfeld JD. Lessons Learned from Deescalation Trials in Favorable Risk HPV-Associated Squamous Cell Head and Neck Cancer-A Perspective on Future Trial Designs. *Clin Cancer Res* (2019) 25(24):7281–6. doi: 10.1158/1078-0432.CCR-19-0945
18. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. *PloS Pathog* (2013) 9(5):e1003384. doi: 10.1371/journal.ppat.1003384
19. Ma W, Melief CJ, van der Burg SH. Control of immune escaped human papilloma virus is regained after therapeutic vaccination. *Curr Opin Virol* (2017) 23:16–22. doi: 10.1016/j.coviro.2017.02.005
20. Tummers B, et Goedemans R, Pelascini LP, Jordanova ES, van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFkappaB activation. *Nat Commun* (2015) 6:6537. doi: 10.1038/ncomms7537
21. Steinbach A, Riemer AB. Immune evasion mechanisms of human papillomavirus: An update. *Int J Cancer* (2018) 142(2):224–9. doi: 10.1002/ijc.31027
22. Zhou C, Tuong ZK, Frazer IH. Papillomavirus Immune Evasion Strategies Target the Infected Cell and the Local Immune System. *Front Oncol* (2019) 9:682. doi: 10.3389/fonc.2019.00682
23. Matlung SE, Wilhelmina van Kempen PM, Bovenschen N, van Baarle D, Willems SM. Differences in T-cell infiltrates and survival between HPV+ and HPV- oropharyngeal squamous cell carcinoma. *Future Sci OA* (2016) 2(1):FSO88. doi: 10.4155/fso.15.88
24. Santegoets SJ, van Ham VJ, Ehsan I, Charoentong P, Duurland CL, van Unen V, et al. The Anatomical Location Shapes the Immune Infiltrate in Tumors of Same Etiology and Affects Survival. *Clin Cancer Res* (2019) 25(1):240–52. doi: 10.1158/1078-0432.CCR-18-1749
25. Fridman WH, Galon J, Dieu-Nosjean MC, Cremer I, Fisson S, Damotte D, et al. Immune infiltration in human cancer: prognostic significance and disease control. *Curr Top Microbiol Immunol* (2011) 344:1–24. doi: 10.1007/82_2010_46
26. Wallis SP, Stafford ND, Greenman J. Clinical relevance of immune parameters in the tumor microenvironment of head and neck cancers. *Head Neck* (2015) 37(3):449–59. doi: 10.1002/hed.23736
27. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discovery* (2019) 18(3):197–218. doi: 10.1038/s41573-018-0007-y
28. Van Abel KM, Routman DM, Moore EJ, Ma DJ, Yin LX, Fields PA, et al. T cell fraction impacts oncologic outcomes in human papillomavirus associated oropharyngeal squamous cell carcinoma. *Oral Oncol* (2020) 111:104894. doi: 10.1016/j.oraloncology.2020.104894
29. Gillison ML, Blumenschein GJr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. CheckMate 141: 1-Year Update and Subgroup Analysis of Nivolumab as First-Line Therapy in Patients with Recurrent/Metastatic Head and Neck Cancer. *Oncologist* (2018) 23(9):1079–82. doi: 10.1634/theoncologist.2017-0674
30. Giraldo NA, Sanchez-Salas R, Peske JD, Vano Y, Becht E, Petitprez F, et al. The clinical role of the TME in solid cancer. *Br J Cancer* (2019) 120(1):45–53. doi: 10.1038/s41416-018-0327-z
31. Jung AC, Guihard S, Krugell S, Ledrappier S, Brochot A, Dalstein V, et al. CD8-alpha T-cell infiltration in human papillomavirus-related

oropharyngeal carcinoma correlates with improved patient prognosis. *Int J Cancer* (2013) 132(2):E26–36. doi: 10.1002/ijc.27776
32. Ward MJ, Thirdborough SM, Mellows T, Riley C, Harris S, Suchak K, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer* (2014) 110(2):489–500. doi: 10.1038/bjc.2013.639
33. Gurin D, Slavik M, Hermanova M, Selingerova I, Kazda T, Hendrych M, et al. The tumor immune microenvironment and its implications for clinical outcome in patients with oropharyngeal squamous cell carcinoma. *J Oral Pathol Med* (2020) 49(9):886–96. doi: 10.1111/jop.13055
34. Wansom D, Light E, Thomas D, Worden F, Prince M, Urba S, et al. Infiltrating lymphocytes and human papillomavirus-16–associated oropharyngeal cancer. *Laryngoscope* (2012) 122(1):121–7. doi: 10.1002/lary.22133
35. Lee YS, Park JY, Cho KJ, Kim SB, Lee SW, Choi SH, et al. Composition of inflammatory cells regulating the response to concurrent chemoradiation therapy for HPV (+) tonsil cancer. *Oral Oncol* (2015) 51(12):1113–9. doi: 10.1016/j.oraloncology.2015.10.001
36. Poropatich K, Hernandez D, Fontanarosa J, Brown K, Woloschak G, Paintal A, et al. Peritumoral cuffing by T-cell tumor-infiltrating lymphocytes distinguishes HPV-related oropharyngeal squamous cell carcinoma from oral cavity squamous cell carcinoma. *J Oral Pathol Med* (2017) 46(10):972–8. doi: 10.1111/jop.12605
37. Nordfors C, Grun N, Tertipis N, Ahrlund-Richter A, Haeggblom L, Sivars L, et al. CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. *Eur J Cancer* (2013) 49(11):2522–30. doi: 10.1016/j.ejca.2013.03.019
38. Punt S, Dronkers EA, Welters MJ, Goedemans R, Koljenovic S, Bloemena E, et al. A beneficial tumor microenvironment in oropharyngeal squamous cell carcinoma is characterized by a high T cell and low IL-17(+) cell frequency. *Cancer Immunol Immunother* (2016) 65(4):393–403. doi: 10.1007/s00262-016-1805-x
39. Lin CM, Lin LW, Chen YW, Ye YL. The expression and prognostic impact of proinflammatory cytokines and their associations with carcinogens in oropharyngeal squamous cell carcinoma. *Cancer Immunol Immunother* (2020) 69(4):549–58. doi: 10.1007/s00262-020-02488-w
40. Partlova S, Boucek J, Kloudova K, Lukesova E, Zabrodsky M, Grega M, et al. Distinct patterns of intratumoral immune cell infiltrates in patients with HPV-associated compared to non-virally induced head and neck squamous cell carcinoma. *Oncoimmunology* (2015) 4(1):e965570. doi: 10.4161/21624011.2014.965570
41. Solomon B, Young RJ, Bressel M, Cernelc J, Savas P, Liu H, et al. Identification of an excellent prognosis subset of human papillomavirus-associated oropharyngeal cancer patients by quantification of intratumoral CD103(+) immune cell abundance. *Ann Oncol* (2019) 30(10):1638–46. doi: 10.1093/annonc/mdz271
42. Hewavisenti R, Ferguson A, Wang K, Jones D, Gebhardt T, Edwards J, et al. CD103+ tumor-resident CD8+ T cell numbers underlie improved patient survival in oropharyngeal squamous cell carcinoma. *J Immunother Cancer* (2020) 8(1):e000452. doi: 10.1136/jitc-2019-000452
43. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. *Nat Rev Immunol* (2016) 16(2):79–89. doi: 10.1038/nri.2015.3
44. Savas P, Virassamy B, Ye C, Salim A, Mintoff CP, Caramia F, et al. Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nat Med* (2018) 24(7):986–93. doi: 10.1038/s41591-018-0078-7
45. Kortekaas KE, Santegoets SJ, Sturm G, Ehsan I, van Egmond SL, Finotello F, et al. CD39 identifies the CD4+ tumor-specific T cell population in human cancer. *Cancer Immunol Res* (2020) 8(10):1311–21. doi: 10.1158/2326-6066.CIR-20-0270
46. Cioni B, Jordanova ES, Hooijberg E, van der Linden R, de Menezes RX, Tan K, et al. HLA class II expression on tumor cells and low numbers of tumor-associated macrophages predict clinical outcome in oropharyngeal cancer. *Head Neck* (2019) 41(2):463–78. doi: 10.1002/hed.25442
47. Ou D, Adam J, Garberis I, Blanchard P, Nguyen F, Levy A, et al. Influence of tumor-associated macrophages and HLA class I expression according to HPV status in head and neck cancer patients receiving chemo/bioradiotherapy. *Radiother Oncol* (2019) 130:89–96. doi: 10.1016/j.radonc.2018.08.013
48. Reeves E, Wood O, Ottensmeier CH, King EV, Thomas GJ, Elliott T, et al. HPV Epitope Processing Differences Correlate with ERAP1 Allotype and Extent of CD8(+) T-cell Tumor Infiltration in OPSCC. *Cancer Immunol Res* (2019) 7(7):1202–13. doi: 10.1158/2326-6066.CIR-18-0498
49. Näsman A, Andersson E, Marklund L, Tertipis N, Hammarstedt-Nordenvall L, Attner P, et al. HLA class I and II expression in oropharyngeal squamous cell carcinoma in relation to tumor HPV status and clinical outcome. *PloS One* (2013) 8(10):e77025. doi: 10.1371/journal.pone.0077025
50. Näsman A, Andersson E, Nordfors C, Grun N, Johansson H, Munck-Wikland E, et al. MHC class I expression in HPV positive and negative tonsillar squamous cell carcinoma in correlation to clinical outcome. *Int J Cancer* (2013) 132(1):72–81. doi: 10.1002/ijc.27635
51. O'Higgins C, Ward FJ, Abu Eid R. Deciphering the Role of Regulatory CD4 T Cells in Oral and Oropharyngeal Cancer: A Systematic Review. *Front Oncol* (2018) 8:442. doi: 10.3389/fonc.2018.00442
52. Drennan S, Stafford ND, Greenman J, Green VL. Increased frequency and suppressive activity of CD127(low/-) regulatory T cells in the peripheral circulation of patients with head and neck squamous cell carcinoma are associated with advanced stage and nodal involvement. *Immunology* (2013) 140(3):335–43. doi: 10.1111/imm.12144
53. Sun W, Li WJ, Wu CY, Zhong H, Wen WP. CD45RA-Foxp3high but not CD45RA+Foxp3low suppressive T regulatory cells increased in the peripheral circulation of patients with head and neck squamous cell carcinoma and correlated with tumor progression. *J Exp Clin Cancer Res* (2014) 33:35. doi: 10.1186/1756-9966-33-35
54. Distel LV, Fickenscher R, Dietel K, Hung A, Iro H, Zenk J, et al. Tumour infiltrating lymphocytes in squamous cell carcinoma of the oro- and hypopharynx: prognostic impact may depend on type of treatment and stage of disease. *Oral Oncol* (2009) 45(10):e167–74. doi: 10.1016/j.oraloncology.2009.05.640
55. Bron L, Jandus C, Andrejevic-Blant S, Speiser DE, Monnier P, Romero P, et al. Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. *Int J Cancer* (2013) 132(3):E85–93. doi: 10.1002/ijc.27728
56. Lukesova E, Boucek J, Rotnaglova E, Salakova M, Koslabova E, Grega M, et al. High level of Tregs is a positive prognostic marker in patients with HPV-positive oral and oropharyngeal squamous cell carcinomas. *BioMed Res Int* (2014) 2014:303929. doi: 10.1155/2014/303929
57. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. *Clin Cancer* (2012) 18(11):3022–9. doi: 10.1158/1078-0432.CCR-11-3216
58. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* (2009) 27(2):186–92. doi: 10.1200/JCO.2008.18.7229
59. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* (2009) 30(6):899–911. doi: 10.1016/j.immuni.2009.03.019
60. Saito T, Nishikawa H, Wada H, Nagano Y, Sugiyama D, Atarashi K, et al. Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* (2016) 22(6):679–84. doi: 10.1038/nm.4086
61. Sugiyama D, Nishikawa H, Maeda Y, Nishioka M, Tanemura A, Katayama I, et al. Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. *Proc Natl Acad Sci USA* (2013) 110(44):17945–50. doi: 10.1073/pnas.1316796110
62. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. *Cancer Res* (2013) 73(1):128–38. doi: 10.1158/0008-5472.CAN-12-2606
63. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* (2009) 326(5955):986–91. doi: 10.1126/science.1172702
64. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and

function during type 1 inflammation. *Nat Immunol* (2009) 10(6):595–602. doi: 10.1038/ni.1731
65. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity* (2011) 35(3):337–48. doi: 10.1016/j.immuni.2011.08.012
66. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H) 2 responses. *Nature* (2009) 458(7236):351–6. doi: 10.1038/nature07674
67. Santegoets SJ, Duurland CL, Jordanova ES, van Ham JJ, Ehsan I, van Egmond SL, et al. Tbet-positive regulatory T cells accumulate in oropharyngeal cancers with ongoing tumor-specific type 1 T cell responses. *J Immunother Cancer* (2019) 7 (1):14. doi: 10.1186/s40425-019-0497-0
68. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* (2017) 14(7):399–416. doi: 10.1038/nrclinonc.2016.217
69. Nakamura K, Smyth MJ. Myeloid immunosuppression and immune checkpoints in the tumor microenvironment. *Cell Mol Immunol* (2020) 17 (1):1–12. doi: 10.1038/s41423-019-0306-1
70. Locati M, Curtale G, Mantovani A. Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Annu Rev Pathol* (2020) 15:123–47. doi: 10.1146/annurev-pathmechdis-012418-012718
71. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* (2010) 11 (10):889–96. doi: 10.1038/ni.1937
72. Anfray C, Ummarino A, Andon FT, Allavena P. Current Strategies to Target Tumor-Associated-Macrophages to Improve Anti-Tumor Immune Responses. *Cells* (2019) 9(1):46. doi: 10.3390/cells9010046
73. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, Ramwadhdoebe TH, Gorter A, Welters MJ, et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J Immunol* (2011) 187(3):1157–65. doi: 10.4049/jimmunol.1100889
74. Santegoets SJ, Duurland CL, Jordanova EJ, van Ham VJ, Ehsan I, Loof NM, et al. CD163(+) cytokine-producing cDC2 stimulate intratumoral type 1 T cell responses in HPV16-induced oropharyngeal cancer. *J Immunother Cancer* (2020) 8(2):e001053. doi: 10.1136/jitc-2020-001053
75. Balermpas P, Rodel F, Liberz R, Oppermann J, Wagenblast J, Ghanaati S, et al. Head and neck cancer relapse after chemoradiotherapy correlates with CD163+ macrophages in primary tumour and CD11b+ myeloid cells in recurrences. *Br J Cancer* (2014) 111(8):1509–18. doi: 10.1038/bjc.2014.446
76. de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, Doorduijn EM, van Ham JJ, Gorter A, et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. *Int J Cancer* (2013) 133(12):2884–94. doi: 10.1002/ijc.28309
77. Heusinkveld M, van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. *J Transl Med* (2011) 9:216. doi: 10.1186/1479-5876-9-216
78. Etzerodt A, Tsalkitzi K, Maniecki M, Damsky W, Delfini M, Baudoin E, et al. Specific targeting of CD163(+) TAMs mobilizes inflammatory monocytes and promotes T cell-mediated tumor regression. *J Exp Med* (2019) 216 (10):2394–411. doi: 10.1084/jem.20182124
79. van der Sluis TC, Sluijter M, van Duikeren S, West BL, Melief CJ, Arens R, et al. Therapeutic Peptide Vaccine-Induced CD8 T Cells Strongly Modulate Intratumoral Macrophages Required for Tumor Regression. *Cancer Immunol Res* (2015) 3(9):1042–51. doi: 10.1158/2326-6066.CIR-15-0052
80. Jahchan NS, Mujal AM, Pollack JL, Binnewies M, Sriram V, Reyno L, et al. Tuning the Tumor Myeloid Microenvironment to Fight Cancer. *Front Immunol* (2019) 10:1611. doi: 10.3389/fimmu.2019.01611
81. Mandruzzato S, Brandau S, Britten CM, Bronte V, Damuzzo V, Gouttefangeas C, et al. Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. *Cancer Immunol Immunother* (2016) 65(2):161–9. doi: 10.1007/s00262-015-1782-5
82. Cassetta L, Baekkevold ES, Brandau S, Bujko A, Cassatella MA, Dorhoi A, et al. Deciphering myeloid-derived suppressor cells: isolation and markers in humans, mice and non-human primates. *Cancer Immunol Immunother* (2019) 68(4):687–97. doi: 10.1007/s00262-019-02302-2
83. Lang S, Bruderek K, Kaspar C, Hoing B, Kanaan O, Dominas N, et al. Clinical Relevance and Suppressive Capacity of Human Myeloid-Derived Suppressor Cell Subsets. *Clin Cancer Res* (2018) 24(19):4834–44. doi: 10.1158/1078-0432.CCR-17-3726
84. Califano JA, Khan Z, Noonan KA, Rudraraju L, Zhang Z, Wang H, et al. Tadalafil augments tumor specific immunity in patients with head and neck squamous cell carcinoma. *Clin Cancer Res* (2015) 21(1):30–8. doi: 10.1158/1078-0432.CCR-14-1716
85. Davis RJ, Van Waes C, Allen CT. Overcoming barriers to effective immunotherapy: MDSCs, TAMs, and Tregs as mediators of the immunosuppressive microenvironment in head and neck cancer. *Oral Oncol* (2016) 58:59–70. doi: 10.1016/j.oraloncology.2016.05.002
86. Dutertre CA, Becht E, Irac SE, Khalilnezhad A, Narang V, Khalilnezhad S, et al. Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. *Immunity* (2019) 51(3):573–89.e578. doi: 10.1016/j.immuni.2019.08.008
87. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* (2017) 356(6335):eaah4573. doi: 10.1126/science.aah4573
88. Bourdely P, Anselmi G, Vaivode K, Ramos RN, Missolo-Koussou Y, Hidalgo S, et al. Transcriptional and Functional Analysis of CD1c(+) Human Dendritic Cells Identifies a CD163(+) Subset Priming CD8(+)CD103(+) T Cells. *Immunity* (2020) 53(2):335–52.e8. doi: 10.1016/j.immuni.2020.06.002
89. De Meulenaere A, Vermassen T, Aspeslagh S, Deron P, Duprez F, Laukens D, et al. Tumor PD-L1 status and CD8(+) tumor-infiltrating T cells: markers of improved prognosis in oropharyngeal cancer. *Oncotarget* (2017) 8 (46):80443–52. doi: 10.18632/oncotarget.19045
90. Fukushima Y, Someya M, Nakata K, Hori M, Kitagawa M, Hasegawa T, et al. Influence of PD-L1 expression in immune cells on the response to radiation therapy in patients with oropharyngeal squamous cell carcinoma. *Radiother Oncol* (2018) 129(2):409–14. doi: 10.1016/j.radonc.2018.08.023
91. Lecerf C, Kamal M, Vacher S, Chemlali W, Schnitzler A, Morel C, et al. Immune gene expression in head and neck squamous cell carcinoma patients. *Eur J Cancer* (2019) 121:210–23. doi: 10.1016/j.ejca.2019.08.028
92. Sato F, Ono T, Kawahara A, Kawaguchi T, Tanaka H, Shimamatsu K, et al. Prognostic impact of p16 and PD-L1 expression in patients with oropharyngeal squamous cell carcinoma receiving a definitive treatment. *J Clin Pathol* (2019) 72(8):542–9. doi: 10.1136/jclinpath-2019-205818
93. Young RJ, Bressel M, Porceddu S, Cernelc J, Savas P, Liu H, et al. Validation and characterisation of prognostically significant PD-L1(+) immune cells in HPV+ oropharyngeal squamous cell carcinoma. *Oral Oncol* (2020) 101:104516. doi: 10.1016/j.oraloncology.2019.104516
94. Kim MH, Kim JH, Lee JM, Choi JW, Jung D, Cho H, et al. Molecular subtypes of oropharyngeal cancer show distinct immune microenvironment related with immune checkpoint blockade response. *Br J Cancer* (2020) 122 (11):1649–60. doi: 10.1038/s41416-020-0796-8
95. Oguejiofor K, Galletta-Williams H, Dovedi SJ, Roberts DL, Stern PL, West CM. Distinct patterns of infiltrating CD8+ T cells in HPV+ and CD68 macrophages in HPV- oropharyngeal squamous cell carcinomas are associated with better clinical outcome but PD-L1 expression is not prognostic. *Oncotarget* (2017) 8(9):14416–27. doi: 10.18632/oncotarget.14796
96. Andre P, Denis C, Soulas C, Bourbon-Caillet C, Lopez J, Arnoux T, et al. Anti-NKG2A mAb Is a Checkpoint Inhibitor that Promotes Anti-tumor Immunity by Unleashing Both T and NK Cells. *Cell* (2018) 175(7):1731–43.e1713. doi: 10.1016/j.cell.2018.10.014
97. van Montfoort N, Borst L, Korrer MJ, Sluijter M, Marijt KA, Santegoets SJ, et al. NKG2A Blockade Potentiates CD8 T Cell Immunity Induced by Cancer Vaccines. *Cell* (2018) 175(7):1744–55.e1715. doi: 10.1016/j.cell.2018.10.028
98. Gooden M, Lampen M, Jordanova ES, Leffers N, Trimbos JB, van der Burg SH, et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8(+) T lymphocytes. *Proc Natl Acad Sci USA* (2011) 108 (26):10656–61. doi: 10.1073/pnas.1100354108
99. Talebian Yazdi M, van Riet S, van Schadewijk A, Fiocco M, van Hall T, Taube C, et al. The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell

lung carcinoma. *Oncotarget* (2016) 7(3):3477–88. doi: 10.18632/oncotarget.6506
100. van Hall T, Andre P, Horowitz A, Ruan DF, Borst L, Zerbib R, et al. Monalizumab: inhibiting the novel immune checkpoint NKG2A. *J Immunother Cancer* (2019) 7(1):263. doi: 10.1186/s40425-019-0761-3
101. Gameiro SF, Ghasemi F, Barrett JW, Koropatnick J, Nichols AC, Mymryk JS, et al. Treatment-naive HPV+ head and neck cancers display a T-cell-inflamed phenotype distinct from their HPV- counterparts that has implications for immunotherapy. *Oncoimmunology* (2018) 7(10):e1498439. doi: 10.1080/2162402X.2018.1498439
102. Huang SH, Waldron JN, Milosevic M, Shen X, Ringash J, Su J, et al. Prognostic value of pretreatment circulating neutrophils, monocytes, and lymphocytes in oropharyngeal cancer stratified by human papillomavirus status. *Cancer* (2015) 121(4):545–55. doi: 10.1002/cncr.29100
103. Panje C, Riesterer O, Glanzmann C, Studer G. Neutrophil-lymphocyte ratio complements volumetric staging as prognostic factor in patients treated with definitive radiotherapy for oropharyngeal cancer. *BMC Cancer* (2017) 17 (1):643. doi: 10.1186/s12885-017-3590-0
104. So YK, Lee G, Oh D, Byeon S, Park W, Chung MK. Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Patients with Human Papillomavirus-Positive Oropharyngeal Cancer. *Otolaryngol Head Neck Surg* (2018) 159 (2):303–9. doi: 10.1177/0194599818764651
105. Sumner WA, Stokes WA, Oweida A, Berggren KL, McDermott JD, Raben D, et al. Survival impact of pre-treatment neutrophils on oropharyngeal and laryngeal cancer patients undergoing definitive radiotherapy. *J Transl Med* (2017) 15(1):168. doi: 10.1186/s12967-017-1268-7
106. Trellakis S, Farjah H, Bruderek K, Dumitru CA, Hoffmann TK, Lang S, et al. Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. *Int J Immunopathol Pharmacol* (2011) 24(3):683–93. doi: 10.1177/039463201102400314
107. Takahashi H, Sakakura K, Tada H, Kaira K, Oyama T, Chikamatsu K. Prognostic significance and population dynamics of peripheral monocytes in patients with oropharyngeal squamous cell carcinoma. *Head Neck* (2019) 41 (6):1880–8. doi: 10.1002/hed.25625
108. Santegoets S, de Groot AF, Dijkgraaf EM, Simoes AMC, van der Noord VE, van Ham JJ, et al. The blood mMDSC to DC ratio is a sensitive and easy to assess independent predictive factor for epithelial ovarian cancer survival. *Oncoimmunology* (2018) 7(8):e1465166. doi: 10.1080/2162402X.2018.1465166
109. Kano S, Homma A, Hatakeyama H, Mizumachi T, Sakashita T, Kakizaki T, et al. Pretreatment lymphocyte-to-monocyte ratio as an independent prognostic factor for head and neck cancer. *Head Neck* (2017) 39(2):247–53. doi: 10.1002/hed.24576
110. Heusinkveld M, Goedemans R, Briet RJ, Gelderblom H, Nortier JW, Gorter A, et al. Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. *Int J Cancer* (2012) 131 (2):E74–85. doi: 10.1002/ijc.26497
111. Masterson L, Lechner M, Loewenbein S, Mohammed H, Davies-Husband C, Fenton T, et al. CD8(+) T cell response to human papillomavirus 16 E7 is able to predict survival outcome in oropharyngeal cancer. *Eur J Cancer* (2016) 67:141–51. doi: 10.1016/j.ejca.2016.08.012
112. Bhatt KH, Neller MA, Srihari S, Crooks P, Lekieffre L, Aftab BT, et al. Profiling HPV-16-specific T cell responses reveals broad antigen reactivities in oropharyngeal cancer patients. *J Exp Med* (2020) 217(10):e20200389. doi: 10.1084/jem.20200389
113. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res* (2002) 62(2):472–9.
114. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. *Cancer Res* (2004) 64(15):5449–55. doi: 10.1158/0008-5472.CAN-04-0831
115. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. *Cancer Res* (2003) 63(3):636–41.
116. Angell H, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Curr Opin Immunol* (2013) 25(2):261–7. doi: 10.1016/j.coi.2013.03.004
117. Licitra L, Perrone F, Bossi P, Suardi S, Mariani L, Artusi R, et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol* (2006) 24(36):5630–6. doi: 10.1200/JCO.2005.04.6136
118. Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G, et al. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. *J Clin Oncol* (2010) 28(17):2881–8. doi: 10.1200/JCO.2009.24.8724
119. Al-Taei S, Banner R, Powell N, Evans M, Palaniappan N, Tabi Z, et al. Decreased HPV-specific T cell responses and accumulation of immunosuppressive influences in oropharyngeal cancer patients following radical therapy. *Cancer Immunol Immunother* (2013) 62(12):1821–30. doi: 10.1007/s00262-013-1488-5
120. Hladikova K, Partlova S, Koucky V, Boucek J, Fonteneau JF, Zabrodsky M, et al. Dysfunction of HPV16-specific CD8+ T cells derived from oropharyngeal tumors is related to the expression of Tim-3 but not PD-1. *Oral Oncol* (2018) 82:75–82. doi: 10.1016/j.oraloncology.2018.05.010
121. Parikh F, Duluc D, Imai N, Clark A, Misiukiewicz K, Bonomi M, et al. Chemoradiotherapy-induced upregulation of PD-1 antagonizes immunity to HPV-related oropharyngeal cancer. *Cancer Res* (2014) 74(24):7205–16. doi: 10.1158/0008-5472.CAN-14-1913
122. Ramos CA, Narala N, Vyas GM, Leen AM, Gerdemann U, Sturgis EM, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes for adoptive immunotherapy of HPV-associated malignancies. *J Immunother* (2013) 36(1):66–76. doi: 10.1097/CJI.0b013e318279652e
123. Smith EM, Pawlita M, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer. *Int J Cancer* (2010) 127 (1):111–7. doi: 10.1002/ijc.25015
124. Nasman A, Romanitan M, Nordfors C, Grun N, Johansson H, Hammarstedt L, et al. Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. *PloS One* (2012) 7(6):e38711. doi: 10.1371/journal.pone.0038711
125. Brown SD, Warren RL, Gibb EA, Martin SD, Spinelli JJ, Nelson BH, et al. Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. *Genome Res* (2014) 24(5):743–50. doi: 10.1101/gr.165985.113
126. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science* (2011) 333(6046):1157–60. doi: 10.1126/science.1208130
127. Ren L, Leisegang M, Deng B, Matsuda T, Kiyotani K, Kato T, et al. Identification of neoantigen-specific T cells and their targets: implications for immunotherapy of head and neck squamous cell carcinoma. *Oncoimmunology* (2019) 8(4): e1568813. doi: 10.1080/2162402X.2019.1568813
128. Yang W, Lee KW, Srivastava RM, Kuo F, Krishna C, Chowell D, et al. Immunogenic neoantigens derived from gene fusions stimulate T cell responses. *Nat Med* (2019) 25(5):767–75. doi: 10.1038/s41591-019-0434-2
129. van den Bulk J, Verdegaal EME, Ruano D, Ijsselsteijn ME, Visser M, van der Breggen R, et al. Neoantigen-specific immunity in low mutation burden colorectal cancers of the consensus molecular subtype 4. *Genome Med* (2019) 11(1):87. doi: 10.1186/s13073-019-0697-8
130. De Stefani A, Forni G, Ragona R, Cavallo G, Bussi M, Usai A, et al. Improved survival with perilymphatic interleukin 2 in patients with resectable squamous cell carcinoma of the oral cavity and oropharynx. *Cancer* (2002) 95(1):90–7. doi: 10.1002/cncr.10654
131. van Poelgeest MI, Visconti VV, Aghai Z, van Ham VJ, Heusinkveld M, Zandvliet ML, et al. Potential use of lymph node-derived HPV-specific T cells for adoptive cell therapy of cervical cancer. *Cancer Immunol Immunother* (2016) 65(12):1451–63. doi: 10.1007/s00262-016-1892-8
132. Wolf GT, Ee WEJr., Dolan RW, Moyer JS, Kaplan MJ, Spring PM, et al. Novel neoadjuvant immunotherapy regimen safety and survival in head and neck squamous cell cancer. *Head Neck* (2011) 33(12):1666–74. doi: 10.1002/hed.21660

133. Berinstein NL, Wolf GT, Naylor PH, Baltzer L, Egan JE, Brandwein HJ, et al. Increased lymphocyte infiltration in patients with head and neck cancer treated with the IRX-2 immunotherapy regimen. *Cancer Immunol Immunother* (2012) 61(6):771–82. doi: 10.1007/s00262-011-1134-z
134. Berinstein NL, McNamara M, Nguyen A, Egan J, Wolf GT. Increased immune infiltration and chemokine receptor expression in head and neck epithelial tumors after neoadjuvant immunotherapy with the IRX-2 regimen. *Oncoimmunology* (2018) 7(5):e1423173. doi: 10.1080/2162402X.2017.1423173
135. Lu H, Dietsch GN, Matthews MA, Yang Y, Ghanekar S, Inokuma M, et al. VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res* (2012) 18(2):499–509. doi: 10.1158/1078-0432.CCR-11-1625
136. Ferris RL, Saba NF, Gitlitz BJ, Haddad R, Sukari A, Neupane P, et al. Effect of Adding Motolimod to Standard Combination Chemotherapy and Cetuximab Treatment of Patients With Squamous Cell Carcinoma of the Head and Neck: The Active8 Randomized Clinical Trial. *JAMA Oncol* (2018) 4(11):1583–8. doi: 10.1001/jamaoncol.2018.1888
137. Noto H, Goto A, Tsujimoto T, Noda M. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. *PloS One* (2012) 7(3):e33411. doi: 10.1371/journal.pone.0033411
138. Eikawa S, Nishida M, Mizukami S, Yamazaki C, Nakayama E, Udono H. Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc Natl Acad Sci U.S.A.* (2015) 112(6):1809–14. doi: 10.1073/pnas.1417636112
139. Amin D, Richa T, Mollaee M, Zhan T, Tassone P, Johnson J, et al. Metformin effects on FOXP3(+) and CD8(+) T cell infiltrates of head and neck squamous cell carcinoma. *Laryngoscope* (2019) 130(9):E490–8. doi: 10.1002/lary.28336
140. Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med* (2015) 373(2):123–35. doi: 10.1056/NEJMoa1504627
141. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* (2015) 373(1):23–34. doi: 10.1056/NEJMoa1504030
142. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, et al. PD-1 Blockade with Pembrolizumab in Advanced Merkel-Cell Carcinoma. *N Engl J Med* (2016) 374(26):2542–52. doi: 10.1056/NEJMoa1603702
143. Ferris RL, Blumenschein GJr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *N Engl J Med* (2016) 375(19):1856–67. doi: 10.1056/NEJMoa1602252
144. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *Lancet Oncol* (2016) 17(7):956–65. doi: 10.1016/S1470-2045(16)30066-3
145. Segal NH, Ou SI, Balmanoukian A, Fury MG, Massarelli E, Brahmer JR, et al. Safety and efficacy of durvalumab in patients with head and neck squamous cell carcinoma: results from a phase I/II expansion cohort. *Eur J Cancer* (2019) 109:154–61. doi: 10.1016/j.ejca.2018.12.029
146. Massarelli E, William W, Johnson F, Kies M, Ferrarotto R, Guo M, et al. Combining Immune Checkpoint Blockade and Tumor-Specific Vaccine for Patients With Incurable Human Papillomavirus 16-Related Cancer: A Phase 2 Clinical Trial. *JAMA Oncol* (2019) 5(1):67–73. doi: 10.1001/jamaoncol.2018.4051
147. Powell SF, Gold KA, Gitau MM, Sumey CJ, Lohr MM, McGraw SC, et al. Safety and Efficacy of Pembrolizumab With Chemoradiotherapy in Locally Advanced Head and Neck Squamous Cell Carcinoma: A Phase IB Study. *J Clin Oncol* (2020) 38(21):2427–37. doi: 10.1200/JCO.19.03156
148. Ferris RL, Blumenschein GJr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. *Oral Oncol* (2018) 81:45–51. doi: 10.1016/j.oraloncology.2018.04.008
149. Haddad R, Concha-Benavente F, Blumenschein GJr., Fayette J, Guigay J, Colevas AD, et al. Nivolumab treatment beyond RECIST-defined progression in recurrent or metastatic squamous cell carcinoma of the head and neck in CheckMate 141: A subgroup analysis of a randomized phase 3 clinical trial. *Cancer* (2019) 125 (18):3208–18. doi: 10.1002/cncr.32190
150. Concha-Benavente F, Srivastava RM, Trivedi S, Lei Y, Chandran U, Seethala RR, et al. Identification of the Cell-Intrinsic and -Extrinsic Pathways Downstream of EGFR and IFNgamma That Induce PD-L1 Expression in Head and Neck Cancer. *Cancer Res* (2016) 76(5):1031–43. doi: 10.1158/0008-5472.CAN-15-2001
151. Jie HB, Srivastava RM, Argiris A, Bauman JE, Kane LP, Ferris RL. Increased PD-1(+) and TIM-3(+) TILs during Cetuximab Therapy Inversely Correlate with Response in Head and Neck Cancer Patients. *Cancer Immunol Res* (2017) 5(5):408–16. doi: 10.1158/2326-6066.CIR-16-0333
152. Ma W, Concha-Benavente F, Santegoets S, Welters MJP, Ehsan I, Ferris RL, et al. EGFR signaling suppresses type 1 cytokine-induced T-cell attracting chemokine secretion in head and neck cancer. *PloS One* (2018) 13(9): e0203402. doi: 10.1371/journal.pone.0203402
153. Trivedi S, Srivastava RM, Concha-Benavente F, Ferrone S, Garcia-Bates TM, Li J, et al. Anti-EGFR Targeted Monoclonal Antibody Isotype Influences Antitumor Cellular Immunity in Head and Neck Cancer Patients. *Clin Cancer Res* (2016) 22(21):5229–37. doi: 10.1158/1078-0432.CCR-15-2971
154. Ferris RL, Licitra L, Fayette J, Even C, Blumenschein GJr., Harrington KJ, et al. Nivolumab in Patients with Recurrent or Metastatic Squamous Cell Carcinoma of the Head and Neck: Efficacy and Safety in CheckMate 141 by Prior Cetuximab Use. *Clin Cancer Res* (2019) 25(17):5221–30. doi: 10.1158/1078-0432.CCR-18-3944
155. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* (2014) 515(7528):568–71. doi: 10.1038/nature13954
156. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science* (2018) 359(6382):1361–5. doi: 10.1126/science.aar6711
157. Wang X, Riviere I. Manufacture of tumor- and virus-specific T lymphocytes for adoptive cell therapies. *Cancer Gene Ther* (2015) 22(2):85–94. doi: 10.1038/cgt.2014.81
158. Yang JC, Rosenberg SA. Adoptive T-Cell Therapy for Cancer. *Adv Immunol* (2016) 130:279–94. doi: 10.1016/bs.ai.2015.12.006
159. Draper LM, Kwong ML, Gros A, Stevanovic S, Tran E, Kerkar S, et al. Targeting of HPV-16+ Epithelial Cancer Cells by TCR Gene Engineered T Cells Directed against E6. *Clin Cancer Res* (2015) 21(19):4431–9. doi: 10.1158/1078-0432.CCR-14-3341
160. Mercier-Letondal P, Marton C, Deschamps M, Ferrand C, Vauchy C, Chenut C, et al. Isolation and Characterization of an HLA-DRB1*04-Restricted HPV16-E7 T Cell Receptor for Cancer Immunotherapy. *Hum Gene Ther* (2018) 29(10):1202–12. doi: 10.1089/hum.2018.091
161. Ali M, Foldvari Z, Giannakopoulou E, Boschen ML, Stronen E, Yang W, et al. Induction of neoantigen-reactive T cells from healthy donors. *Nat Protoc* (2019) 14(6):1926–43. doi: 10.1038/s41596-019-0170-6
162. van der Burg SH. Correlates of immune and clinical activity of novel cancer vaccines. *Semin Immunol* (2018) 39:119–36. doi: 10.1016/j.smim.2018.04.001
163. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJ. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer* (2016) 16(4):219–33. doi: 10.1038/nrc.2016.16
164. Voskens CJ, Sewell D, Hertzano R, DeSanto J, Rollins S, Lee M, et al. Induction of MAGE-A3 and HPV-16 immunity by Trojan vaccines in patients with head and neck carcinoma. *Head Neck* (2012) 34(12):1734–46. doi: 10.1002/hed.22004
165. Schuler PJ, Harasymczuk M, Visus C, Deleo A, Trivedi S, Lei Y, et al. Phase I dendritic cell p53 peptide vaccine for head and neck cancer. *Clin Cancer Res* (2014) 20(9):2433–44. doi: 10.1158/1078-0432.CCR-13-2617
166. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* (2009) 361(19):1838–47. doi: 10.1056/NEJMoa0810097
167. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends-van der Meer DM, et al. Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. *Clin Cancer Res* (2016) 22 (10):2342–50. doi: 10.1158/1078-0432.CCR-15-2594
168. Hilf N, Kuttruff-Coqui S, Frenzel K, Bukur V, Stevanovic S, Gouttefangeas C, et al. Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature* (2019) 565(7738):240–5. doi: 10.1038/s41586-018-0810-y

169. Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nat Rev Immunol* (2018) 18(3):168–82. doi: 10.1038/nri.2017.131
170. Sahin U, Derhovanessian E, Miller M, Kloke BP, Simon P, Lower M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* (2017) 547(7662):222–6. doi: 10.1038/nature23003
171. Demaria S, Golden EB, Formenti SC. Role of Local Radiation Therapy in Cancer Immunotherapy. *JAMA Oncol* (2015) 1(9):1325–32. doi: 10.1001/jamaoncol.2015.2756
172. Shaverdian N, Lisberg AE, Bornazyan K, Veruttipong D, Goldman JW, Formenti SC, et al. Previous radiotherapy and the clinical activity and toxicity of pembrolizumab in the treatment of non-small-cell lung cancer: a secondary analysis of the KEYNOTE-001 phase 1 trial. *Lancet Oncol* (2017) 18(7):895–903. doi: 10.1016/S1470-2045(17)30380-7
173. Bonomo P, Desideri I, Loi M, Mangoni M, Sottili M, Marrazzo L, et al. Anti PD-L1 DUrvalumab combined with Cetuximab and RadiOtherapy in locally advanced squamous cell carcinoma of the head and neck: A phase I/II study (DUCRO). *Clin Transl Radiat Oncol* (2018) 9:42–7. doi: 10.1016/j.ctro.2018.01.005
174. Bahig H, Aubin F, Stagg J, Gologan O, Ballivy O, Bissada E, et al. Phase I/II trial of Durvalumab plus Tremelimumab and stereotactic body radiotherapy for metastatic head and neck carcinoma. *BMC Cancer* (2019) 19(1):68. doi: 10.1186/s12885-019-5266-4
175. Yu Y, Lee NY. JAVELIN Head and Neck 100: a Phase III trial of avelumab and chemoradiation for locally advanced head and neck cancer. *Future Oncol* (2019) 15(7):687–94. doi: 10.2217/fon-2018-0405
176. Elbers JBW, Al-Mamgani A, Tesseslaar MET, van den Brekel MWM, Lange CAH, van der Wal JE, et al. Immuno-radiotherapy with cetuximab and avelumab for advanced stage head and neck squamous cell carcinoma: Results from a phase-I trial. *Radiother Oncol* (2020) 142:79–84. doi: 10.1016/j.radonc.2019.08.007
177. Vermeer DW, Spanos WC, Vermeer PD, Bruns AM, Lee KM, Lee JH. Radiation-induced loss of cell surface CD47 enhances immune-mediated clearance of human papillomavirus-positive cancer. *Int J Cancer* (2013) 133(1):120–9. doi: 10.1002/ijc.28015
178. Advani R, Flinn I, Popplewell L, Forero A, Bartlett NL, Ghosh N, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med* (2018) 379(18):1711–21. doi: 10.1056/NEJMoa1807315
179. Logtenberg MEW, Jansen JHM, Raaben M, Toebes M, Franke K, Brandsma AM, et al. Glutaminyl cyclase is an enzymatic modifier of the CD47-SIRPalpha axis and a target for cancer immunotherapy. *Nat Med* (2019) 25(4):612–9. doi: 10.1038/s41591-019-0356-z
180. Hanoteau A, Newton JM, Krupar R, Huang C, Liu HC, Gaspero A, et al. Tumor microenvironment modulation enhances immunologic benefit of chemoradiotherapy. *J Immunother Cancer* (2019) 7(1):10. doi: 10.1186/s40425-018-0485-9
181. Beyranvand Nejad E, van der Sluis TC, van Duikeren S, Yagita H, Janssen GM, van Veelen PA, et al. Tumor Eradication by Cisplatin Is Sustained by CD80/86-Mediated Costimulation of CD8+ T Cells. *Cancer Res* (2016) 76(20):6017–29. doi: 10.1158/0008-5472.CAN-16-0881
182. van der Sluis TC, van Duikeren S, Huppelschoten S, Jordanova ES, Beyranvand Nejad E, Sloots A, et al. Vaccine-induced tumor necrosis factor-producing T cells synergize with cisplatin to promote tumor cell death. *Clin Cancer Res* (2015) 21(4):781–94. doi: 10.1158/1078-0432.CCR-14-2142
183. Goh AR, Shin SP, Jung NR, Ryu CH, Eom HS, Lee JH, et al. Low-dose cisplatin converts the tumor microenvironment into a permissive state for HSVtk-induced antitumor immunity in HPV16-related tonsillar carcinoma. *Cancer Lett* (2015) 356(2 Pt B):743–50. doi: 10.1016/j.canlet.2014.10.022
184. Dorta-Estremera S, Hegde VL, Slay RB, Sun R, Yanamandra AV, Nicholas C, et al. Targeting interferon signaling and CTLA-4 enhance the therapeutic efficacy of anti-PD-1 immunotherapy in preclinical model of HPV(+) oral cancer. *J Immunother Cancer* (2019) 7(1):252. doi: 10.1186/s40425-019-0728-4
185. Cillo AR, Kurten CHL, Tabib T, Qi Z, Onkar S, Wang T, et al. Immune Landscape of Viral- and Carcinogen-Driven Head and Neck Cancer. *Immunity* (2020) 52(1):183–99.e189. doi: 10.1016/j.immuni.2019.11.014
186. Ijsselsteijn ME, van der Breggen R, Farina Sarasqueta A, Koning F, de Miranda N. A 40-Marker Panel for High Dimensional Characterization of Cancer Immune Microenvironments by Imaging Mass Cytometry. *Front Immunol* (2019) 10:2534. doi: 10.3389/fimmu.2019.02534
187. Welters MJ, van der Sluis TC, van Meir H, Loof NM, van Ham VJ, van Duikeren S, et al. Vaccination during myeloid cell depletion by cancer chemotherapy fosters robust T cell responses. *Sci Transl Med* (2016) 8(334):334ra352. doi: 10.1126/scitranslmed.aad8307
188. Melief CJM, Welters MJP, Vergote I, Kroep JR, Kenter GG, Ottevanger PB, et al. Strong vaccine responses during chemotherapy are associated with prolonged cancer survival. *Sci Transl Med* (2020) 12(535):eaaz8235. doi: 10.1126/scitranslmed.aaz8235
189. Abdulrahman Z, De Miranda NF, Van Esch EM, De Vos van Steenwijk PJ, Nijman HW, Welters MJP, et al. A pre-existing inflammatory immune microenvironment predicts the clinical response of vulvar high-grade squamous intraepithelial lesions to therapeutic HPV16 vaccination. *J Immunother Cancer* (2020) 8(1):e000563. doi: 10.1136/jitc-2020-000563
190. Duhen T, Duhen R, Montler R, Moses J, Moudgil T, de Miranda NF, et al. Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat Commun* (2018) 9(1):2724. doi: 10.1038/s41467-018-05072-0
191. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* (2014) 124(5):2246–59. doi: 10.1172/JCI73639
192. Parkhurst M, Gros A, Pasetto A, Prickett T, Crystal JS, Robbins P, et al. Isolation of T-Cell Receptors Specifically Reactive with Mutated Tumor-Associated Antigens from Tumor-Infiltrating Lymphocytes Based on CD137 Expression. *Clin Cancer Res* (2017) 23(10):2491–505. doi: 10.1158/1078-0432.CCR-16-2680
193. Ye Q, Song DG, Poussin M, Yamamoto T, Best A, Li C, et al. CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. *Clin Cancer Res* (2014) 20(1):44–55. doi: 10.1158/1078-0432.CCR-13-0945
194. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med* (1998) 188(12):2357–68. doi: 10.1084/jem.188.12.2357
195. Levitsky HI, Lazenby A, Hayashi RJ, Pardoll DM. In vivo priming of two distinct antitumor effector populations: the role of MHC class I expression. *J Exp Med* (1994) 179(4):1215–24. doi: 10.1084/jem.179.4.1215
196. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* (2001) 410(6832):1107–11. doi: 10.1038/35074122
197. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* (2008) 358(25):2698–703. doi: 10.1056/NEJMoa0800251
198. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* (2010) 207(3):637–50. doi: 10.1084/jem.20091918
199. Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* (2014) 344(6184):641–5. doi: 10.1126/science.1251102

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Presence of Human Papillomavirus and Epstein–Barr Virus, but Absence of Merkel Cell Polyomavirus, in Head and Neck Cancer of Non-Smokers and Non-Drinkers

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Objective: Determine the presence and prognostic value of human papillomavirus (HPV), Epstein-Barr virus (EBV), Merkel cell polyomavirus (MCPyV), and cell cycle proteins in head and neck squamous cell carcinoma (HNSCC) of non-smokers and non-drinkers (NSND).

Methods: Clinical characteristics and tumors of 119 NSND with HNSCC were retrospectively collected and analyzed on tissue microarrays. RNAscope *in situ* hybridization (ISH) was used to screen for the presence of HPV and MCPyV mRNA. Immunohistochemistry was performed for expression of p16 as surrogate marker for HPV, Large T-antigen for MCPyV, and cell cycle proteins p53 and pRb. Positive virus results were confirmed with polymerase chain reaction. For EBV, EBV encoded RNA ISH was performed. Differences in 5-year survival between virus positive and negative tumors were determined by log rank analysis.

Results: All oropharyngeal tumors (OPSCC) (n = 10) were HPV-positive, in addition to one oral (OSCC) and one nasopharyngeal tumor (NPSCC). The other three NPSCC were EBV-positive. MCPyV was not detected. Patients with HPV or EBV positive tumors did not have a significantly better 5-year disease free or overall survival. Over 70% of virus negative OSCC showed mutant-type p53 expression.

Conclusion: In this cohort, all OPSCC and NPSCC showed HPV or EBV presence. Besides one OSCC, all other oral (n = 94), hypopharyngeal (n = 1), and laryngeal (n = 9) tumors were HPV, EBV, and MCPyV negative. This argues against a central role of these viruses in the ethiopathogenesis of tumors outside the oro- and nasopharynx in NSND. So,

for the majority of NSND with virus negative OSCC, more research is needed to understand the carcinogenic mechanisms in order to consider targeted therapeutic options.

Keywords: head and neck cancer, human papillomavirus, Epstein–Barr virus, polyomavirus, non-smokers, non-drinkers, cell cycle protein, *in situ* hybridization

INTRODUCTION

Viruses play an increasing role in head and neck squamous cell carcinoma (HNSCC). High-risk human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) has been identified as an entity with a different carcinogenesis than traditional HNSCC resulting from excessive tobacco and alcohol consumption. HPV is also an independent prognostic factor for a better disease free survival (DFS) and overall survival (OS), which has led to a down staging of these tumors in the eighth edition of the American Joint Committee on Cancer (AJCC) and union for International Cancer Control tumor-node-metastasis (TNM) classification (1–3). Because of the better prognosis, de-escalation strategies are proposed for HPV-positive OPSCC patients (4). The prevalence of HPV-positive OPSCC is rising in the Western World. A HPV prevalence above 50% has already been reported in America, Europe, and Australia, based on HPV DNA in combination with either E6*I mRNA or p16 immunohistochemistry (IHC) detection (5, 6). Combining these HPV detection methods has been recommended because only OPSCC with transcriptionally active HPV is related to a better survival compared to biologically inactive infections (7, 8).

Another virus known for its carcinogenic potential in the head and neck region is the Epstein-Barr virus (EBV). EBV has a strong association with nasopharyngeal squamous cell carcinoma (NPSCC), approaching a prevalence of 100% in these tumors, and is endemic in Southern China, Southeast Asia, Northern Africa, and the Mediterranean basin (9, 10). It is suggested to cause an immunosuppressive microenvironment in these tumors, among others *via* PD-L1 overexpression, making these patients interesting candidates for checkpoint blockade therapy (10). Detection of EBV presence can be performed reliably with EBV encoded RNA (EBER) *in situ* hybridization (ISH) (9, 11).

Lately, besides these acknowledged oncogenic viruses, there is attention for polyomaviruses in HNSCC. Merkel cell polyomavirus (MCPyV) has not only been detected by digital transcriptome subtraction and polymerase chain reaction (PCR) in up to 80% of Merkel cell carcinoma of the skin, but also in non-malignant tonsillar tissue, oral squamous cell carcinoma (OSCC), and pharyngeal cancer, with a reported prevalence of 23, 6.6–29, and 50% respectively (12–17). Although it was thought not to play a role in oral carcinogenesis because of low viral loads detected with quantitative real-time PCR, the presence of MCPyV appears to be predictive for a better DFS (16).

Cell cycle deregulation plays a central role in head and neck carcinogenesis, with frequent inactivation of *TP53* and *CDKN2A*, leading to cell proliferation and prevention of apoptosis, among others. In HPV-related OPSCC, HPV integration in the host cell DNA genome leads to deregulation of oncoproteins E6 and E7, resulting in inactivity of p53 and retinoblastoma tumor suppressor gene product pRb, respectively. The negative feedback of pRb inactivation leads to p16 overexpression (18). In EBV infected NPSCC, it has been suggested that the cell cycle pathway is the most deregulated pathway, promoting the progression of the G1/S phase *via* inhibition of p16 expression and pRb overexpression (19). For MCPyV, oncogenetic transformation requires both integration of the viral genome into the host genome and truncation of the Large T-antigen (LTAg) to render the viral genome replication deficient (20). LTAg mutations disrupt the DNA binding domain and the helicase domain distal to the pRb-binding motif, thereby promoting cell cycle progression by retaining its ability to bind to pRb (20, 21).

There is a small group of HNSCC patients without any exposure to the traditional risk factors. The mechanisms underlying carcinogenesis in these non-smokers and non-drinkers (NSND) remain largely unclear, but a significant role of oncogenic viruses would be expected. Indeed, a higher prevalence of HPV in these tumors has been reported in several studies, though in small numbers of patients (22–25). Therefore, the goal of this study was to determine the presence of HPV, EBV, and MCPyV in a series of 119 well-characterized NSND with HNSCC. Secondary analyses evaluate differences in tumor suppressor proteins p16, p53, and pRb expression regarding viral presence and whether the presence of these viruses is predictive for a better DFS and OS.

MATERIALS AND METHODS

Patients

Consecutive patients with HNSCC were selected at the University Medical Center Utrecht (UMCU) and Maastricht University Medical Center (MUMC). In the UMCU, patients were prospectively selected between 1980 and 2004, as described previously (26). In the MUMC, HNSCC patients have been selected retrospectively between 2011 and 2016, in addition to all patients with OPSCC between 2003 and 2010. Inclusion criteria were: ≥18-years-old NSND patients with HNSCC, available formalin fixated and paraffin embedded (FFPE) tumor tissue, and >2 years follow up. Patient characteristics, risk factors, World Health Organization tumor classification, AJCC seventh edition staging, and information concerning recurrent disease or death were collected from the medical records. Non-smoking was defined as having no history of smoking, non-drinking as having no history of alcohol consumption (not even 'sporadic' alcohol consumption), as reported in the patients' medical records during both their first presentation at the Head and Neck outpatient clinic, as well as during the anesthesiological screening before panendoscopy or surgical resection. Patients with a second

primary tumor in the head and neck region, tumors outside the upper aerodigestive tract, a cervical metastasis of unknown origin, or a histopathologic diagnosis other than squamous cell carcinoma were excluded.

The Medical Ethics Review Committee of the MUMC (2018-0567) has approved this study and the principles outlined in the Declaration of Helsinki were followed. All data and tissues were handled according to General Data Protection Regulation.

Tissue Microarrays

FFPE blocks of either the diagnostic biopsy or tumor resection were retrieved and hematoxylin and eosin sections were digitally evaluated with a senior head and neck pathologist (SW or MH), using Pannoramic viewer (3DHISTEC, Budapest, Hungary). Per patient, three 0.6mm tumor tissue cores and one normal epithelium core were selected, placed in a tissue microarray (TMAs), and cut into 5 µm sections.

RNA *In Situ* Hybridization

To screen for the presence of HPV and MCPyV mRNA, the RNAscope 2.5 RED assay kit and HPV-16/18 or V-MCPyV-LT-ST-Ag probe cocktails (Advanced Cell Diagnostics, Newark, California) were used according to the manufacturer's instructions. In short, TMA sections were deparaffinized and pretreated with RNAscope Hydrogen Peroxide for 10 min. Antigen retrieval comprised of boiling the slide sections in the provided Target Retrieval Reagents solution at 100°C for 15 min. After washing, the TMAs were dried over night at room temperature and treated for 30 min with RNAscope Protease Plus. In situ hybridization was performed applying four droplets of the provided probes prior to each of the six amplification steps (30 min at 40°C, 15 min at 40°C, 30 min at 40°C, 15 min at 40°C, 30 min at room temperature, and 15 min at room temperature, respectively). After each hybridization step, the slides were washed in the RNAscope wash buffer for 2 min at room temperature. Subsequent to alkaline phosphatase Fast Red chromogenic visualization of hybridized probes, the slides were counterstained with hematoxylin and assessed under a bright field microscope at 200x magnification. Tissue with at least 1 red punctate signal dot in the cytoplasm and/or nucleus of malignant cells was considered to be positive, as suggested by the manufacturer for genes with an expression level varying between 1 to >10 copies per cell. Probes for housekeeping gene transcript human peptidylprolyl isomerase B (Hs-PPIB) and bacterial dihydrodipicolinate reductase gene (dapB) transcript were used as positive and negative controls, respectively. As virus specific positive controls, virus positive tumor tissue was used, in addition to the HPV-18 positive cell line HeLa, HPV-16 positive cell lines SiHa and Caski, and MCPyV positive cell lines MKL-1, MKL-2, and WaGa (**Figure 1**). Negative controls were tumor tissue of a virus negative patient, cell lines MKL-1 and MKL-2 for HPV, and cell lines MCC13 and MCC26 for MCPyV (27).

EBER-ISH was performed using the Dako fluorescein-labeled EBV peptide nucleic acid (PNA) probe mixture and PNA ISH Detection kit (Agilent Technologies, Santa Clara, California) according to the manufacturer's instructions. Briefly, following TMA section deparaffinization, target retrieval was performed with the Dako Omnis ISH Pre-Treatment solution for 5 min. Subsequently, enzyme pre-treatment was carried out using two steps of 3 min ethanol 96% application and one step of Dako ISH Pepsin for 15 min. Once the provided EBER RNA CISH probe was applied, denaturation at 66°C for 10 min, and hybridization at 45°C for 90 min followed. The slides were washed with the ISH Stringent Wash Buffer for 3 min, and the provided reagents were applied for staining: CISH Endogenous Enzyme Block for 3 min, Anti-FITc-AP for 30 min, and BCIP-NBT Substrate for 15 min. Sections were counterstained with Nuclear Fast Red and analyzed under a bright field microscope at 200x magnification. Strong blue staining of more than 50% of tumor nuclei was considered to be positive. In parallel, a probe for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to ensure the presence of mRNA in the TMA and a case of EBV-positive infectious mononucleosis served as a positive control (**Figure 1**).

Immunohistochemistry

Three-µm FFPE TMA sections were subjected to IHC, using primary monoclonal antibodies directed against p16, p53, pRb, and the LTAg of MCPyV (**Table 1**). Immunostainings were performed on a Dako Omnis autostainer (Agilent Technologies) using the EnVision FLEX+ Mouse (LINKER) kit. In short, antigen retrieval was performed on the TMA with sodium citrate-solution (pH 6.0) for p16, or a high pH-buffer (pH 9.0) for p53, pRb, and MCPyV. Endogenous peroxidase was blocked with Dako REAL Peroxidase-Blocking Solution prior to 20 minutes of incubation with the primary antibody. Binding of the antibodies was visualized by an enzymatic reaction with horseradish peroxidase and 3,3'-Diaminobenzidine as substrate, producing a brown precipitate (**Figure 1**). Slides were counterstained with hematoxylin and evaluated under a bright field microscope by two independent assessors, blinded for patients' clinical characteristics. In case of dissonance between the assessors, a third assessor evaluated the staining and agreement was reached by discussion. For p16, strong homogenous staining in the cytoplasm and nuclei of >70% of the tumor cells was considered to be overexpression (28). p53 staining was assessed as 0-mutant type (0% nuclear staining), mutant-type overexpression (>70% strong nuclear staining in the non-keratinizing tumor cells), or wild-type (heterogeneous nuclear staining) (29). For pRb, nuclear staining in <25% of tumors cells was evaluated as loss of pRb (30). MCPyV LTAg was considered positive if >10% of nuclei were stained (31). For p16, a case of HPV-positive OPSCC was used as a control, and for p53 and pRb normal tonsil tissue was used. MCPyV positive cell lines MKL-1, MKL-2, and WaGa served as positive controls for the LTAg of MCPyV.

Human Papillomavirus-Specific Polymerase Chain Reaction

Of patients with a positive result for HPV RNA-ISH and/or p16 IHC, DNA was isolated from eight 5-µm FFPE whole tissue sections with the Maxwell RSC DNA FFPE kit (Promega, Madison, Wisconsin). DNA concentrations were determined using the Quantus Fluorometer and the QuantiFluor ONE

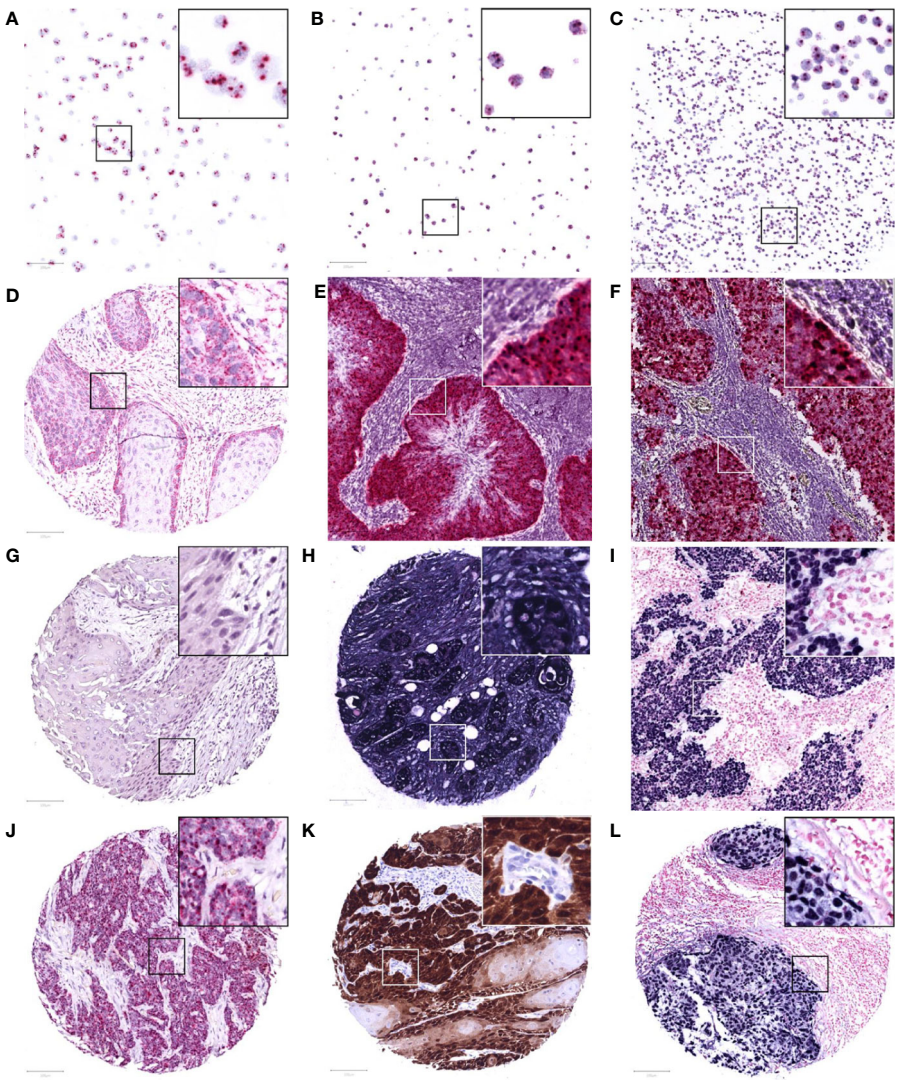

FIGURE 1 | Representative images of RNAscope *in situ* hybridization on cell lines Caski **(A)**, HeLa **(B)**, and WaGa **(C)**, positive for HPV-16, HPV-18, and MCPyV, respectively. A positive and negative control with housekeeping gene transcript PPIB **(D)** and bacterial transcript dapB **(G)** on the TMA, and a control patient positive for HPV-16 and MCPyV **(E, F)**. Positive internal control for housekeeping gene GAPDH **(H)** and a control case of EBV-positive infectious mononucleosis **(I)** following Epstein-Barr virus encoded RNA *in situ* hybridization. Study TMA cores of patients positive for HPV-16 mRNA (at least 1 red punctate dot per tumor cell), p16 immunohistochemistry (>70% strong brown staining of tumor nuclei and cytoplasm), and EBV mRNA (>50% strong blue staining of tumor nuclei) are presented in **(J–L)**, respectively. The images were taken at 200x magnification, an area of 100 μm^2 is marked in each image and 3x magnified in its top right corner.

TABLE 1 | Immunohistochemistry primary antibodies and evaluation criteria.

Antibody characteristics		Source	Clone	Dilution	Retrieval	Localization	Evaluation criteria	
Antibody	**Company**						**Cut off**	**References**
p16	Immunologic	Monoclonal, Mouse	MX007	1:200	Citrate (pH 6.0)	Nuclear and cytoplasmic	>70%	(28)
p53	Dako Omnis	Monoclonal, Mouse	DO-7	Ready-to-use	High pH buffer (pH 9.0)	Nuclear	0% >70%	(29)
pRb	Leica Biosystems	Monoclonal, Mouse	13a10	1:100	High pH buffer (pH 9.0)	Nuclear	≥25%	(30)
Large T-antigen	Santa Cruz Biotechnology	Monoclonal, Mouse	CM2B4	1:50	High pH buffer (pH 9.0)	Nuclear	>10%	(31)

dsDNA system (Promega). Next, 250 ng DNA was added to 1 ml SurePath preservative fluid (VWR International, Amsterdam, Netherlands) and used for HPV-DNA analysis utilizing the COBAS 4800 platform (Roche, Basel, Switzerland), according to the manufacturer's instructions. The COBAS 4800 tests specifically for HPV-16, HPV-18, and a combination of 12 other HR-HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Results were considered reliable when the controls were labeled "valid". Housekeeping gene β-globin was used as a control for the human DNA, in addition to DNA samples of an HPV-positive and HPV-negative tumor.

Statistical Analysis

Patients were considered to be HPV or MCPyV positive when the virus was detected with at least two techniques (ISH, IHC and/or PCR). EBV presence was based on the EBER-ISH result. Differences in clinical parameters between virus positive and virus negative patients were evaluated by Mann-Whitney U test for age because of a non-normal distribution, Fisher's exact test for binominal variables (sex, M-stage, recurrence, p16, pRb), and the Fisher-Freeman-Halton exact test for tumor location, T-stage, N-stage, and p53 because of expected counts of less than five. The 5-year DFS and OS were estimated with Kaplan-Meier curves and differences between virus positive and negative tumors were determined by log rank test. DFS was defined as the last date of treatment until the biopsy date of a histologically proven recurrence or second primary tumor in the head and neck region. OS was defined as the time between the primary tumor biopsy date and death. Censoring took place when patients were lost to follow-up, deceased without recurrent disease for DFS, or at the cut-off point of 60 months. All clinical and pathological parameters were assessed in bivariate analysis regarding survival. Variables with significant ($p < 0.05$) or near significant ($p < 0.1$) relationships were evaluated in multinomial logistic regression to assess predictors for DFS and OS. Analyses were performed using IBM SPSS Statistics 25.0 (IMB corp., Armonk, NY) and a p-value of <0.05 was considered to be statistically significant.

RESULTS

A total of 119 patients were included in this study. These patients had a median age of 74.9 years (inter quartile range = 14.6 years) and were mainly women (78%) with a tumor of the oral cavity (80%) and no regional or distant metastases (66 and 95%, respectively). Thirty-one patients (26%) had recurrent disease within 5 years (**Table 2**).

Human Papillomavirus

ISH on the TMAs showed HPV-16/18 mRNA expression in tumors of ten patients. All of these tumors showed p16 overexpression by IHC as well, in addition to five tumors with no HPV-16/18 mRNA expression. COBAS analysis on HR-HPV DNA in these 15 patients detected the presence of HPV-16 in ten tumors and another HR-HPV type in one other case. For patient 61, the quality of the DNA was insufficient for COBAS analysis. This resulted in a total of 12 tumors being HPV-positive based on at least two detection techniques (**Table 3**).

Compared to HPV-negative tumors, HPV-positive tumors were associated with lower age (67.3 versus 76.2 years old, $p = 0.003$), oropharyngeal origin (83% versus 0%, $p < 0.001$), and N2-stage (58% versus 13%, $p = 0.004$) (**Table 2**). All oropharyngeal

TABLE 2 | Comparison of clinical characteristics between human papillomavirus (HPV) and Epstein–Barr virus (EBV) positive and negative head and neck squamous cell carcinoma in non-smokers and non-drinkers.

Clinical characteristics		Total (n = 119)		HPV-positive (n = 12)		HPV-negative (n = 107)		p-value	EBV-positive (n = 3)		EBV-negative (n = 116)		p-value
Age (years)	Median (interquartile range)	74.9	(14.6)	67.3	(13.2)	76.2	(14.7)	0.003	48.0	(NA*)	75.3	(14.1)	0.062
		n	*(%)*	*n*	*(%)*	*n*	*(%)*		*n*	*(%)*	*n*	*(%)*	
Sex	Female	93	(78)	10	(83)	83	(78)	1.0	2	(67)	91	(78)	0.53
	Male	26	(22)	2	(17)	24	(22)		1	(33)	25	(22)	
Location	Hypopharynx	1	(0.8)	0	(0)	1	(0.9)	<0.001	0	(0)	1	(0.9)	<0.001
	Larynx	9	(7.6)	0	(0)	9	(8.4)		0	(0)	9	(7.8)	
	Nasopharynx	4	(3.4)	1	(8.3)	3	(2.8)		3	(100)	1	(0.9)	
	Oral cavity	95	(80)	1	(8.3)	94	(88)		0	(0)	95	(82)	
	Oropharynx	10	(8.4)	10	(83)	0	(0)		0	(0)	10	(8.6)	
T-stage	1	33	(28)	3	(25)	30	(28)	0.35	1	(33)	32	(28)	1.0
	2	36	(30)	2	(17)	34	(32)		1	(33)	35	(30)	
	3	13	(11)	3	(25)	10	(9.3)		0	(0)	13	(11)	
	4	37	(31)	4	(33)	33	(31)		1	(33)	1	(31)	
N-stage	0	78	(66)	5	(42)	73	(68)	0.004	0	(0)	78	(67)	0.061
	1	18	(15)	0	(0)	18	(17)		1	(33)	17	(15)	
	2	21	(18)	7	(58)	14	(13)		2	(67)	19	(16)	
	3	2	(1.7)	0	(0)	2	(1.9)		0	(0)	2	(1.7)	
M-stage	0	113	(95)	11	(92)	102	(95)	0.42	3	(100)	111	(96)	1.0
	1	6	(5.0)	1	(8.3)	5	(4.7)		0	(0)	5	(4.3)	
Recurrence	Yes	31	(26)	2	(17)	29	(27)	0.73	2	(67)	29	(25)	0.17
	No	88	(74)	10	(83)	78	(73)		1	(33)	87	(75)	

**Not applicable because of small number of patients.*

TABLE 3 | Demographics and viral analysis results of 15 virus positive tumors in non-smokers and non-drinkers.

Virus	Study ID	Age (years)	Sex	Tumor location	T	N	M	Recurrence	ISH	p16 IHC	COBAS PCR
HPV	1	56.4	Male	Oropharynx	3	2	0	No	+*	+	HPV-16
	3	53.9	Female	Oropharynx	1	2	0	No	+*	+	HPV-16
	4	71.5	Male	Oropharynx	4	2	0	No	+*	+	HPV-16
	5	76.6	Female	Oropharynx	2	2	0	No	+*	+	HPV-16
	9	69.5	Female	Oropharynx	3	0	0	No	-*	+	HR-HPV
	16	68.3	Female	Oropharynx	1	0	0	No	+*	+	HPV-16
	21	71.8	Female	Oropharynx	4	2	1	Yes	+*	+	HPV-16
	29	57.8	Female	Nasopharynx	2	0	0	No	+*	+	HPV-16
	32	63.5	Female	Oral cavity	4	0	0	No	-*	+	HPV-16
	40	67.9	Female	Oropharynx	4	2	0	No	+*	+	HPV-16
	61	66.6	Female	Oropharynx	3	0	0	Yes	+*	+	Invalid
	122	57.9	Female	Oropharynx	1	2	0	No	+*	+	HPV-16
EBV	6	48.0	Male	Nasopharynx	4	2	0	Yes	+†	NA	NA
	13	47.3	Female	Nasopharynx	2	1	0	No	+†	NA	NA
	18	74.6	Female	Nasopharynx	1	2	0	Yes	+†	NA	NA

*ISH, in situ hybridization; IHC, immunohistochemistry; PCR, polymerase chain reaction; +, positive; -, negative; *, using RNAscope; †, using EBER-ISH; HR-HPV, high-risk human papilloma virus; EBV, Epstein-Barr virus; NA, not applicable.*

tumors (n = 10) were HPV-positive, in addition to one OSCC of the alveolar process and a NPSCC. Although the OSCC case showed HPV-16 DNA and p16 overexpression, no mRNA was detected with ISH, neither on the TMA nor on a whole section.

Four of the twelve patients with HPV-positive tumors (33%) died within five years, two of which had recurrent disease. For patient 1 there were no details recorded on the cause of death (OS = 59.6 months), patient 5 died of the complications of an aortic valve prosthesis endocarditis (OS = 23.7 months), patient 21 received palliative treatment because of distant metastasis (DFS = 1.6 months), and patient 61 developed liver metastases 20.1 months after initial therapy (**Table 3**). The presence of HPV was no predictor for a better DFS or OS ($p = 0.33$ and $p = 0.27$, respectively), compared to HPV-negative HNSCC in NSND (**Figures 2A, B**). A younger age at cancer diagnosis ($p = 0.008$), T1 stage ($p = 0.0047$), and N0 or N1 stage (both $p < 0.001$) were

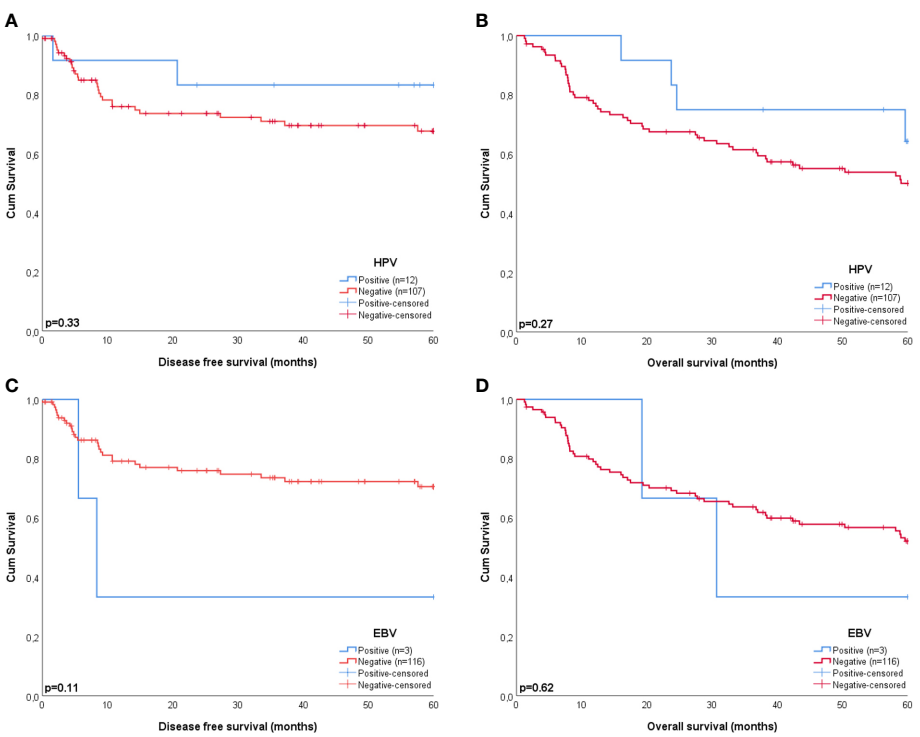

FIGURE 2 | Kaplan-Meier curves estimating the survival of patients with HPV **(A, B)** and EBV **(C, D)** positive and negative tumors. HPV and EBV were no significant predictors for a better disease free ($p = 0.33$ and $p = 0.11$, respectively) or overall survival ($p = 0.27$ and $p = 0.62$, respectively).

TABLE 4 | Multivariable analysis of predictors for 5-year overall survival in non-smokers and non-drinkers with head and neck squamous cell carcinoma.

Parameter	Coefficient (β)	Standard error	Wald X^2	OR	95% CI		p-value
					Lower	Upper	
Age	0.053	0.020	7.0	1.05	1.0	1.1	0.008
T-stage (reference T4)							
T1	-1.2	0.86	3.9	0.31	0.099	0.99	0.047
T2	-0.67	0.54	1.5	0.51	0.18	1.5	0.22
T3	0.79	0.74	1.1	2.2	0.52	9.4	0.29
N-stage (reference N3)							
N0	-19	0.59	1081	<0.001	<0.001	<0.001	<0.001
N1	-17	0.78	506	<0.001	<0.001	<0.001	<0.001
N2	-18	0.00	NA	<0.001	<0.001	<0.001	NA

NA, not available because of small number of cases.
Nagelkerke R^2 = 0.35.

TABLE 5 | Expression of cell cycle proteins p16, p53, and pRb in virus positive and negative tumors.

Cell cycle protein		Virus positive		Virus negative		p-value	HPV-positive		HPV-negative		p-value	EBV-positive		EBV-negative		p-value
		n	(%)	n	(%)		n	(%)	n	(%)		n	(%)	n	(%)	
p16	Overexpression	12	(80)	3	(2.9)	<0.001	12	(100)	3	(2.8)	<0.001	0	(0)	15	(13)	1.0
	No overexpression	3	(20)	101	(97)		0	(0)	104	(97)		3	(100)	101	(87)	
p53	Mutant-type overexpression	2	(13)	51	(49)	0.002	0	(0)	53	(50)	<0.001	2	(67)	51	(44)	0.80
	Wild-type	11	(73)	28	(27)		10	(83)	29	(27)		1	(33)	38	(33)	
	0-mutant type	2	(13)	25	(24)		2	(17)	25	(23)		0	(0)	27	(23)	
pRb	Positive (preserved expression)	5	(33)	84	(81)	<0.001	2	(17)	87	(81)	<0.001	3	(100)	86	(74)	0.57
	Loss	10	(67)	20	(19)		10	(83)	20	(19)		0	(0)	30	(26)	

HPV, human papillomavirus; EBV, Epstein–Barr virus.

retained in the best multivariable model as predictors for OS in HNSCC of NSND (**Supplementary table 1**, **Table 4**). The model explained 35% of the variation (model fit: omnibus test of model coefficients: $X^2 = 36.0$ and $p < 0.001$; M-stage was omitted because of low case numbers).

HPV-positive tumors showed significantly more often p16 overexpression, p53 wild-type expression, and loss of pRb than HPV-negative tumors (p16: 100 versus 2.8%, $p < 0.001$; p53: 83 versus 27%, $p < 0.001$; pRb: 83 versus 19%, $p < 0.001$) (**Table 5**).

Epstein–Barr Virus

Three tumors were EBV positive as detected by EBER-ISH. These patients all had a tumor of the nasopharynx (100 versus 0.9% in EBV-negative tumors, $p < 0.001$), resulting in virus positivity of all four nasopharyngeal tumors in this cohort (three containing EBV and one HPV). Two of the three patients (67%) with EBV-positive tumors were below 50 years of age and non-Caucasian (Northern African and East Asian) and they all had regional metastases (**Tables 2**, **3**).

Patient 6 and 18 were both diagnosed with recurrent disease, the former with distant metastases 8.3 months after chemoradiotherapy and the latter with regional metastases 5.5 months after locoregional radiotherapy, which eventually led to their death. Although this resulted in a 33% 5-year survival for patients with EBV-positive tumors, EBV was no significant predictor for DFS or OS in this cohort, compared to EBV-negative HNSCC ($p = 0.11$ and $p = 0.62$, respectively) (**Figures 2C, D**).

None of the EBV-positive tumors showed p16 overexpression, all were positive for pRb and two of the three tumors (66%) showed p53 mutant-type overexpression. This did not differ significantly from cell cycle protein expression in EBV-negative tumors, most probably because of the small number of EBV-positive tumors (**Table 5**).

Merkel Cell Polyomavirus

MCPyV was not detected in any of the samples with RNA-ISH or IHC against the LTAg of MCPyV.

Virus Negative Tumors

None of the squamous cell carcinomas of the oral tongue (OTSCC) (n = 39), larynx (LSCC) (n = 9), or hypopharynx (HPSCC) (n = 1) showed involvement of HPV, EBV, or MCPyV. Except for one tumor of the alveolar process, all other OSCC (n = 54) were virus negative as well (**Table 2**). Although the patients with virus negative tumors were relatively old (mean age of 75 years) when being diagnosed with HNSCC, the 5-year OS of these patients was still 50% (**Figure 2**).

The three patients with virus negative OSCC containing p16 overexpression all had recurrent disease within 6 months after surgical resection [a T4N1M0 floor of mouth tumor, after an irradical resection the patient wished no further treatment (DFS = 0 months), OS = 27.4 months; a T4N0M0 oral cavity tumor (not otherwise specified), DFS = 5.4 months after resection, OS = 8.2 months; a T2N1M0 retromolar triangle

TABLE 6 | Multivariable analysis of predictors for 5-year overall survival in non-smokers and non-drinkers with virus negative oral squamous cell carcinoma.

Parameter	Coefficient (β)	Standard error	Wald X^2	OR	95% CI		p-value
					Lower	Upper	
Age	0.051	0.022	5.3	1.05	1.0	1.1	0.021
N-stage (reference N3)							
N0	-20	0.72	803	<0.001	<0.001	<0.001	<0.001
N1	-18	0.89	414	<0.001	<0.001	<0.001	<0.001
N2	-19	0.00	NA	<0.001	<0.001	<0.001	NA

NA, not available because of small number of cases.
Nagelkerke R^2 = 0.31.

tumor, DFS 4.8 months after resection, OS = 11.8 months]. Two of these tumors showed loss of pRb expression. A poor DFS (2.3, 8.8, and 20.3 months) was also found in three other tumors in this cohort with some p16 expression (>50%): all OTSCC with a pRb expression above 50%. Apart from one LSCC, all tumors with loss of pRb were OSCC (19/20) without any expression of p16. A younger age at cancer diagnosis (p = 0.021) and N0 or N1 stage (both $p < 0.001$) were retained in the best multivariable model as predictors for OS in NSND with virus negative OSCC (**Supplementary Table 2**, **Table 6**). The model explained 31% of the variation (model fit: omnibus test of model coefficients: $X^2 = 24.4$ and $p < 0.001$; M-stage and p16 were omitted because of low case numbers).

Over 70% (76/104) of virus negative tumors showed mutant-type p53 expression, with 0-type mutant expression in 20% (19/94) of OSCC, 56% (5/9) of LSCC, and 100% (1/1) of HPSCC, and mutant-type overexpression in 52% (49/94) of OSCC and 22% (2/9) of LSCC (**Table 5**). Cell cycle protein expression was no predictor for a better DFS or OS in patients with virus negative tumors (data not displayed).

DISCUSSION

The objective of this study was to determine if HPV, EBV, and MCPyV play a role in head and neck carcinogenesis of NSND, the role of cell cycle proteins p16, p53, and pRb regarding viral presence, and the influence of these viruses and proteins on patient survival. In this cohort of 119 NSND, the ten oropharyngeal (100%) and four nasopharyngeal (100%) tumors contained either HPV or EBV. Besides one oral cavity tumor, all other specimens of the oral cavity, hypopharynx, and larynx were HPV, EBV, and MCPyV-negative. Virus positivity did not predict better disease free or overall survival. Regarding cell cycle protein expression, HPV-positive tumors showed more p16 overexpression, wild-type p53 expression, and loss of pRb compared to HPV-negative tumors. OSCC with >70% p16 expression had a poor DFS and OS, with loss of pRb in two of the three cases. The other pRb negative tumors were mainly OSCC as well and did not show p16 expression. Mutant type p53 expression was observed in over 70% of virus negative HNSCC.

As the worldwide HPV prevalence is rising, a wide range has been reported in the literature, ranging from 20% in OPSCC patients from Eastern Asia or Central America, to over 50% in Europe and Australia, based on HPV DNA combined with E6*I mRNA or p16 IHC (5, 6). A recent systematic review specifically analyzing patients without tobacco or alcohol consumption reported an OPSCC HPV prevalence of over 60% in non-smokers and over 40% in non-drinkers, compared to 20% in smokers and drinkers, based on at least two detection techniques (combining PCR, ISH, IHC, or sequencing) (25). This is a lower prevalence than the 100% (10/10) HPV infections of OPSCC in the current study. Possibly, the low number of OPSCC in the current study explains the 100% prevalence, as it could be a coincidence that they were all HPV-positive. Nonetheless, it is acknowledged that HPV plays an increasingly substantial role in OPSCC carcinogenesis of patients without the traditional risk factors. The HPV prevalence of 1.8% (2/109) in non-OPSCC as found in the current study is comparable to the low prevalence in other studies (32–35).

In this study, 4.2% (4/95) of OSCC showed p16 overexpression with IHC, and one of those contained HPV-16 DNA following COBAS analysis resulting in HPV-positivity according to detection by two methods. p16 overexpression could result from loss of pRb function *via* structural alterations, or maybe as a result of other oncogenic viruses affecting pRb expression that we are not aware of (36). Lechner and colleagues speculated that high levels of protein p16 could also occur in cells irrespective of pRb expression or HPV-positivity, as a result of enrichment for *NSD1* mutations in *CDKN2A* wild-type tumors (36, 37). *NSD1* is coding for Histone H3K36 methyltransferase, which is associated with DNA hypomethylation, resulting in p16 overexpression when mutated by not being able to regulate its expression *via* methylation anymore (36). Indeed, p16 overexpression has been reported in non-OPSCC, without a correlation to HPV infection nor as a predictor for survival (34, 38). Therefore, The College of American Pathologists does not endorse routine p16 screening for non-OPSCC (39). As the one HPV-positive OSCC in this cohort had no loss of pRb and lacked HPV mRNA in a whole section following RNAscope ISH analysis, the p16 overexpression could be a result of *CDKN2A* mutation, and HPV a commensal with the HPV DNA not located in the tumor cells but in the adjacent mucosal epithelial cells (40). The three HPV-negative OSCC with p16 overexpression in the current study had a poor DFS. This could be a result of pRb loss, although for the whole study group p16 overexpression was no significant predictor for survival. Additionally, HNSCC could be the result of a genetic predisposition. OSCC has been associated with specific *CDKN2A*

germline mutations, accompanied with loss of heterogeneity of the wild-type allele, in a small fraction of young NSND (41).

The 5-year OS of HNSCC patients in general is 40–50%, whereas it is 70–80% for patients with HPV-positive OPSCC (2, 42). In this study, there was no significant difference in OS or DFS between virus positive and virus negative patients. However, it is not certain if the survival comparison of HPV-positive versus HPV-negative HNSCC was one of HPV-positivity or of tumor location (OPSCC versus non-OPSCC), as in this cohort HPV-negative tumors were exclusively non-OPSCC. The same applies to EBV-positive tumors, which were all NPSCC. Therefore, these survival analyses should be interpreted with caution. The 5-year OS of 67% for patients with HPV-positive tumors was lower than expected, but these patients were relatively old with a median age of 67.3 years. Patients with HPV-negative HNSCC (53%) did not differ significantly in 5-year OS from patients with HPV-positive OPSCC, even though they had a median age of almost 9 years older than the patients with HPV-positive tumors (76.2 versus 67.3 years). So, considering their age at the time of HNSCC diagnosis, the HPV-negative NSND, mainly with OSCC, had a relatively good 5-year OS. Nevertheless, a young age at the time of cancer diagnosis was predictive of a better OS in both the virus negative OSCC group and the whole NSND cohort, besides a T1 stage and a N0 or N1 stage.

Viral association in all four NPSCC patients of this cohort was as expected. Although the worldwide incidence of EBV related NPSCC has been decreasing over the past decade, the prevalence is still high with almost 100% in endemic regions (southern China, Southeast Asia, Northern Africa, and the Mediterranean basin), and 60–85% in non-endemic regions (9, 43–46). NPSCC infections with HPV are less common, and have been reported in studies from non-endemic regions like West Africa and Europe (outside the Mediterranean basin), with a prevalence between 1.6–16% (43, 45, 47, 48). Based on 517 U.S. NPSCC patients with known HPV testing (34.8% HPV-positive) in the Surveillance, Epidemiology and End Results database, predictors for HPV-positivity in NPSCC have been established: being younger than 25-years-old, Caucasian (rather than East-Asian or other ethnicities), an AJCC-7 stage other than stage 1, and no distant metastases (M0) (49). Indeed, the one patient in our cohort with HPV-positive NPSCC was a Caucasian patient with stage 2 disease.

Expression of cell cycle proteins p16 and pRb was as expected in the HPV-positive OPSCC of this cohort, with overexpression of the former and loss of the latter. Although p53 is usually degraded by HPV's viral oncoprotein E6, only two HPV-positive tumors showed 0-mutant type p53 expression, whereas the other 10 showed wild-type p53 expression. This is in agreement with earlier observations, where there was p53 expression in 7/10 HPV-positive tumors of non-smokers, despite absence of *TP53* mutations (50). Possible explanations for this finding are virally induced processes, such as hypoxia, oxidative stress, or impaired repair of double strand DNA breaks (50, 51). On the other hand, up to 55–75% of HPV-negative OSCC contain *TP53* mutations, which is comparable to the 72% of HPV-negative OSCC showing mutant-type p53 expression (either 0-type or overexpression) in the current study (36, 52). The EBV-positive tumors were all without p16 overexpression and with preserved pRb expression. Zhang and colleagues reported that the cell cycle pathway is the most deregulated pathway in NPSCC in comparison to non-tumor nasopharyngeal epithelium, with down-regulation of p16 and up-regulation of pRb (19). Although the precise mechanism of p16 inactivation by EBV in NPSCC remains unclear, it has been suggested that the Late Membrane Protein 1 of EBV could inhibit p16 expression and induce pRb phosphorylation, promoting the progression of the G1/S phase (19). This is in accordance with the cell cycle protein expressions found in the current study.

No MCPyV was detected in the current study, which contrasts earlier findings. Hamiter and colleagues performed PCR using specific primers for the regulatory and LTAg of MCPyV, followed by DNA sequence analysis to confirm viral presence in 6/21 (29%, three were NSND) patients with OTSCC (16). Although the OTSCC group in the current study was almost twice the size (n = 39), no MCPyV presence was detected. Other studies report MCPyV DNA in 4–50% of HNSCC based on quantitative real-time PCR, though with low viral loads (14, 15, 17). This discrepancy could be the result of differences in sensitivity between the used detection methods (RNA-ISH and LTAg IHC versus DNA PCR and sequencing). However, the MCPyV presence in the literature was solely based on PCR and was not confirmed with another detection method. Nevertheless, the significance of MCPyV presence in HNSCC has yet to be determined, but our data strengthen the premise that MCPyV is not likely to play an important role in head and neck carcinogenesis.

HPV has its clinical relevance in routine practice as a prognostic marker in OPSCC, with a better DFS and OS, in addition to an improved radiosensitivity compared to HPV-negative OPSCC because of altered DNA repair, reduced hypoxic regions, and an increased cellular immune response (3, 53). With the conduction of multiple phase III de-escalation trials for HPV-positive OPSCC, and the high HPV prevalence in OPSCC of NSND, the treatment of NSND may be affected in the near future (4). For EBV-positive NPSCC, treatment usually consists of radiotherapy, with or without chemotherapy. Besides the anti-tumor effects of radiotherapy based on direct and indirect DNA damage, it also induces an immune response comprising of a network of immune-stimulatory and –inhibitory signals like up-regulation of immune checkpoint proteins such as PD-1/PD-L1 (54). Consequently, there are a number of clinical trials evaluating the incorporation of immune checkpoint inhibitors in the treatment of EBV-positive NPSCC (10, 54). Mutant p53 has been associated with resistance to chemoradiation in OSCC and an increased risk of locoregional recurrence and metastases (55). Since NSND mainly have OSCC and frequent mutant-type p53 expression (as presented in this study), p53 could be used to predict therapy failure in case of recurrent disease.

One of the limitations of this study was that the definition of when a patient was a non-smoker and non-drinker was collected retrospectively from their medical records. Nevertheless, a strict definition was used (for example with exclusion of patients with

"sporadic" alcohol consumption), based on a standard history taking template including specific questions on any current or previous tobacco and alcohol consumption, during two separate hospital visits (Head and Neck outpatient clinic and Anesthesiology screening). Secondly, there was a small group of patients with tumors at anatomical sites other than the oral cavity (OSCC). In combination with the strict NSND definition, this resulted in no virus negative OPSCC and NPSCC. However, it has been reported that NSND are mainly patients with OSCC, so a small number of tumors outside the oral cavity was expected (25, 56). Thirdly, there might be a higher percentage of HNSCC positive for HPV DNA in this cohort, because COBAS PCR analysis was not performed on all samples. Conversely, HPV is only considered to be predictive for survival when being transcriptionally active, and since all tumors were tested on HPV-16/18 mRNA and p16 IHC, we expect to have detected the tumors with biologically active HPV infection (7, 8). Finally, some of the FFPE material was rather old (>25 years of storage), which is known to often result in breakdown of the nucleic acids. Indeed, DNA quality of DNA extracted from one tumor was insufficient for COBAS analysis. Nevertheless, the TMA blocks were freshly sectioned before ISH, IHC, or PCR, and all the positive controls were adequate.

CONCLUSION

A high prevalence of HPV and EBV was observed in OPSCC and NPSCC of NSND respectively, but not in HNSCC outside these locations. Although a significant role of oncogenic viruses would be assumed in this specific patient group lacking the traditional risk factors for developing HNSCC, HPV, EBV, and MCPyV were not detected in this relatively large cohort of 95 OSCC apart from one case, using clinically relevant cut-off values. This argues against a central role of these viruses in the etiopathogenesis of oral, hypopharyngeal, and laryngeal squamous cell carcinoma in this specific patient group. With ongoing de-escalation trials for HPV-positive OPSCC and trials for immune checkpoint inhibitors in the treatment of EBV-positive NPSCC, the treatment of NSND with tumors at those locations may change in the near future. However, for the majority of NSND with virus negative OSCC, more research is needed to understand the carcinogenic mechanisms in order to consider targeted therapeutic options.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Maastricht University Medical Center (METc 2018-0567). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors have contributed substantially to the conception and design (FM, SW, BK, and ES), acquisition of data (FM, FK, FJ, and FF), analysis and interpretation of data (FM, SW, RB, AH, MH, BK, and ES); drafting the article (FM, FJ, and ES) or revising it critically for important intellectual content (FK, FF, SW, RB, AH, MH, and BK); final approval of the version to be published (FM, FK, FJ, FF, SW, RB, AH, MH, BK, and ES); and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.560434/full#supplementary-material

REFERENCES

1. Brierley JDG, Gospodarowicz MK, Wittekind C. *TNM Classification of Malignant Tumours 8th Edition*. New Jersey: Wiley-Blackwell (2016).
2. O'Rorke MA, Ellison MV, Murray LJ, Moran M, James J, Anderson LA. Human papillomavirus related head and neck cancer survival: a systematic review and meta-analysis. *Oral Oncol* (2012) 48(12):1191–201. doi: 10.1016/j.oraloncology.2012.06.019
3. 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):1813–20. doi: 10.1002/ijc.22851
4. Mirghani H, Blanchard P. Treatment de-escalation for HPV-driven oropharyngeal cancer: Where do we stand? *Clin Trans Radiat Oncol* (2018) 8:4–11. doi: 10.1016/j.ctro.2017.10.005
5. Castellsague X, Alemany L, Quer M, Halec G, Quiros B, Tous S, et al. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients. *J Natl Cancer Institute* (2016) 108(6):djv403. doi: 10.1093/jnci/djv403
6. Hong A, Lee CS, Jones D, Veillard AS, Zhang M, Zhang X, et al. Rising prevalence of human papillomavirus-related oropharyngeal cancer in Australia over the last 2 decades. *Head Neck* (2016) 38(5):743–50. doi: 10.1002/hed.23942
7. Jung AC, Briolat J, Millon R, de Reynies A, Rickman D, Thomas E, et al. Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. *Int J Cancer* (2010) 126(8):1882–94. doi: 10.1002/ijc.24911
8. Mena M, Taberna M, Tous S, Marquez S, Clavero O, Quiros B, et al. Double positivity for HPV-DNA/p16ink4a is the biomarker with strongest diagnostic accuracy and prognostic value for human papillomavirus related

oropharyngeal cancer patients. *Oral Oncol* (2018) 78:137–44. doi: 10.1016/j.oraloncology.2018.01.010
9. Chan JK. Virus-associated neoplasms of the nasopharynx and sinonasal tract: diagnostic problems. *Modern Pathol* (2017) 30(s1):S68–s83. doi: 10.1038/modpathol.2016.189
10. Outh-Gauer S, Alt M, Le Tourneau C, Augustin J, Broudin C, Gasne C, et al. Immunotherapy in head and neck cancers: A new challenge for immunologists, pathologists and clinicians. *Cancer Treat Rev* (2018) 65:54–64. doi: 10.1016/j.ctrv.2018.02.008
11. Yu F, Lu Y, Petersson F, Wang DY, Loh KS. Presence of lytic Epstein-Barr virus infection in nasopharyngeal carcinoma. *Head Neck* (2018) 40(7):1515–23. doi: 10.1002/hed.25131
12. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Sci (N Y NY)* (2008) 319(5866):1096–100. doi: 10.1126/science.1152586
13. Kassem A, Schopflin A, Diaz C, Weyers W, Stickeler E, Werner M, et al. Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene. *Cancer Res* (2008) 68(13):5009–13. doi: 10.1158/0008-5472.CAN-08-0949
14. Tanio S, Matsushita M, Kuwamoto S, Horie Y, Kodani I, Murakami I, et al. Low prevalence of Merkel cell polyomavirus with low viral loads in oral and maxillofacial tumours or tumour-like lesions from immunocompetent patients: Absence of Merkel cell polyomavirus-associated neoplasms. *Mol Clin Oncol* (2015) 3(6):1301–6. doi: 10.3892/mco.2015.629
15. Salakova M, Koslabova E, Vojtechova Z, Tachezy R, Sroller V. Detection of human polyomaviruses MCPyV, HPyV6, and HPyV7 in malignant and non-malignant tonsillar tissues. *J Med Virol* (2016) 88(4):695–702. doi: 10.1002/jmv.24385
16. Hamiter M, Asarkar A, Rogers D, Moore-Medlin T, McClure G, Ma X, et al. A pilot study of Merkel cell polyomavirus in squamous cell carcinoma of the tongue. *Oral Oncol* (2017) 74:111–4. doi: 10.1016/j.oraloncology.2017.09.021
17. Mohebbi E, Noormohamadi Z, Sadeghi-Rad H, Sadeghi F, Yahyapour Y, Vaziri F, et al. Low viral load of Merkel cell polyomavirus in Iranian patients with head and neck squamous cell carcinoma: Is it clinically important? *J Med Virol* (2018) 90(2):344–50. doi: 10.1002/jmv.24953
18. Angiero F, Gatta LB, Seramondi R, Berenzi A, Benetti A, Magistro S, et al. Frequency and role of HPV in the progression of epithelial dysplasia to oral cancer. *Anticancer Res* (2010) 30(9):3435–40.
19. Zhang W, Zeng Z, Zhou Y, Xiong W, Fan S, Xiao L, et al. Identification of aberrant cell cycle regulation in Epstein-Barr virus-associated nasopharyngeal carcinoma by cDNA microarray and gene set enrichment analysis. *Acta Biochim Biophys Sin (Shanghai)* (2009) 41(5):414–28. doi: 10.1093/abbs/gmp025
20. Harms PW, Harms KL, Moore PS, DeCaprio JA, Nghiem P, Wong MKK, et al. The biology and treatment of Merkel cell carcinoma: current understanding and research priorities. *Nat Rev Clin Oncol* (2018) 15(12):763–76. doi: 10.1038/s41571-018-0103-2
21. Tornesello ML, Annunziata C, Tornesello AL, Buonaguro L, Buonaguro FM. Human Oncoviruses and p53 Tumor Suppressor Pathway Deregulation at the Origin of Human Cancers. *Cancers* (2018) 10(7):213. doi: 10.3390/cancers10070213
22. Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: a role for HPV. *Oral Oncol* (2009) 45(6):486–91. doi: 10.1016/j.oraloncology.2008.07.008
23. Farshadpour F, Konings S, Speel EJ, Hordijk GJ, Koole R, van Blokland M, et al. Human Papillomavirus and Oropharyngeal Squamous Cell Carcinoma: A Case-Control Study regarding Tobacco and Alcohol Consumption. *Pathol Res Int* (2011) 2011:806345. doi: 10.4061/2011/806345
24. Laco J, Vosmikova H, Novakova V, Celakovsky P, Dolezalova H, Tucek L, et al. The role of high-risk human papillomavirus infection in oral and oropharyngeal squamous cell carcinoma in non-smoking and non-drinking patients: a clinicopathological and molecular study of 46 cases. *Virchows Archiv An Int J Pathol* (2011) 458(2):179–87. doi: 10.1007/s00428-010-1037-y
25. Mulder FJ, Pierssens D, Baijens LWJ, Kremer B, Speel EM. Evidence for different molecular parameters in head and neck squamous cell carcinoma of nonsmokers and nondrinkers: Systematic review and meta-analysis on HPV, p16, and TP53. *Head Neck* (2020) 41(1):303–22. doi: 10.1002/hed.26513
26. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Head and neck squamous cell carcinoma in non-smoking and non-drinking patients with multiple tumors: etiologic significance of p53 and Ki-67 in non-tumorous epithelium. *J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol* (2008) 37(9):549–54. doi: 10.1111/j.1600-0714.2008.00657.x
27. Chteinberg E, Sauer CM, Rennspiess D, Beumers L, Schiffelers L, Eben J, et al. Neuroendocrine Key Regulator Gene Expression in Merkel Cell Carcinoma. *Neoplasia* (2018) 20(12):1227–35. doi: 10.1016/j.neo.2018.10.003
28. Mooren JJ, Gultekin SE, Straetmans JM, Haesevoets A, Peutz-Kootstra CJ, Huebbers CU, et al. 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(9):2108–17. doi: 10.1002/ijc.28534
29. Li Y, Zhang J. Expression of mutant p53 in oral squamous cell carcinoma is correlated with the effectiveness of intra-arterial chemotherapy. *Oncol Lett* (2015) 10(5):2883–7. doi: 10.3892/ol.2015.3651
30. Plath M, Broglie MA, Forbs D, Stoeckli SJ, Jochum W. Prognostic significance of cell cycle-associated proteins p16, pRB, cyclin D1 and p53 in resected oropharyngeal carcinoma. *J Otolaryngol Head Neck Surg* (2018) 47(1):53. doi: 10.1186/s40463-018-0298-3
31. Leroux-Kozal V, Leveque N, Brodard V, Lesage C, Dudez O, Makeieff M, et al. Merkel cell carcinoma: histopathologic and prognostic features according to the immunohistochemical expression of Merkel cell polyomavirus large T antigen correlated with viral load. *Hum Pathol* (2015) 46(3):443–53. doi: 10.1016/j.humpath.2014.12.001
32. Brägelmann J, Dagogo-Jack I, El Dinali M, Stricker T, Brown CD, Zuo Z, et al. Oral cavity tumors in younger patients show a poor prognosis and do not contain viral RNA. *Oral Oncol* (2013) 49(6):525–33. doi: 10.1016/j.oraloncology.2013.02.003
33. Braakhuis BJ, Rietbergen MM, Buijze M, Snijders PJ, Bloemena E, Brakenhoff RH, et al. TP53 mutation and human papilloma virus status of oral squamous cell carcinomas in young adult patients. *Oral Dis* (2014) 20(6):602–8. doi: 10.1111/odi.12178
34. Ni Y, Zhang X, Wan Y, Dun Tang K, Xiao Y, Jing Y, et al. Relationship between p16 expression and prognosis in different anatomic subsites of OSCC. *Cancer Biomark* (2019) 26(3):375–83. doi: 10.3233/CBM-192402
35. Yang LQ, Xiao X, Li CX, Wu WY, Shen XM, Zhou ZT, et al. Human papillomavirus genotypes and p16 expression in oral leukoplakia and squamous cell carcinoma. *Int J Clin Exp Pathol* (2019) 12(3):1022–8.
36. The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* (2015) 517(7536):576–82. doi: 10.1038/nature14129
37. Lechner M, Chakravarthy AR, Walter V, Masterson L, Feber A, Jay A, et al. Frequent HPV-independent p16/INK4A overexpression in head and neck cancer. *Oral Oncol* (2018) 83:32–7. doi: 10.1016/j.oraloncology.2018.06.006
38. Rooper LM, Windon MJ, Hernandez T, Miles B, Ha PK, Ryan WR, et al. HPV-positive Squamous Cell Carcinoma of the Larynx, Oral Cavity, and Hypopharynx: Clinicopathologic Characterization With Recognition of a Novel Warty Variant. *Am J Surg Pathol* (2020) 44(5):691–702. doi: 10.1097/PAS.0000000000001433
39. Fakhry C, Lacchetti C, Rooper LM, Jordan RC, Rischin D, Sturgis EM, et al. Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. *J Clin Oncol* (2018) 36(31):3152–61. doi: 10.1200/JCO.18.00684
40. Boy S, Van Rensburg EJ, Engelbrecht S, Dreyer L, van Heerden M, van Heerden W. HPV detection in primary intra-oral squamous cell carcinomas–commensal, aetiological agent or contamination? *J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol* (2006) 35(2):86–90. doi: 10.1111/j.1600-0714.2006.00385.x
41. Fostira F, Koutsodontis G, Vagia E, Economopoulou P, Kotsantis I, Sasaki C, et al. Predisposing Germline Mutations in Young Patients With Squamous Cell Cancer of the Oral Cavity. *JCO Precis Oncol* (2018) 2):1–8. doi: 10.1200/PO.18.00022
42. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* (2011) 11(1):9–22. doi: 10.1038/nrc2982
43. Svajdler MJr., Kaspirkova J, Mezencev R, Laco J, Torday T, Dubinsky P, et al. Human papillomavirus and Epstein-Barr virus in nasopharyngeal carcinoma in a non-endemic eastern european population. *Neoplasma* (2016) 63(1):107–14. doi: 10.4149/neo_2016_013

44. Tang LL, Chen WQ, Xue WQ, He YQ, Zheng RS, Zeng YX, et al. Global trends in incidence and mortality of nasopharyngeal carcinoma. *Cancer Lett* (2016) 374(1):22–30. doi: 10.1016/j.canlet.2016.01.040
45. Ruuskanen M, Irjala H, Minn H, Vahlberg T, Randen-Brady R, Hagstrom J, et al. Epstein-Barr virus and human papillomaviruses as favorable prognostic factors in nasopharyngeal carcinoma: A nationwide study in Finland. *Head Neck* (2019) 41(2):349–57. doi: 10.1002/hed.25450
46. Chen YP, Chan ATC, Le QT, Blanchard P, Sun Y, Ma J. Nasopharyngeal carcinoma. *Lancet* (2019) 394(10192):64–80. doi: 10.1016/S0140-6736(19)30956-0
47. Asante DB, Asmah RH, Adjei AA, Kyei F, Simpong DL, Brown CA, et al. Detection of Human Papillomavirus Genotypes and Epstein-Barr Virus in Nasopharyngeal Carcinomas at the Korle-Bu Teaching Hospital, Ghana. *ScientificWorldJournal* (2017) 2017:2721367. doi: 10.1155/2017/2721367
48. Robinson M, Suh YE, Paleri V, Devlin D, Ayaz B, Pertl L, et al. Oncogenic human papillomavirus-associated nasopharyngeal carcinoma: an observational study of correlation with ethnicity, histological subtype and outcome in a UK population. *Infect Agent Cancer* (2013) 8(1):30. doi: 10.1186/1750-9378-8-30
49. Wotman M, Oh EJ, Ahn S, Kraus D, Costantino P, Tham T. HPV status in patients with nasopharyngeal carcinoma in the United States: A SEER database study. *Am J Otolaryngol* (2019) 40(5):705–10. doi: 10.1016/j.amjoto.2019.06.007
50. Hafkamp HC, Speel EJ, Haesevoets A, Bot FJ, Dinjens WN, Ramaekers FC, et al. A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5-8. *Int J Cancer* (2003) 107(3):394–400. doi: 10.1002/ijc.11389
51. Verhees F, Legemaate D, Demers I, Jacobs R, Haakma WE, Rousch M, et al. The Antiviral Agent Cidofovir Induces DNA Damage and Mitotic Catastrophe in HPV-Positive and -Negative Head and Neck Squamous Cell Carcinomas In Vitro. *Cancers* (2019) 11(7):919. doi: 10.3390/cancers11070919
52. Azulay EE, Cooks T, Elkabets M. Potential oncogenic roles of mutant-p53-derived exosomes in the tumor-host interaction of head and neck cancers. *Cancer Immunol Immunother* (2020) 69(2):285–92. doi: 10.1007/s00262-019-02450-5
53. Liu C, Mann D, Sinha UK, Kokot NC. The molecular mechanisms of increased radiosensitivity of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC): an extensive review. *J Otolaryngol Head Neck Surg* (2018) 47(1):59. doi: 10.1186/s40463-018-0302-y
54. Yeo ELL, Li YQ, Soo KC, Wee JTS, Chua MLK. Combinatorial strategies of radiotherapy and immunotherapy in nasopharyngeal carcinoma. *Chin Clin Oncol* (2018) 7(2):15. doi: 10.21037/cco.2018.04.05
55. Gupta S, Kushwaha VS, Verma S, Khan H, Bhatt ML, Husain N, et al. Understanding molecular markers in recurrent oral squamous cell carcinoma treated with chemoradiation. *Heliyon* (2016) 2(12):e00206. doi: 10.1016/j.heliyon.2016.e00206
56. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. *Oral Dis* (2007) 13(2):239–43. doi: 10.1111/j.1601-0825.2006.01274.x

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Correlation of HPV16 Gene Status and Gene Expression With Antibody Seropositivity and TIL Status in OPSCC

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Introduction: Human papillomavirus 16 (HPV16) is the main cause of oropharyngeal squamous cell carcinoma (OPSCC). To date, the links between HPV16 gene expression and adaptive immune responses have not been investigated. We evaluated the correlation of HPV16 DNA, RNA transcripts and features of adaptive immune response by evaluating antibody isotypes against E2, E7 antigens and density of tumor-infiltrating lymphocytes (TIL).

Material and Methods: FFPE-tissue from 27/77 p16-positive OPSCC patients was available. DNA and RNA were extracted and quantified using qPCR for all HPV16 genes. The TIL status was assessed. Immune responses against E2 and E7 were quantified by ELISA (IgG, IgA, and IgM; 77 serum samples pre-treatment, 36 matched post-treatment).

Results: Amounts of HPV16 genes were highly correlated at DNA and RNA levels. RNA co-expression of all genes was detected in 37% (7/19). E7 qPCR results were correlated with higher anti-E7 antibody (IgG, IgA) level in the blood. Patients with high anti-E2 IgG antibody (>median) had better overall survival (p=0.0311); anti-E2 and anti-E7 IgA levels had no detectable effect. During the first 6 months after treatment, IgA but not IgG increased significantly, and >6 months both antibody classes declined over time. Patients with immune cell-rich tumors had higher levels of circulating antibodies against HPV antigens.

Conclusion: We describe an HPV16 qPCR assay to quantify genomic and transcriptomic expression and correlate this with serum antibody levels against HPV16 oncoproteins. Understanding DNA/RNA expression, relationship to the antibody response in patients regarding treatment and outcome offers an attractive tool to improve patient care.

Keywords: oropharyngeal squamous cell carcinoma, human papillomavirus 16, antibody isotype, gene expression, immune response

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer in the western world (1, 2). 25% of all cases of HNSCC are associated with human papillomavirus (HPV) and 60% of oropharyngeal squamous cell carcinomas (OPSCC) are HPV-driven (3, 4). Infection with a high-risk HPV sub-type can result in the development of OPSCC in individuals irrespective of classical risk factors such as tobacco use and alcohol consumption (5).

The coding regions of the HPV genome consist of an early region (E) with six open reading frames (ORF) E1, E2, E4, E5, E6, E7, and a late region (L) with the ORFs, L1 and L2. The viral oncoproteins E6 and E7, interact with tumor suppressors p53 and Rb, respectively, inactivating their protective function and resulting in aberrant cell cycle control (6). As a result of the functional inactivation of pRb by E7, p16 is upregulated; hence high expression of p16 is often used as a surrogate diagnostic marker for HPV16 (7). E2 has important regulatory function in E6 and E7 gene transcription (8) and viral replication, this function can be disrupted through integration (9). The viral oncoprotein E5 can reduce cell surface expression of the HLA class I, thereby promoting tumor escape from immune control by $CD8^+$ T cells (10). Nonetheless, patients with HPV-positive (HPV^{pos}) OPSCC have a better prognosis in comparison to those with HPV-negative (HPV^{neg}) OPSCC (5). This is most likely attributable to the patient's anti-tumor immune response and is independent of individual treatment regimens (11, 12). The immunological 'visibility' to T cells can be assessed by counting the number of tumor-infiltrating lymphocytes (TILs), higher TIL-density correlates with better survival. In contrast, patients with HPV^{pos} OPSCC but a low TIL-density have a disease-related survival resembling that of $HPV16^{neg}$ OPSCC (13). HPV16 antigens can also be recognized by B cells. Seropositivity to the early genes E1, E2, E4, E6, E7, and L1 has been described as a serological markers for the presence of $HPV16^{pos}$ OPSCC (14, 15). $HPV16^{pos}$ OPSCC patients show increased levels of E6 and E7 antibody in the blood independent of the viral load (16). Humoral immune responses also link to outcome: in OPSCC, seropositivity to HPV16 E6 is associated with a 68% reduction in the risk of death (17). Survival benefit was also reported in OPSCC patients who were found to be seropositive to HPV16 E1, E2, or E6 (18).

Systematic analyses of HPV16 genes at the DNA and RNA level, and their correlation to TIL status and humoral immune responses have not yet been published; it further remains unclear how often antibody class switching occurs in HPV^{pos} HNSCC patients.

In this study, we aimed to evaluate the expression of HPV16 genes at a genomic and transcriptomic level, using a qPCR assay. This helps to understand, which genes of HPV16 genome are present in the tumor at the DNA level, and which of them are transcribed in HNSCC. The data were related to the presence of IgG, IgA and IgM antibodies against the HPV16 E2 and E7 antigens, as determined by ELISA. We used paired serum samples before and after treatment, to measure IgG and IgA antibody response to HPV16 E2 and E7 to determine whether treatment influences the antibody response and how the removal of the cancer as the source of antigen affects antibody levels over time. Additionally, we assessed the TIL status for those tumors and related this to the HPV16 expression and antibody responses.

MATERIAL AND METHODS

Study Ethics

The study given UK Medical Research and Ethics Committee (MREC 09/H0501/90) and institutional approval at Southampton University Hospitals NHS Foundation Trust, Southampton, UK. Written informed consent was obtained from all patients.

Patient Cohort

Patient samples were collected between 2011 and 2018 (**Supplementary Table 1**). Perioperative serum samples (n=77) were selected from patients with $p16^+$ oropharyngeal squamous cell carcinoma (OPSCC). Stored serum samples were available for all patients, 73/77 patients had pre-treatment samples, 40/77 patients had post-treatment samples, 36/77 patients had both pre and post-treatment samples available.

Where available, formalin fixed, paraffin embedded (FFPE) material (n=27) from the primary tumor were retrieved from pathology archives. Snap-frozen tissue samples were available for 4/77 patients. TNM was re-staged according to the 8^{th} edition (AJCC).

DNA, RNA Extraction From FFPE Tissue and RT-PCR

DNA and RNA were extracted from FFPE tissue in accordance to the manufacturer's protocol (Maxwell® RSC DNA FFPE Kit, AS1450, Maxwell® RSC RNA FFPE Kit, AS1440, Promega, Southampton, UK). The concentrations of DNA and RNA were measured using the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). All samples were diluted to a final concentration of 10 ng/µl (DNA), 20 ng/µl (RNA). 2 µl RNA was used for reverse transcription (20 µl, Superscript III first-strand synthesis system, Thermo Scientific, Waltham, MA, USA).

qPCR for HPV16

Quantitative polymerase chain reaction (qPCR) was performed (GoTaq qPCR systems assay, Promega, Southampton, UK) using 1 µl each of DNA (10ng) and 1 µl of cDNA. Samples were loaded onto 384 well plates in triplicates, read on the Applied Biosystems 7900HT workstation and analyzed using SDS 2.3 software (Thermo Fisher, Waltham, MA, USA). The qPCR settings on the workstation were adjusted according to the manufacturer's protocol for the GoTaq® assay.

Published primer pairs for all HPV16 genes used are shown in **Table 1** (19–26). Primer pair annealing to the HPV16 genome NC_001526.4 was confirmed using Primer-BLAST (27, 28). Primer pairs were confirmed *in silico* to be specific for HPV16 and to not bind to other high-risk virus genomes. Specificity was verified using NCBI without detecting any unintended templates of common human viruses (taxid: 10239). Four E5 primer pairs were evaluated as we could not generate a PCR product using the

TABLE 1 | Primer pairs (custom DNA primer, Sigma-Aldrich) used for qPCR with GoTag qPCR kit (Promega) and SYBR settings including their sequence in 5'-3' direction, the length of the PCR product, location on the HPV16 genome (NC_001526.4), and the citation to the originate paper.

Gene	Forward Primer 5'->3'	Reverse Primer 5'->3'	Size (bp)	Location (HPV16 genome)	Citation
E1	AGTAGAGCTGCAAAAAGGAGATTA	CTGACTACATGGTGTTTCAGTCTC	123	355-454	Nilsson et al. (19)
E2	AACGAAGTATCCTCTCCTGAAATTATTAG	CCAAGGCGACGGCTTTG	82	2498-2563	Peitsaro et al. (20)
E4	GACTATCCAGCGACCAAGATCAG	CTGAGTCTCTGTGCAACAACTTAGTG	77	2599-2650	Egawa et al. (21)
E5	GCGACGTGAGAGCAACG	AGGGGTTTCCGGTGTCTGG	na	Not found	Paolini et al. (22)
E5	GCATCCACAACATTACTGGCG	GTAGACACAGACAAAAGCAGCGG	95	3004-3076	Um et al. (23)
E5	CTTTGCTTTTGTGTGCTTTTGTGTG	AAAGCGTGCATGTGTATGTATTAAA	192	3034-3201	Sahab et al. (24)
E5	ATGACAAATCTTGATACTGCA	AATGATGTGTATGTAGACACAG	125	2986-3089	Campo et al. (25)
E6	GAGAACTGCAATGTTTCAGGACC	TGTATAGTTGTTTGCAGCTCTGTGC	81	7136-7192	Peitsaro et al. (20)
E7	AAGTGTGACTCTACGCTTCGGTT	GCCCATTAACAGGTCTTCCAAA	78	7781-7837	Wang-Johanning et al. (26)
L1	TTAGGTGTGGGCATTAGTGG	TCCCCTATAGGTGGTTTGCA	164	5111-5255	Nilsson et al. (19)
L2	GACCCTGCTTTTGTAACCACTC	ATGCTGGCCTATGTAAAGCAAC	166	4087-4231	Nilsson et al. (19)
ß-actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	295	n.a.	Wang-Johanning et al. (26)

initial primer pair [Paolini et al. (22)], because of missing genomic complementary sequence.

The expression values of the samples (s) were calculated with the modified ΔΔCt relative expression method (29, 30). The median Ct(cycle threshold)-value of the gene of interest (GOI) was normalized against the reference mean (ß-actin) (26). ΔΔCt relative values were calculated of the positive control (pc) and subtracted from the total number of cycles to obtain positively correlation values:

$$40 - \Delta\Delta Ct(GOI) = 40 - \left(\left(\mathbf{meanCt}[\mathbf{GOI}]_s - \mathbf{meanCt}[\mathbf{REF}]_s\right) - \left(\mathbf{meanCt}[\mathbf{GOI}]_{pc} - \mathbf{meanCt}[\mathbf{REF}]_{pc}\right)\right)$$

This is a semi-quantitative method; therefore, the relative values are reported as "amount" or "levels" of DNA and of RNA expression.

The RNA qPCR was not successful in a subgroup of samples. We were not able to detect the ß-actin gene, predominantly from older FFPE pathological blocks (>5 years).

HPV16 Viral Genome Level Analysis

RNA was extracted from snap-frozen tissue of four patients (Maxwell® RSC simplyRNA Tissue Kit(AS1340), Promega, Southampton, UK). RNA concentration and quality were analyzed using the RNA Nano Kit for the 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). RNA-sequencing was undertaken by Edinburgh Genomics (University of Edinburgh, Edinburgh, UK). An automated TruSeq stranded mRNA-seq library preparation from total RNA and the NovaSeq sequencing-system was used (100 bp paired-end; 1,750M+1,750M reads, Illumina, San Diego, CA, USA). This RNA sequencing dataset was generated in an independent collaboration with Transgene, and the fact that Transgene funded the RNA sequencing for four cases of the study, did not influence the study design, execution, and results interpretation.

The "viral genome level analysis module" of the bioinformatics pipeline viGen (31) was used to align the FASTQ files against the human reference genome (hg19), filter out human RNA-sequences and map un-aligned reads against the viral reference. Access to the raw and processed data of this RNA sequencing set is possible *via* the gene expression omnibus (GEO accession number: GSE160008).

Immunohistochemistry

p16 IHC on formalin-fixed, paraffin-embedded (FFPE) tumor tissue was performed as part of the routine diagnostics process (**Supplementary Table 1**). Additional IHC was performed on FFPE tissue using standard protocols for the automated platforms Dako PT Link for Heat Induced Epitope Retrieval and Dako Autostainer 48S Link (Agilent, Santa Clara, CA, USA) with staining of tumor-infiltrating lymphocytes using anti-CD8 antibodies (Anti-Human CD8, Clone C8, 144B, Dako, concentration: 1:100, Agilent, Santa Clara, CA, USA). An unsuccessful immunohistochemical approach for HPV16 E2 and E7 was performed with following antibodies [Anti-HPV16 E2, NBP2-53115, NovusBio (no literature), Centennial, CO, USA; Anti-HPV16 E7, ab20191, Abcam, Cambridge, UK (32); Anti-HPV16 E7 (716–325), sc-51951, Santa Cruz Biotechnology, Dallas, TX, USA (33)]. Different concentrations and antigen retrieval techniques revealed still unspecific staining.

Frequency and density of CD8 positive T cells were determined as previously described (34, 35). One case was excluded in the further IHC analysis due to non-specific IHC staining.

Expression and Purification of HVP16 E2 and E7 Protein

HPV16 E2 and E7 protein were produced in the protein core facility of the CRUK Experimental Cancer Sciences Center, Faculty of Medicine, Southampton University.

Control-GST and GST-E2 were expressed as soluble proteins in bacteria, purified on GSTrap column (17528101, GE Healthcare, Chicago, IL, USA) and dialyzed in PBS. GST-E7 was expressed as an insoluble protein in bacteria. The protein was first purified under denaturing conditions in the presence of urea on a HiTrap Q HP anion exchange column (17115301, GE Healthcare, Chicago, IL, USA) and dialyzed in buffer without urea. The dialyzed material was then purified under native conditions on a HiTrap Q HP anion exchange column prior to gel filtration in PBS.

The following plasmids were used: p3187 HPV-16 E2 was a gift from Peter Howley (Addgene plasmid#10846; http://n2t.net/addgene:10846;RRID : Addgene_10846) (36). pGEX2T E7 was a gift from Karl Munger (Addgene plasmid#13634; http://n2t.net/

addgene:13634;RRID : Addgene_13634) (37). Different E6 protein expression and purification methods failed, as non-reliable ELISA results were detected.

ELISA

A standard ELISA was performed in triplicates including a standard curve and negative control. Plates were coated with GST HPV16 E2 and E7 with a working concentration of 2µg/mL at 4°C overnight. Total IgG (goat anti-human (Fc), HRP, A0179, Sigma, St. Louis, MO, USA), IgA (Goat anti-human IgA (Heavy chain), HRP, PA1-74395), and IgM (Goat anti-human IgM (Heavy chain), HRP, A18835, Thermo Scientific, Waltham, MA, USA) were used as secondary antibodies. The Multiskan FC Microplate Photometer was used to analyze the plates, and OD values were exported using the SkanIt software (Thermo Scientific, Waltham, MA, USA).

A standard curve was generated by seven steps of doubling dilutions starting at a concentration of 1:100 using a pool of positive sera. Antibody titers were measured in arbitrary units (AU) using this standard curve. The negative control serum pool was used to set the positive cut-off point (mean+1.645 standard deviations).

Analysis and Statistics

The statistical evaluations were undertaken and graphed using Microsoft Excel (version 16.33) and GraphPad Prism (version 8.4.2). Nonparametric unpaired (Mann-Whitney test) or paired tests (Wilcoxon signed-rank test) were used. Correlations are reported using the nonparametric Spearman's correlation coefficient. An r-value <0.3 was deemed as very weak, 0.3–<0.5 as weak, 0.5–0.7 moderate and >0.7 as strong (38). The survival analysis was performed using the Kaplan-Meier estimator and a Mantel-Cox log-rank test to compare survival curves. P-values less than 0.05 was considered statistically significant.

RESULTS

Patient Summary

Our clinical cohort of 77 patients included only $p16^+$ oropharyngeal tumors (patient characteristics shown in **Table 2**). Most of the tumors were histologically non-keratinizing squamous cell carcinoma (58/77, 75%). Higher tumor stage was associated with poor survival (p=0.0133, **Figure S1**). The patient's treatment is summarized in **Table 2**. Three patients had after primary CRT/RT a surgical salvage (only preoperative serum samples were evaluated). The median follow-up time was 4 years and 5 months (Overall survival: 2-year: 94%, 5-year: 85.3%). No significant difference in survival according to gender, age, tumor site or nodal status was found.

TABLE 2 | Summary of the patient characteristics.

Descriptive Statistics		n=	Percentage
Patients	All	77	100%
Gender	Male	67	87%
	Female	10	13%
Age	Median (years)	56	
	Range (years)	35-79	
Tumor	Oropharynx	77	100%
	Base of Tongue	28	36%
	Tonsil	49	64%
T status	T1	15	19%
	T2	36	47%
	T3	19	25%
	T4	7	9%
N Status	N0	6	8%
	N1	14	18%
	N2	53	69%
	N3	4	5%
Treatment	Surgery +/- adj. CRT/RT	23	30%
	CRT/RT	51	66%
	CRT/RT + Surgery	3	4%

Analysis of HPV16 DNA and RNA

The HPV16 genes E1, E2, E4, E5, E6, E7, L1, and L2, were assessed using qPCR assays on DNA and cDNA from 27 $p16^+$ OPSCC patients. We could not detect the complementary sequence in the HPV16 genome for the primer pair published by Paolini et al. (22) using primer-BLAST (28). This primer pair was not used in further analyses. Additional three published E5 primer sets were evaluated (**Supplementary Table 1**). A concordant correlation of E5 DNA with the other HPV16 genes was observed using these primer pairs (**Figure S2**). For further analysis we chose to proceed with the E5 primer pair which had previously been used in HNSCC (Um et al.) (23).

The quantity of DNA identified for the early and late genes is shown in **Figure 1A**, expressed as 40-ΔΔCt values and ordered by the amount of DNA of the oncogenic E6. In three patients (Case 25, 26, 27) no DNA was detected for any HPV16 gene; these cases were excluded from further analysis and classified as false positive (HPV-status) p16 IHC results.

In the remaining 24 samples, HPV16 DNA was present (40-ΔΔCT range: 22.96-40) (**Figure 1A**). 40-ΔΔCT was highly consistent for E6, E1, E2, E4, L1, and L2 within any individual patient. By contrast, the 40-ΔΔCT detection thresholds of DNA for E7 and E5 genes were less correlated with the other HPV16 genes (in particular case 3). No E5 DNA was detected in case 24 (**Figure 1A**), despite this patient having the highest 40-ΔΔCT values for the other HPV16 genes.

The correlation matrix in **Figure 1B** shows the Spearman's correlation coefficients (r-values) and the respective p-values. The analysis showed a high correlation of E6 DNA to that of the other genes (strongest for E1 and E6, r=0.97, p<0.0001). In contrast, the amounts of E7 DNA were less correlated with other HPV genes, especially with E5 DNA (r=0.22, not significant) (**Figure 1B**). This is consistent with the visual impression in **Figure 1A**. E7 and E5 were only modestly correlated with the 40-ΔΔCT values from other HPV16 genes; the r-values ranged from 0.45 to 0.66 (p<0.05, **Figure 1B**). A highly significant correlation (r>0.85, p<0.001) was observed for

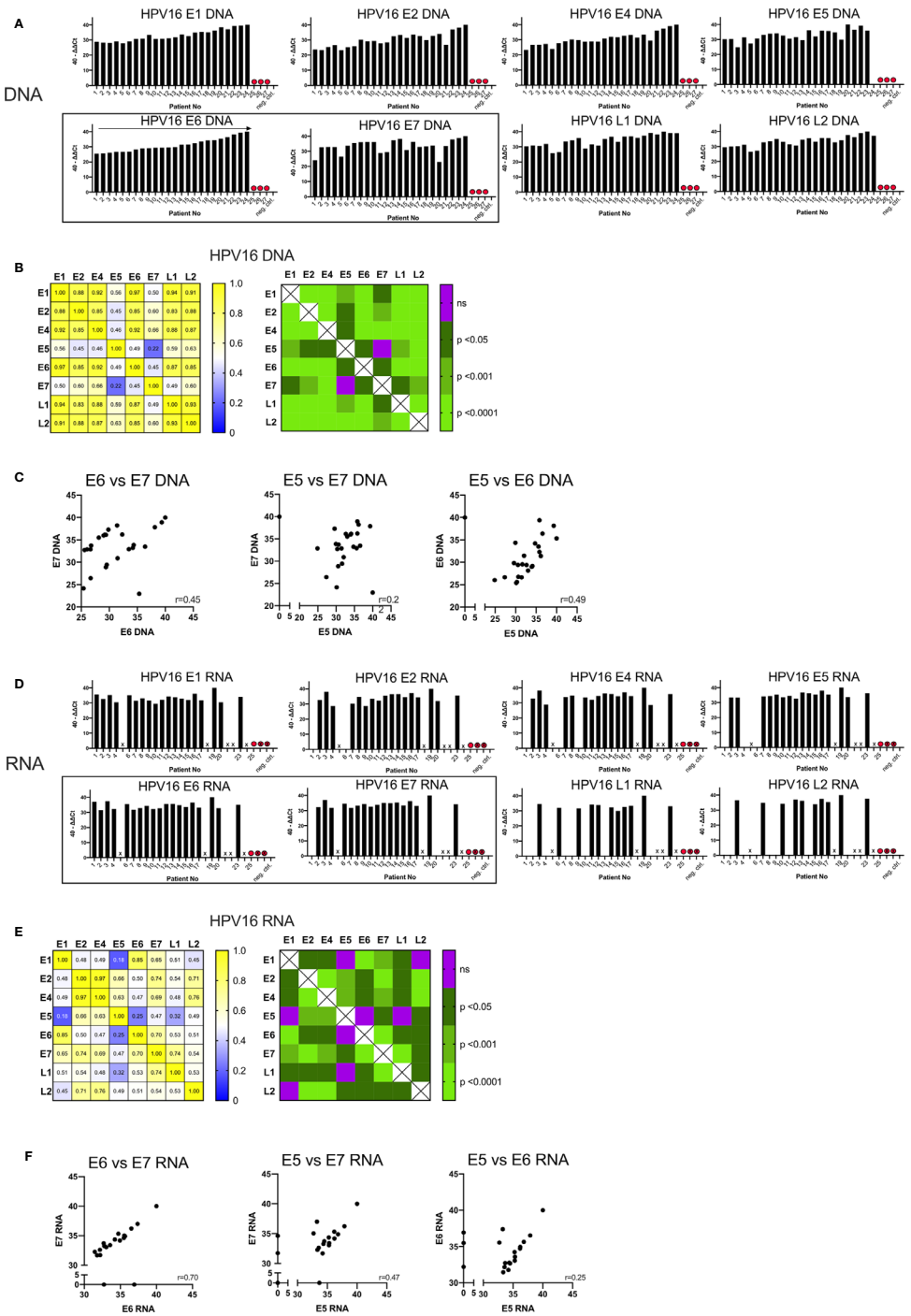

FIGURE 1 | Graphs showing the qPCR data of 27 p16^{+} HNSCC patients analyzed. **(A)** Bar graph displaying 40-ΔΔCT values of DNA for all HPV16 genes of all patients including the blank and negative control (p16 and HPV16 negative non oropharyngeal HNSCC) ordered by the amount of E6 DNA. HPV16^{neg} false p16 positive cases are marked with a red dot. **(B)** The correlation matrix on the left shows the Spearman's correlation coefficients (r) of each HPV16 gene DNA quantity compared to each other. The right matrix displays the corresponding significance levels. **(C)** Scatter plot displaying individual correlations between the DNA levels of the oncogenic genes E5, E6, and E7. **(D)** Bar graph displaying 40-ΔΔCT values of RNA for all HPV16 genes of all patients including the blank and negative control (p16 and HPV16 negative non oropharyngeal HNSCC) ordered by the amount of E6 DNA **(A)**. HPV16^{neg} false p16 positive cases are marked with a red dot and not analyzable cases are not labelled on the x-axis and are marked with a cross. **(E)** The correlation matrix on the left shows the Spearman's correlation coefficients (r) of each HPV16 gene RNA expression compared to each other. The right matrix displays the corresponding significance levels. **(F)** Scatter plot displaying individual correlations between the RNA expression of the oncogenic genes E5, E6, and E7.

DNA amounts among the early genes E1, E2, E4, and E6 with each other and the late genes L1 and L2 (**Figure 1B**). Per-patient correlations between the DNA levels of the oncogenic genes E5, E6, and E7 are displayed in a scatterplot in **Figure 1C** with an r-value of 0.45 for E6 vs E7.

To study transcription of the HPV16 genes, we performed qPCR analysis on cDNA. In seven cases (5, 18, 21, 22, 24, 26, and 27) the quality and amount of RNA was too low to detect the housekeeping gene, β-actin and these are marked with a cross on **Figure 1D**. As expected in case 25, (previously identified as HPV16 negative) we did not detect HPV16 RNA. Overall, a total of 19 samples yielded product in the qPCR RNA expression analysis and we detected 40-ΔΔCt values ranging from 28.6–40.

RNA expression of all HPV16 genes were found in 7/19 (37%) patients. In five patients we detected all early genes but only one late gene (26%); in two cases we found all early genes but no late genes (11%) (**Figure 1D**). In case 4 we found RNA for all early genes except E5; and in case 20 we found no E7 RNA despite the detection of DNA. Overall, expression of RNA for late genes was detected in 11/19 cases (58%) for L1, 10/19 cases (53%) for L2, and seven cases had a co-expression of L1 and L2 (37%).

Using Spearman's correlation coefficient to assess HPV16 genes (**Figure 1E**), E6 transcripts were highly correlated with those for E1 ($r=0.85$, $p<0.0001$) and E7 ($r=0.7$, $p<0.0001$), while E7 transcripts were highly correlated with those for E2/L1 ($r=0.74$, $p<0.0001$). Poor correlation was shown between E5/E6 ($r=0.25$, not significant), E5/E7 ($r=0.47$, $p<0.05$) and E5/E1 transcripts ($r=0.18$, not significant). However, E5 RNA was highly correlated with E2 ($r=0.66$, $p<0.001$) and E4 RNA ($r=0.63$, $p<0.001$). **Figure 1E** also shows the strongest correlation between the transcripts for E2/E4 ($r=0.97$).

Per-patient correlations between the RNA expression for the oncogenic genes E5, E6 and E7 are shown in **Figure 1F**. For both E6/E7 and E5/E7 the correlation between RNA expression of those oncogenes has a moderate ($r=0.47$) and high ($r=0.70$) correlation value respectively. Whereas the E5/E6 correlation was low and not statistically significant.

We quantified the HPV16 viral transcripts in 4 cases (15, 16, 23, and 27) using RNA-sequencing and compared this to the qPCR results. The 40-ΔΔCt values assessed by qPCR for DNA and RNA are displayed together with the HPV16 genome alignment using RNA-sequencing data in **Figure 2**. Consistent with data from qPCR, case 27 did not have any expressed HPV16 genes detected by RNA-sequencing either (previously identified as $HPV16^{neg}$). The other three cases showed high amount for all HPV16 genes and RNA transcripts assessed by qPCR as well as by using RNA-sequencing alignment.

Correlation of IgG and IgA Antibodies to HPV16 E2 and E7

Total IgM, IgG, and IgA antibody responses to E2, E6, and E7 antigens were evaluated using ELISA. E6 protein showed unreliable and inconsistent results, most likely due to problems in protein folding in the protein expression system (data not shown). 3/73 pre-treatment samples (4%) contained strongly detectable IgM antibodies against E2 and E7 simultaneously, perhaps reflecting recent re-exposure of HPV16. Interestingly, the HPV^{neg} case 25 had low levels of circulating E2 IgG (AU=0.2) and E7 IgA (AU=0.8), likely reflecting an unrelated, cleared infection with HPV16. The other two HPV^{neg} cases (26 and 27), had no detectable antibodies to E2 and E7.

Of the remaining 70 pre-treatment serum samples, 46 were positive for E2 IgG, 56 for E7 IgG, 28 for E2 IgA, and 63 for E7 IgA. Thirty-nine cases were positive for E2 and E7 IgG, while 24 had positive ELISA results for E2 and E7 IgA. Positive results for all different antigen-antibody combinations were found in 20 cases. We assessed the relationship between IgA and IgG antibody levels (including the post-treatment samples; **Figure S3**). The antibody levels were correlated with weak to high correlation values.

Antibody levels were not related to the location of the primary tumor (**Figure 3A**), nodal status or primary treatment strategy (not shown). **Figure 3B** shows that T2 and T3 tumors appear to have higher levels of E2 and E7 IgG, and E7 IgA antibodies, but these differences did not reach significance.

IgA Antibody Levels Are Increased Early After Treatment

We compared pairs (n=32) with an interval of ≤6 months or >6 months between samples to understand the post-treatment kinetics. IgA antibody levels against E2 and E7 increased within the first 6 months post-treatment ($p=0.0232$ and $p=0.034$, respectively), while this was not seen for IgG (**Figures 3C, D**).

We compared all baseline samples (n=67) against all post-treatment samples (n=36). IgG and IgA antibody levels remained stable ≤6 months after treatment. A significant decrease in IgG antibody levels >6 months after treatment was seen (**Figures 3C, D**). This was also the case for anti-E7 IgA antibodies, but it did not reach significance.

Patients With High Levels of Anti-E2 IgG Have a Better Overall Survival

Using ELISA and follow-up data from 77 patients, we performed Kaplan-Meier survival analysis. When the cohort was divided into E2 IgG high vs E2 IgG low, according to the median AU values, patients with high E2 IgG antibody levels showed a significantly longer survival than those with low levels ($p=0.0321$). Exclusion of HPV^{neg} qPCR cases did not change the survival analysis, on the contrary, it increased the significance slightly to $p=0.0311$ (**Figure 3E**). A similar trend was shown for E7 IgG, but we did not detect a link between IgA antibody levels and outcome (**Figure 3E**).

High HPV16 DNA Levels and RNA Expression for E7 Are Correlated With Antibody Level

AU values were plotted against the amount of E2 and E7 DNA and RNA after classifying them into a low value (40-ΔΔCt <30)

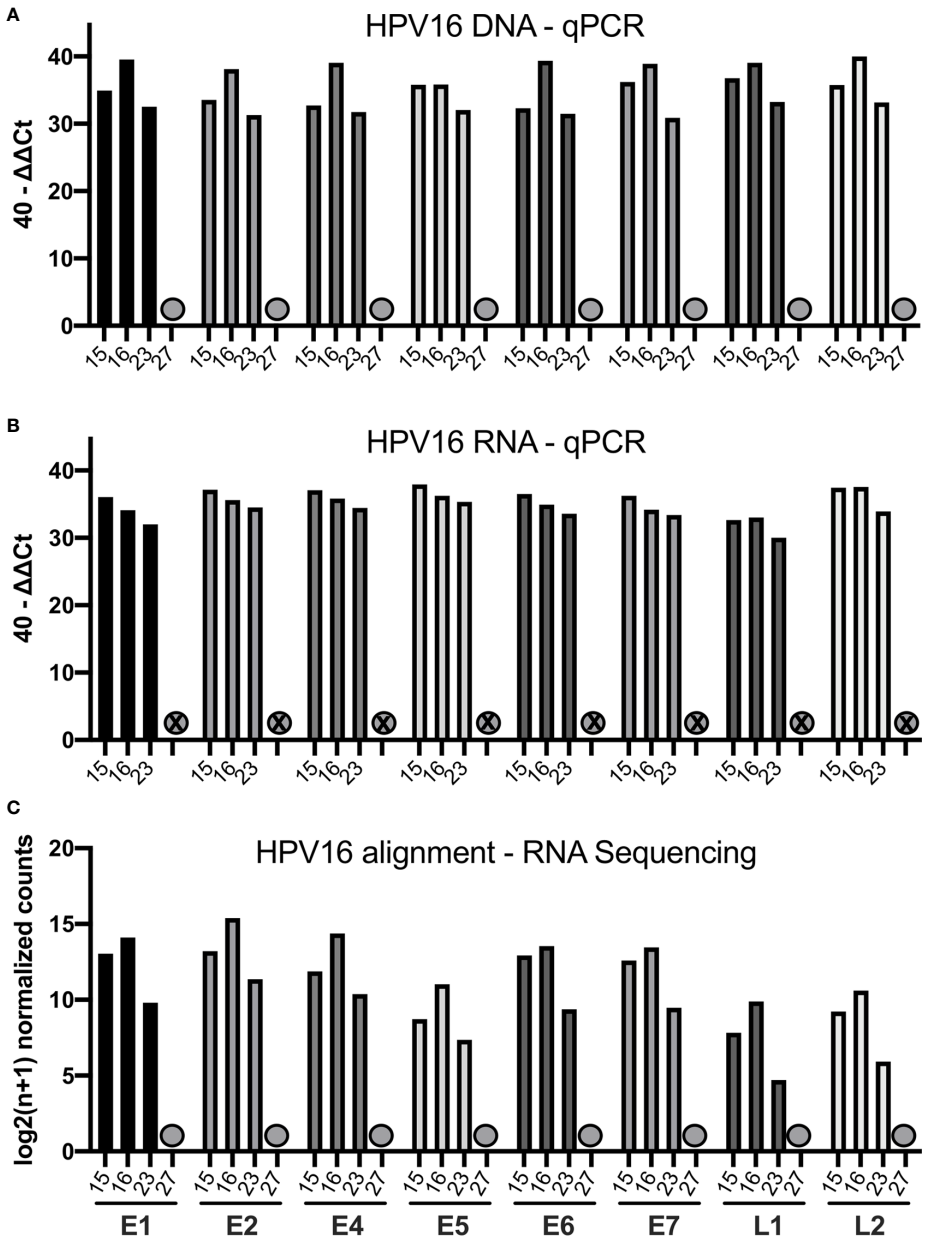

FIGURE 2 | Bar graphs showing the qPCR and RNA-sequencing data of four selected patients (15, 16, 23, 27). **(A)** Bar graph displaying 40-ΔΔCT values of DNA for all HPV16 genes of those patients. The HPV16^{neg} (false p16 positive case 27) is marked with a grey dot. **(B)** Bar graph showing the corresponding 40-ΔΔCT values of RNA for the respective patients **(C)** Bar graph showing the log2(n+1) normalized counts for the HPV16 genome using RNA-sequencing data of respective patients.

or high value (40-ΔΔCt ≥30) group (DNA: **Figure 4A**, RNA: **Figure 4B**). The cut-off was based on the rounded average 40-ΔΔCt values of 30.42 on DNA level. Detection of more E2 DNA or transcripts did not relate to higher serum levels of anti-E2 IgG or IgA antibody. However, E7 DNA and RNA transcripts were associated with a higher AU value for anti-E7 IgG and IgA, although the effect was not statistically significant, most likely due to low case numbers. Grouping the ELISA results showed that high amounts of DNA were significantly correlated with high AU values (p=0.035).

TIL Status Has an Influence on Antibody Levels Against HPV16 E2 and E7

The TIL status of patients was determined using IHC staining specific for CD8$^+$ cells and categorized into high, moderate and low as previously published (13). In the HPV16^{pos} qPCR cohort (n=24) 15 patients had a high TIL count for CD8. The rest showed a moderate or low TIL count (**Figures 4C, D**).

AU were plotted in the three TIL categories according to the number of CD8$^+$ cells (**Figure 4C**). Due to the small numbers in the low expression group we combined all ELISA data (E2 IgG,

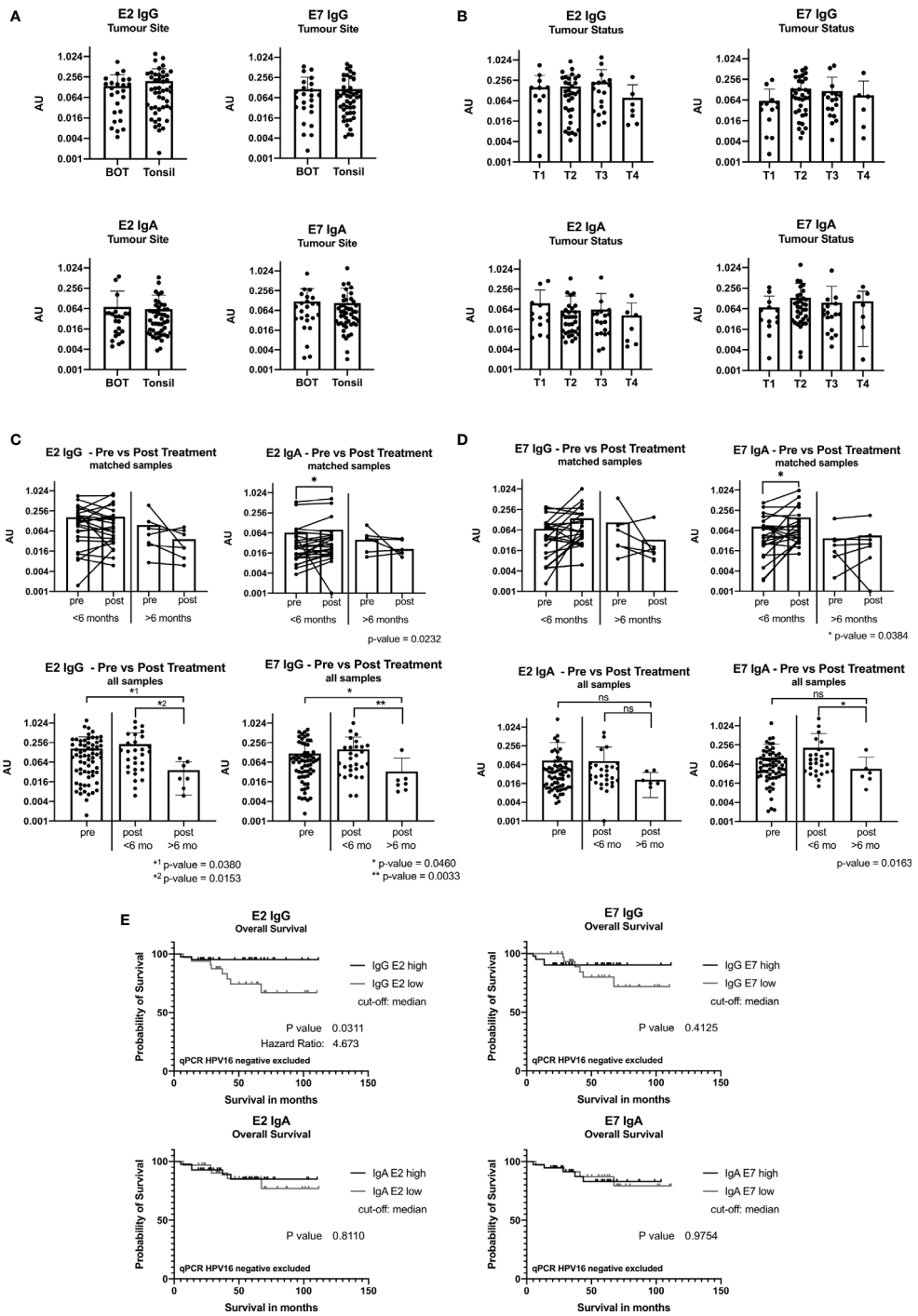

FIGURE 3 | ELISA results reported in arbitrary units (AU) from samples taken at baseline before treatment. **(A)** Bar graph showing AU values for the tumor site base of tongue (BOT) and tonsil for the antibody classes IgG and IgA. **(B)** Bar graph displaying AU values depending on the tumor size [small tumor size (T1) and advanced tumor status (T4)]. **(C)** Graph showing ELISA AU values of pre- and post-treatment samples for IgG. The top two figures show the paired E2 IgG data grouped by time in months when the second sample was taken after completion of treatment, less than 6 months (<6 months) and more than 6 months (>6 months). The bottom graphs display all pre-treatment and post-treatment samples and are plotted separated by their second sample date. Significance levels are shown for E2 IgG and E7 IgG ($p<0.05$). **(D)** Graphs showing the ELISA results for IgA with a statistically significant increase in the group with sample <6 months after treatment end (E2: p=0.0232, E7: p=0.0384). (ns, not significant; *: $p\leq0.05$, **: $p\leq0.01$). **(E)** Kaplan-Meier graph of overall survival of 74 patients (n=3 qPCR HPV16 negative excluded) grouping in IgG E2 low (n=33) and IgG E2 high (n=41) using median antibody values. The IgG E2 high group shows a significant better overall survival than the low AU value group (OS after 5 years: 95% vs 74%, p=0.0.311). E7 IgG shows a not statistically significant difference of the overall survival.

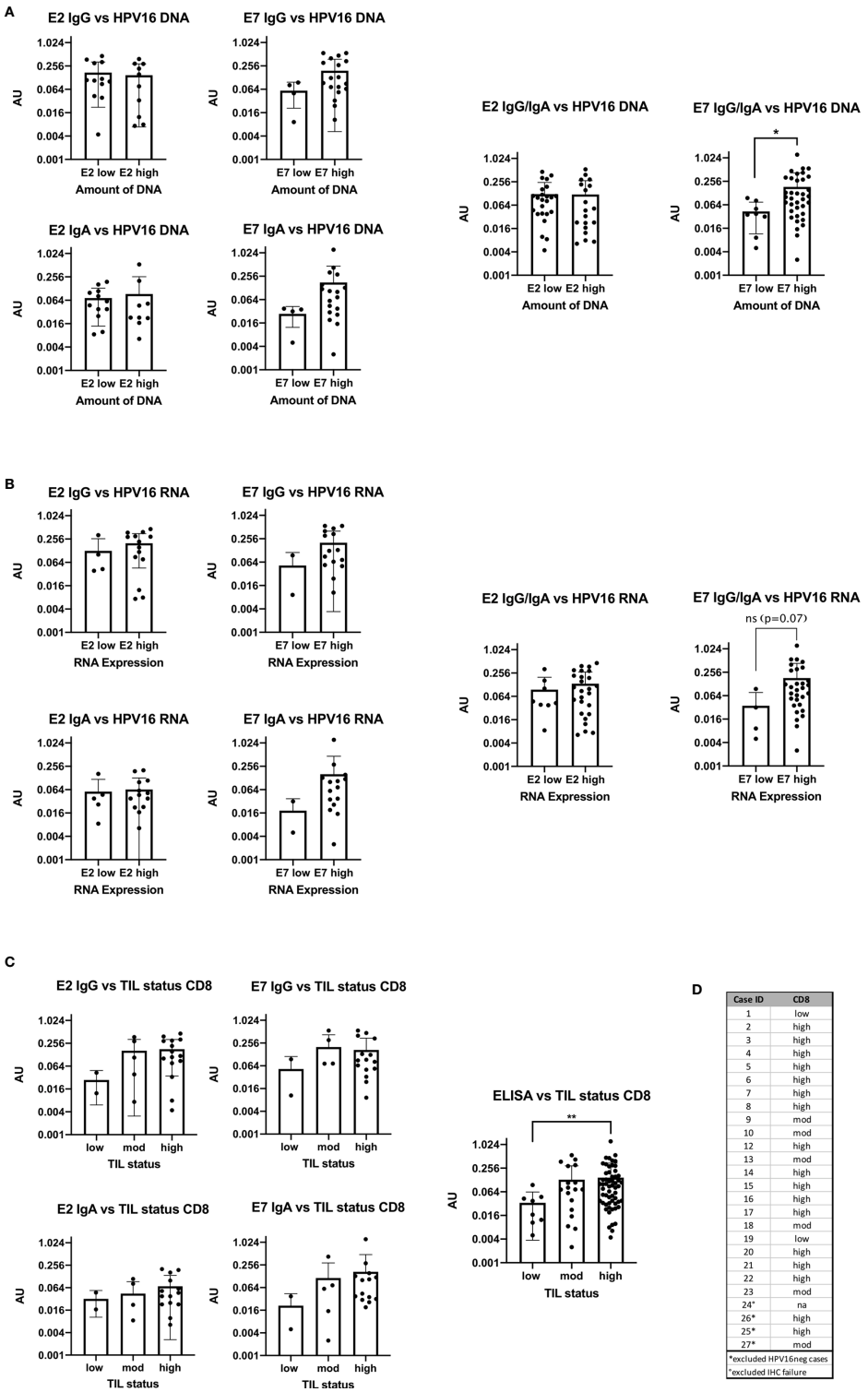

FIGURE 4 | Bar plots showing the comparison of antibody levels with E2 and E7 expression levels on DNA, RNA and with the TIL status **(A)** High DNA expression level of E7 is accompanied with statistically significant higher AU values (p=0.0245) of E7 IgG in the serum **(B)** the RNA expression level and ELISA antibody units are congruent to the DNA results, but not statistically significant (p=0.07). **(C)** Patients with high or moderate TIL status assessed by CD8 have higher AU values in the serum for E2 and E7 IgG than patients with TIL^{low} tumors. The same trend is seen for E2 and E7 IgA. Combining all ELISA data to a summarized seropositivity the TIL^{high} HNSCC show significantly higher AU values than the low. (**: p≤0.01). **(D)** Table showing the case numbers and their TIL status for CD103 and CD8.

E2 IgA, E7 IgG, and E7 IgA) and compared the overall seropositivity with the TIL status. Significant more circulating antibodies in TIL^{high} tumors were found (p=0.0097).

DISCUSSION

Several diagnostic approaches for HPV16 detection are described. The assessments include HPV-PCR, HPV-*in situ hybridization* (ISH) and the hybrid-capture HPV DNA Test which can be used to detect a whole group of high-risk and low-risk HPV types (39). Hitherto, only few methods for the detection of HPV16 in FFPE material have been published (40). Systematic assessment of the HPV16 genome, quantifying all genes separately, had not yet been undertaken. The primer pairs we used in the qPCR assay for HPV16 detection were rigorously tested to be specific for each HPV16 gene, without cross reactivities to other high-risk HPV types, or other human viruses. The assay specificity was also supported by using the viGen bioinformatic pipeline (31) to detect HPV16 genes, mapping the RNA-sequencing data onto HPV16 genome. This separate analysis using different starting material revealed the same findings and confirms the validity of our qPCR assay.

Evaluation of all HPV16 genes in OPSCC with qPCR revealed highly correlated amounts of HPV16 gene DNA with the lowest values for E5 and E7. Intriguingly, we could detect all HPV16 genes in most cases. E5 expression displayed the lowest correlation with other HPV16 genes and was absent in case 24. E5 is composed of 83 amino acids, making it the smallest of all HPV16 genes. E5 has been shown to be necessary for early cancer development and can be lost during the course of cancer development (41). Our case is likely an example of this process. We identified 3 HPV^{neg} cases in our cohort (n=27), which expressed high levels of p16 protein. This is approximately 11% and is in the lower range of published percentages of 15%–20% for $p16^{+}$ but HPV-ISH^{neg} HNSCC (7).

The 40-ΔΔCT values for DNA differ from those identified for RNA expression, reflecting variable transcriptional activity between cases. The late genes L1 and L2 are both transcribed from the late promotor and are only expressed in the surface of the stratified squamous epithelium, while in the basal cells only early genes are transcribed (42). The assembly and dissemination of HPV16 from the superficial epithelial cells happens after late antigen expression. However, in the case of tumor formation, viral assembly is not critical once transformation has occurred and hence, the late genes are likely redundant. Intriguingly, the late antigens are still transcribed into RNA in 58% of cases for L1, 53% for L2 and 37% expressed both. This finding underpins the possibility of complete viral reassembly in more than one third of cases, supporting the risk of viral transmission between partners after the cancer has been established (43). If true, this has important social implications for the patient and their partner(s). This question should be evaluated formally in prospective work to assess whether a prophylactic vaccine may be indicated for partners and patients.

We describe two cases where E7 DNA is detected, but not transcribed. Both oncogenes E5 and E6 however are transcribed in those cases. It is not clear if E7 was expressed during cancer initiation, and then lost during cancer growth, or if there is an immunological mechanism (one case was TIL^{high}, the other TIL^{low}). In contrast to published data, we could detect E2 RNA at the same time as RNA for E6 and E7 (9) suggesting that E2 does not necessarily exert inhibitory transcriptional control over E6/E7 expression (8).

It has been reported that patients with $HPV16^{pos}$ OPSCC show increased levels of E6 and E7 antibodies in the blood, independent of the viral load (16). However, we find a correlation between E7 DNA and RNA expression and higher serum levels of IgG and IgA antibodies against E7, but not E2: thus, individual genes of HPV16 appear to be differentially expressed, transcribed and could be differentially immunogenic. Therefore, a detailed analysis on a gene by gene basis is important for being able to interpret immunological data.

E2 has previously been reported to be associated with a greater cytotoxic T lymphocyte response compared to E7 (44). We did not directly assess T cell reactivity in our cases; the humoral responses we observe however raise the possibility that T helper cells are less activated in response to E2 than E7, and therefore lead to less B cell activation and antibody production. The relationship between HPV16 gene expression and antigenicity requires further investigation. This would also have important implications for vaccine development.

The presence of viral antigen leads to the activation of immune responses aimed at clearing the infection. This clearance requires both T and B cell activation. T cells activated against HPV antigens are able to recognize the intracellular virus through presentation of viral peptides in MHC molecules, and this has been shown by the presence of virus-specific T cells in HPV^{pos} HNSCC (45). These T helper cells activate B cells to produce specific antibodies which can then be detected in the serum of patients e.g. by ELISA. ELISA is a rapid and inexpensive assay which can give additional information about the patients' prognosis beforehand and could be a useful additional diagnostic tool.

Antibodies directed against HPV early antigens have been proposed as a prognostic biomarker before and after removal of the tumor (46). If treatment is successful it would remove the source of HPV antigen, limiting B cell responses. Therefore, following effective CRT or complete surgical resection of all disease, a significant decrease or loss of antibodies over time is expected. Continued detection of antibodies may indicate either residual tumor (post-CRT) or distant metastasis (post-complete surgical resection). 15%–20% of HPV^{pos} patients die from residual or recurrent disease within 2-years and a simple blood based biomarker would be clinically relevant (47). While pre- and post-surgery levels of E6 antibody have been described as a prognostic indicator of recurrence (48), pre and post-treatment levels of antibody to E7 appear to serve as a biomarker. In our study, the pre- and post-treatment antigen levels were different in the two groups sampled at different times (<6/>6 months). However, we could not assess the relevance of antibodies as a

biomarker of recurrence in our cohort as no recurrences occurred during the period of sample collection.

We saw stable antibody levels for E2 IgG before treatment and in serum samples taken in the first 6 months after treatment, while there was a slight increase in anti-E7 IgG; after that timepoint antibody levels decreased. This finding is consistent with the published data by Fakhry et al. (48). For IgA, in the first 6 months after surgery, we saw increasing antibody levels in the blood, but again after 6 months, these levels decreased. Therefore, even after the tumor is completely removed, antibodies persist in the blood. As the half-life of immunoglobulins is much less than 6 months, these findings must mean that HPV16-reactive B cells/ Plasma cells may persist in lymphatic structures, for example the bone marrow. Nonetheless, these B cells must be relatively short lived as, following tumor removal, we find that after 6 months antibody levels decline. As expected, the kinetics for E6 and E7 DNA are different and decrease rapidly after treatment in oral rinse samples in contrast to the antibody responses (49), demonstrating that both tests offer different biological insights in to the success of treatment.

Consistent with published data (17, 50) we confirmed better survival for OPSCC patients with IgG responses to the E2 gene it is thought that the antigenic determinant is located at the N terminal region of E2 (18). A fascinating new observation from our study is that the presence of IgA antibodies does not appear to link to survival benefit. This is intriguing, as IgA responses have been accused of dismantling adaptive T cell responses in human liver cancer (51). Formal study of the biological function of IgA^+ B cells and plasma cells is needed to understand the underpinning biology. Consistent with the published data, there was no significant survival benefit seen for E7 antibody seropositivity, although a trend emerged after longer follow-up.

Generally, patients with HPV^{pos} OPSCC have a better outcome than HPV^{neg} patients, but those HPV^{pos} with low TIL status have the same poor outcome as HPV^{neg} OPSCC (13). Patients with immune-cold tumors (TIL^{low}) had less antibody production, while immune-hot tumors (TIL^{high}) were associated with greater antibody production. Our data is consistent with the observation that B cells and T cells are abundant in the same cancers (52), the antibody levels demonstrate the functional link between these cell populations, most likely anatomically located together in tertiary lymphatic structures in the cancer microenvironment. Importantly, the circulating antibody levels may be a potential biomarker to stratify patients in clinical trials evaluating immunotherapies.

Caveats for generalizing our data include the small size of our qPCR cohort, as some samples were too low-quality for further analysis. This might also be a limitation in using FFPE material: RNA is less stable than DNA and consistent with this is that technical failures occurred in the older FFPE blocks.

In our serum sample cohort, the selection criteria were based on p16, as the commonly available surrogate marker for HPV-driven disease. This may have biased case choice and going forward, we would use the qPCR for DNA in combination with p16 IHC as the more robust tool for identifying $HPV16^{pos}$ tumors.

The ELISA assay had initially been planned to include antibodies against E2, E6 and E7. However, we did not successfully express E6 protein. Nevertheless, including E6 and E5 in future research could unfold additional insights in antibody responses to those oncogenic HPV16 antigens. ELISA findings have to be confirmed prospectively in a larger HPV^{pos} OPSCC cohort with standardized and consecutive serum sample collection. This would validate our results and improve the understanding of HPV16 expression at genomic and transcriptomic levels, as well as antibody responses and their clinical impact.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160008.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UK Medical Research and Ethics Committee and by institutional approval at Southampton University Hospitals NHS Foundation Trust, Southampton, UK. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AW designed the study. AW did the experimental work, analyzed data, and wrote the manuscript. EC co-wrote the manuscript. JT undertook bioinformatic analyses of the HPV16 alignment and RNA-sequencing. OW and LC provided technical and experimental support. GT contributed pathological supervision and supervised the IHC. OA contributed to the method development of the ELISA assay. EK, PF, and SL contributed to writing and ordering of the manuscript. CO designed the study, supervised the experimental work and data analysis and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.591063/full#supplementary-material

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* (2012) 62(1):10–29. doi: 10.3322/caac.20138
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2018) 68(6):394–424. doi: 10.3322/caac.21492
3. D'Souza G, Dempsey A. The role of HPV in head and neck cancer and review of the HPV vaccine. *Prev Med* (2011) 53 Suppl 1:S5–11. doi: 10.1016/j.ypmed.2011.08.001
4. Schache AG, Powell NG, Cuschieri KS, Robinson M, Leary S, Mehanna H, et al. HPV-Related Oropharynx Cancer in the United Kingdom: An Evolution in the Understanding of Disease Etiology. *Cancer Res* (2016) 76(22):6598–606. doi: 10.1158/0008-5472.CAN-16-0633
5. Sethi S, Ali-Fehmi R, Franceschi S, Struijk L, van Doorn LJ, Quint W, et al. Characteristics and survival of head and neck cancer by HPV status: a cancer registry-based study. *Int J Cancer* (2012) 131(5):1179–86. doi: 10.1002/ijc.26500
6. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* (2004) 78(21):11451–60. doi: 10.1128/JVI.78.21.11451-11460.2004
7. Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, 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):2465–72. doi: 10.1002/ijc.22980
8. McBride AA. The papillomavirus E2 proteins. *Virology* (2013) 445(1-2):57–79. doi: 10.1016/j.virol.2013.06.006
9. Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* (1985) 314(6006):111–4. doi: 10.1038/314111a0
10. Gruener M, Bravo IG, Momburg F, Alonso A, Tomakidi P. The E5 protein of the human papillomavirus type 16 down-regulates HLA-I surface expression in calnexin-expressing but not in calnexin-deficient cells. *Virol J* (2007) 4:116. doi: 10.1186/1743-422X-4-116
11. Welters MJP, Ma W, Santegoets SJAM, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral {HPV}16-{Specific} {T} {Cells} {Constitute} a {Type} {I}-{Oriented} {Tumor} {Microenvironment} to {Improve} {Survival} in {HPV}16-{Driven} {Oropharyngeal} {Cancer}. *Clin Cancer Res* (2018) 24(3):634–47. doi: 10.1158/1078-0432.CCR-17-2140
12. Chakravarthy A, Henderson S, Thirdborough SM, Ottensmeier CH, Su X, Lechner M, et al. Human Papillomavirus Drives Tumor Development Throughout the Head and Neck: Improved Prognosis Is Associated With an Immune Response Largely Restricted to the Oropharynx. *J Clin Oncol* (2016) 34(34):4132–41. doi: 10.1200/JCO.2016.68.2955
13. Ward MJ, Thirdborough SM, Mellows T, Riley C, Harris S, Suchak K, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer* (2014) 110(2):489–500. doi: 10.1038/bjc.2013.639
14. Anderson KS, Wong J, D'Souza G, Riemer AB, Lorch J, Haddad R, et al. Serum antibodies to the HPV16 proteome as biomarkers for head and neck cancer. *Br J Cancer* (2011) 104(12):1896–905. doi: 10.1038/bjc.2011.171
15. Holzinger D, Wichmann G, Baboci L, Michel A, Hofler D, Wiesenfarth M, et al. Sensitivity and specificity of antibodies against HPV16 E6 and other early proteins for the detection of HPV16-driven oropharyngeal squamous cell carcinoma. *Int J Cancer* (2017) 140(12):2748–57. doi: 10.1002/ijc.30697
16. Kreimer AR, Clifford GM, Snijders PJ, Castellsague X, Meijer CJ, Pawlita M, et al. HPV16 semiquantitative viral load and serologic biomarkers in oral and oropharyngeal squamous cell carcinomas. *Int J Cancer* (2005) 115(2):329–32. doi: 10.1002/ijc.20872
17. Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *J Clin Oncol* (2013) 31(21):2708–15. doi: 10.1200/JCO.2012.47.2738
18. Dahlstrom KR, Anderson KS, Cheng JN, Chowell D, Li G, Posner M, et al. HPV Serum Antibodies as Predictors of Survival and Disease Progression in Patients with HPV-Positive Squamous Cell Carcinoma of the Oropharynx. *Clin Cancer Res* (2015) 21(12):2861–9. doi: 10.1158/1078-0432.CCR-14-3323
19. Nilsson K, Wu C, Kajitani N, Yu H, Tsimtsirakis E, Gong L, et al. The DNA damage response activates HPV16 late gene expression at the level of RNA processing. *Nucleic Acids Res* (2018) 46(10):5029–49. doi: 10.1093/nar/gky227
20. Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* (2002) 40(3):886–91. doi: 10.1128/JCM.40.3.886-891.2002
21. Egawa N, Wang Q, Griffin HM, Murakami I, Jackson D, Mahmood R, et al. HPV16 and 18 genome amplification show different E4-dependence, with 16E4 enhancing E1 nuclear accumulation and replicative efficiency via its cell cycle arrest and kinase activation functions. *PloS Pathog* (2017) 13(3):e1006282. doi: 10.1371/journal.ppat.1006282
22. Paolini F, Curzio G, Cordeiro MN, Massa S, Mariani L, Pimpinelli F, et al. HPV 16 E5 oncoprotein is expressed in early stage carcinogenesis and can be a target of immunotherapy. *Hum Vaccin Immunother* (2017) 13(2):291–7. doi: 10.1080/21645515.2017.1264777
23. Um SH, Mundi N, Yoo J, Palma DA, Fung K, MacNeil D, et al. Variable expression of the forgotten oncogene E5 in HPV-positive oropharyngeal cancer. *J Clin Virol* (2014) 61(1):94–100. doi: 10.1016/j.jcv.2014.06.019
24. Sahab Z, Sudarshan SR, Liu X, Zhang Y, Kirilyuk A, Kamonjoh CM, et al. Quantitative measurement of human papillomavirus type 16 e5 oncoprotein levels in epithelial cell lines by mass spectrometry. *J Virol* (2012) 86(17):9465–73. doi: 10.1128/JVI.01032-12
25. Campo MS, Graham SV, Cortese MS, Ashrafi GH, Araibi EH, Dornan ES, et al. HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells. *Virology* (2010) 407(1):137–42. doi: 10.1016/j.virol.2010.07.044
26. Wang-Johanning F, Lu DW, Wang Y, Johnson MR, Johanning GL. Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep Papanicolaou tests using real-time polymerase chain reaction analysis. *Cancer* (2002) 94(8):2199–210. doi: 10.1002/cncr.10439
27. Seedorf K, Krammer G, Durst M, Suhai S, Rowekamp WG. Human papillomavirus type 16 DNA sequence. *Virology* (1985) 145(1):181–5. doi: 10.1016/0042-6822(85)90214-4
28. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* (2012) 13:134. doi: 10.1186/1471-2105-13-134

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001) 25(4):402–8. doi: 10.1006/meth.2001.1262
30. Laible M, Schlombs K, Kaiser K, Veltrup E, Herlein S, Lakis S, et al. Technical validation of an RT-qPCR in vitro diagnostic test system for the determination of breast cancer molecular subtypes by quantification of ERBB2, ESR1, PGR and MKI67 mRNA levels from formalin-fixed paraffin-embedded breast tumor specimens. *BMC Cancer* (2016) 16:398. doi: 10.1186/s12885-016-2476-x
31. Bhuvaneshwar K, Song L, Madhavan S, Gusev Y. viGEN: An Open Source Pipeline for the Detection and Quantification of Viral RNA in Human Tumors. *Front Microbiol* (2018) 9:1172. doi: 10.3389/fmicb.2018.01172
32. Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, et al. Immunohistochemical Evaluation of E6/E7 HPV Oncoproteins Staining in Cervical Cancer. *Anticancer Res* (2016) 36(6):3195–8.
33. Wang YX, Zhang ZY, Wang JQ, Qian XL, Cui J. HPV16 E7 increases COX-2 expression and promotes the proliferation of breast cancer. *Oncol Lett* (2018) 16(1):317–25. doi: 10.3892/ol.2018.8624
34. Clarke J, Panwar B, Madrigal A, Singh D, Gujar R, Wood O, et al. Single-cell transcriptomic analysis of tissue-resident memory T cells in human lung cancer. *J Exp Med* (2019) 216(9):2128–49. doi: 10.1084/jem.20190249
35. Ganesan AP, Clarke J, Wood O, Garrido-Martin EM, Chee SJ, Mellows T, et al. Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nat Immunol* (2017) 18(8):940–50. doi: 10.1038/ni.3775
36. Yasugi T, Benson JD, Sakai H, Vidal M, Howley PM. Mapping and characterization of the interaction domains of human papillomavirus type 16 E1 and E2 proteins. *J Virol* (1997) 71(2):891–9. doi: 10.1128/JVI.71.2.891-899.1997
37. Wu EW, Clemens KE, Heck DV, Munger K. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein. *J Virol* (1993) 67(4):2402–7. doi: 10.1128/JVI.67.4.2402-2407.1993
38. Moore DS, Notz WII, Flinger MA. *The basic practice of statistics. 6th ed*. New York, NY, United States: W. H. Freeman and Company (2013).
39. Mandelblatt JS, Lawrence WF, Womack SM, Jacobson D, Yi B, Hwang YT, et al. Benefits and costs of using HPV testing to screen for cervical cancer. *Jama-J Am Med Assoc* (2002) 287(18):2372–81. doi: 10.1001/jama.287.18.2372
40. Kocjan BJ, Maver PJ, Hosnjak L, Zidar N, Odar K, Gale N, et al. Comparative evaluation of the Abbott RealTime High Risk HPV test and INNO-LiPA HPV Genotyping Extra test for detecting and identifying human papillomaviruses in archival tissue specimens of head and neck cancers. *Acta Dermatovenerol Alp Pannonica Adriat* (2012) 21(4):73–5.
41. Venuti A, Paolini F, Nasir L, Corteggio A, Roperto S, Campo MS, et al. Papillomavirus E5: the smallest oncoprotein with many functions. *Mol Cancer* (2011) 10:140. doi: 10.1186/1476-4598-10-140
42. Stubenrauch F, Laimins LA. Human papillomavirus life cycle: active and latent phases. *Semin Cancer Biol* (1999) 9(6):379–86. doi: 10.1006/scbi.1999.0141
43. Sathasivam HP, Bhatia R, Bradley P, Robson A, Paleri V, Cocks H, et al. Concurrent HPV-related oropharyngeal carcinoma in four couples. *Oral Oncol* (2018) 86:33–7. doi: 10.1016/j.oraloncology.2018.09.003
44. Krishna S, Ulrich P, Wilson E, Parikh F, Narang P, Yang S, et al. Human Papilloma Virus Specific Immunogenicity and Dysfunction of CD8(+) T Cells in Head and Neck Cancer. *Cancer Res* (2018) 78(21):6159–70. doi: 10.1158/0008-5472.CAN-18-0163
45. Heusinkveld M, Goedemans R, Briet RJ, Gelderblom H, Nortier JW, Gorter A, et al. Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. *Int J Cancer* (2012) 131(2):E74–85. doi: 10.1002/ijc.26497
46. Mirghani H, Lang Kuhs KA, Waterboer T. Biomarkers for early identification of recurrences in HPV-driven oropharyngeal cancer. *Oral Oncol* (2018) 82:108–14. doi: 10.1016/j.oraloncology.2018.05.015
47. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, 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(4):261–9. doi: 10.1093/jnci/djn011
48. Fakhry C, Qualliotine JR, Zhang Z, Agrawal N, Gaykalova DA, Bishop JA, et al. Serum Antibodies to HPV16 Early Proteins Warrant Investigation as Potential Biomarkers for Risk Stratification and Recurrence of HPV-Associated Oropharyngeal Cancer. *Cancer Prev Res (Phila)* (2016) 9(2):135–41. doi: 10.1158/1940-6207.CAPR-15-0299
49. Fakhry C, Blackford AL, Neuner G, Xiao W, Jiang B, Agrawal A, et al. Association of Oral Human Papillomavirus DNA Persistence With Cancer Progression After Primary Treatment for Oral Cavity and Oropharyngeal Squamous Cell Carcinoma. *JAMA Oncol* (2019) 5(7):985–92. doi: 10.1001/jamaoncol.2019.0439
50. Smith EM, Rubenstein LM, Ritchie JM, Lee JH, Haugen TH, Hamsikova E, et al. Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers? *Cancer Epidemiol Biomarkers Prev* (2008) 17(8):2087–96. doi: 10.1158/1055-9965.EPI-08-0054
51. Shalapour S, Lin XJ, Bastian IN, Brain J, Burt AD, Aksenov AA, et al. Inflammation-induced IgA+ cells dismantle anti-liver cancer immunity. *Nature* (2017) 551(7680):340–5. doi: 10.1038/nature24302
52. Wood O, Clarke J, Woo J, Mirza AH, Woelk CH, Thomas GJ, et al. Head and Neck Squamous Cell Carcinomas Are Characterized by a Stable Immune Signature Within the Primary Tumor Over Time and Space. *Clin Cancer Res* (2017) 23(24):7641–9. doi: 10.1158/1078-0432.CCR-17-0373

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Survival Significance of Number of Positive Lymph Nodes in Oral Squamous Cell Carcinoma Stratified by p16

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Objectives: To analyze the significance of the number of positive lymph nodes in oral squamous cell carcinoma (SCC) stratified by p16.

Methods: A total of 674 patients were retrospectively enrolled and divided into 4 groups based on their number of positive lymph nodes (0 vs. 1–2 vs. 3–4 vs. ≥5). The Kaplan-Meier method was used to calculate the disease-free survival (DFS) and disease-specific survival (DSS) rates. Cox model was used to evaluate the independent risk factor.

Results: p16 showed positivity in 85 patients with a rate of 12.6%. In patients with p16 negativity, the 5-year DFS rates were 52%, 39%, and 21% in patients with 0, 1–2, and 3–4 positive lymph nodes, respectively, in patients with ≥5 positive lymph nodes, all patients developed recurrence within 2 years after operation, the difference was significant; the 5-year DSS rates were 60, 38, and 18% in patients with 0, 1–2, and 3–4 positive lymph nodes, respectively, in patients with ≥5 positive lymph nodes, all patients died within 4-years after operation. The difference was significant. In p16 positivity patients, the 3-year DFS rates were 41% and 17% in patients with 0–2 and ≥3 positive lymph nodes, respectively, the difference was significant; the 3-year DSS rates were 84 and 46% in patients with 0–2 and ≥3 positive lymph nodes, the difference was significant.

Conclusions: The number of positive lymph nodes is significantly associated with the survival in oral SCC, its survival effect is not affected by p16 status.

Keywords: oral squamous cell carcinoma, AJCC classification, number of positive lymph nodes, survival analysis, p16

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INTRODUCTION

Oral squamous cell carcinoma (SCC) is the most common malignancy in the head and neck, and the mainstay of treatment is curative surgery followed by adjuvant treatment (1). Although there has been great progress in medical science, the prognosis of oral SCC has not apparently improved with a 5-year overall survival rate of about 40% (2–4). The most important prognostic factor is cervical nodal metastasis, the survival would decrease by half even if there is only one positive lymph node (5). Much effort has been made to formulate a reliable neck lymph node classification for better guiding treatment and predicting prognosis. The newest version of AJCC classification takes

the size, number, extracapsular spread (ECS), and laterality of positive nodes into consideration during the cervical nodal status definition (6). However, a number of researchers have noted that this classification fails to detect the survival difference between N1 and N2a disease (7), and also between N2b and N2c disease (8). Thus, a proposed nodal system based on the number of positive lymph nodes is suggested, and it is verified to be superior to the 8th AJCC classification (9–11).

HPV-induced cancer is attracting more and more attention, and it contributes to at least 70% of the newly diagnosed oropharynx SCC. p16 over-expression is significantly associated with HPV infection (12), and it usually carries a favorable prognosis in oropharynx SCC. However, the reported rates of HPV infection and p16 over-expression as well as its impact on prognosis in oral SCC varies greatly (13). Therefore, in the current study, we aimed to analyze the significance of the number of positive lymph nodes in oral SCC stratified by p16.

PATIENTS AND METHODS

Ethical Consideration

Henan Cancer Hospital institutional research committee approved our study, and all participants signed an informed consent agreement. All methods were performed in accordance with the relevant guidelines and regulations. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient Selection

Medical records of patients undergoing surgical treatment for primary oral SCC between January 2013 and December 2019 were retrospectively enrolled, included patients needed to meet the following criteria: there was no history of other cancer; there was enough tissue available for HPV analysis; the patient received neck dissection; the number of lymph nodes examined was not <10. Demography and pathologic information, and TNM stage based on the 8th AJCC classification as well as follow-up data was extracted and analyzed.

Important Variable Definition

Drinkers were defined as those who consumed at least one alcoholic drink per day for at least 1 year, and smokers were defined as those who smoked on a daily basis or had quit smoking for <5 years (3), perineural invasion (PNI) was considered to be present if tumor cells were identified within the perineural space and/or nerve bundle; lymphovascular infiltration was positive if tumor cells were noted within the lymphovascular channels (14). The pathologic depth of invasion (DOI) was measured from the level of the adjacent normal mucosa to the deepest point of tumor infiltration, regardless of the presence or absence of ulceration (6). Extracapsular spread (ECS) was positive if there were tumor cells out of the capsular of the positive lymph node (15).

TABLE 1 | Demography and pathologic data in the 674 patients with oral squamous cell carcinoma.

Variables	*N* (%)
Age	
<40	78 (11.6%)
≥40	596 (88.4%)
Gender	
Male	517 (76.7%)
Female	157 (23.3%)
Smoker	519 (77.0%)
Drinker	387 (57.4%)
Primary site	
Tongue	248 (36.8%)
Buccal	177 (26.3%)
Gingiva	132 (19.6%)
The floor of the mouth	117 (17.4%)
Pathologic tumor stage	
T1+T2	385 (57.1%)
T3+T3	289 (42.9%)
Tumor differentiation	
Well	266 (39.5%)
Moderate	300 (44.5%)
Poor	108 (16.0%)
Perineural invasion	273 (40.5%)
Lymphovascular invasion	234 (34.7%)
Positive margin	35 (5.2%)
Pathologic neck lymph node stage	
N0	385 (57.1%)
N1	103 (15.3%)
N2	107 (15.9%)
N3	79 (11.7%)

Immunohistochemical (IHC) Analysis

From July 2013, routine immunohistochemical analysis of p16 was performed for every oral SCC patient. Level of positivity of p16 over expression was consistent with previous studies well (16): 0-+, defined as <25% tumor staining; ++, defined as 25–50% tumor staining; + + +, defined as 50–75% tumor staining; and + + ++: defined as more than 75% tumor staining. Tumors with level + + + and + + ++ classified as positive p16.

HPV Assessment

From July 2013, HPV detection was selectively performed for oral SCC patients in our cancer center by fresh tumor tissue. DNA was extracted using TIANcombi DNA Lyse&Det PCR Kit (TIANGEN Cooperation, Beijing, China), and then submitted to real-time PCR with the INNO-LIPA HPV Genotyping Extra System® kit (Innogenetics), it could detect 7 low-risk HPV types (6, 11, 40, 43, 44, 54, 70), 3 indeterminate-risk types (69, 71, 74), and 18 high risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82). For paraffin-embedded tissue, at least five 10-um thick slices were used for DNA extraction by TIANcombi DNA Lyse&Det PCR Kit (TIANGEN Cooperation,

TABLE 2 | Comparison of clinical and pathologic variables among patients with different numbers of positive lymph nodes.

Variables	Number of positive lymph nodes				*p*
	0 ($n = 385$)	1–2 ($n = 180$)	3–4 ($n = 72$)	≥5 ($n = 37$)	
Age					
<40	38 (9.9%)	22 (12.2%)	13 (18.1%)	5 (13.5%)	
≥40	347 (90.1%)	158 (87.8%)	59 (81.9%)	32 (86.5%)	0.235
Sex					
Male	279 (72.5%)	144 (80.0%)	61 (84.7%)	33 (89.2%)	
Female	106 (27.5%)	36 (20.0%)	11 (15.3%)	4 (10.8%)	0.013
Smoker	277 (71.9%)	143 (79.4%)	62 (86.1%)	37 (100%)	<0.001
Drinker	225 (58.4%)	100 (55.6%)	42 (58.3%)	20 (54.1%)	0.893
Primary site					
Tongue	114 (29.6%)	76 (42.2%)	35(48.6%)	23 (62.2%)	
Buccal	122 (31.7%)	42 (23.3%)	11 (15.3%)	2 (5.4%)	
Gingiva	90 (23.4%)	30 (16.7%)	10 (13.9%)	2 (5.4%)	
The floor of the mouth	59 (15.3%)	32 (17.8%)	16 (22.2%)	10 (27.0%)	<0.001
Pathologic tumor stage					
T1+T2	253 (65.7%)	102 (56.7%)	25 (34.7%)	5 (13.5%)	
T3+T4	132 (34.3%)	78 (43.3%)	47 (65.3%)	32 (86.5%)	<0.001
Tumor differentiation					
Well	197 (51.2%)	50 (27.8%)	15 (20.8%)	4 (10.8%)	
Moderate + poor	188 (48.8%)	130 (72.2%)	57 (79.2%)	33 (89.2%)	<0.001
Perineural invasion	116 (30.1%)	85 (47.2%)	42 (58.3%)	30 (81.1%)	<0.001
Lymphovascular invasion	101 (26.2%)	61 (33.9%)	45 (62.5%)	27 (73.0%)	<0.001
Positive margin	8 (2.1%)	13 (7.2%)	8 (11.1%)	6 (16.2%)	<0.001
Extracapsular spread	–	26 (14.4%)	26 (36.1%)	27 (73.0%)	<0.001
Neck lymph node stage					
N1	–	103 (57.2%)	0	0	
N2	–	59 (32.8%)	36 (50.0%)	12 (32.4%)	
N3	–	18 (10.0%)	36 (50.0%)	25 (67.6%)	<0.001
HPV positivity	37 (9.6%)	20 (11.1%)	8 (11.1%)	4 (10.8%)	0.946
p16 positivity	41 (10.6%)	22 (12.2%)	12 (16.7%)	10 (27.0%)	0.024

Beijing, China) according to the instruction, the following procedures were similar with above-mentioned description.

Treatment Proposal

In our cancer center, preoperative systemic examinations of ultrasound, CT/MRI and/or PET-CT was performed for every patient. Complete resection of primary tumor was achieved with at least 1 cm margin, a free flap or pedicled flap was used to close the defect if necessary. For a cN0 neck, a dissection of level 1 to 3/4 was performed, for a cN+ neck, a modified radical or radical neck dissection of level 1 to 5 was performed. Adjuvant treatment was suggested if there was presence of T3/4 disease, pathologic cervical disease, PNI, LVI, positive margin, and ECS. After discharging, the patient was followed every 3 months for the first 2 years, every 6 months for the third to fifth year, and then once per year. If there was suspicion of disease recurrence, active inference was taken.

Statistic Analysis

The cut-off value of positive lymph nodes was defined according to previous studies (8, 10, 17), the patients were divided into four groups based on the four knots (0 vs. 1–2 vs. 3–4 vs. ≥5). The difference among the four groups was compared using the Chi-square test. However, owing to the small sample size of patients with p16 positivity, these patients were divided into two groups (0–2 vs. ≥3), and also because of their limited follow-up time, prognostic difference of the two groups was compared using the 3-year survival rate. The study endpoints were disease-free survival (DFS) and the disease specific survival (DSS), and they were calculated by the Kaplan-Meier method. The survival time of DFS was calculated from the date of surgery to the date of first locoregional recurrence or distant metastasis or the last follow-up. The survival time of DSS was calculated from the date of surgery to the date of cancer-caused death or the last follow-up. The factors which were significant in univariate analysis were then analyzed in the Cox proportional hazards model to find out the independent factor. The Harrell's C-concordance index was used to compare the model fitness between number of positive lymph nodes model and the 8th AJCC classification, where the higher the value, the better the discrimination among subgroups (18). All statistical analyses were performed using SPSS 20.0, a value of $p < 0.05$ was considered to be significant.

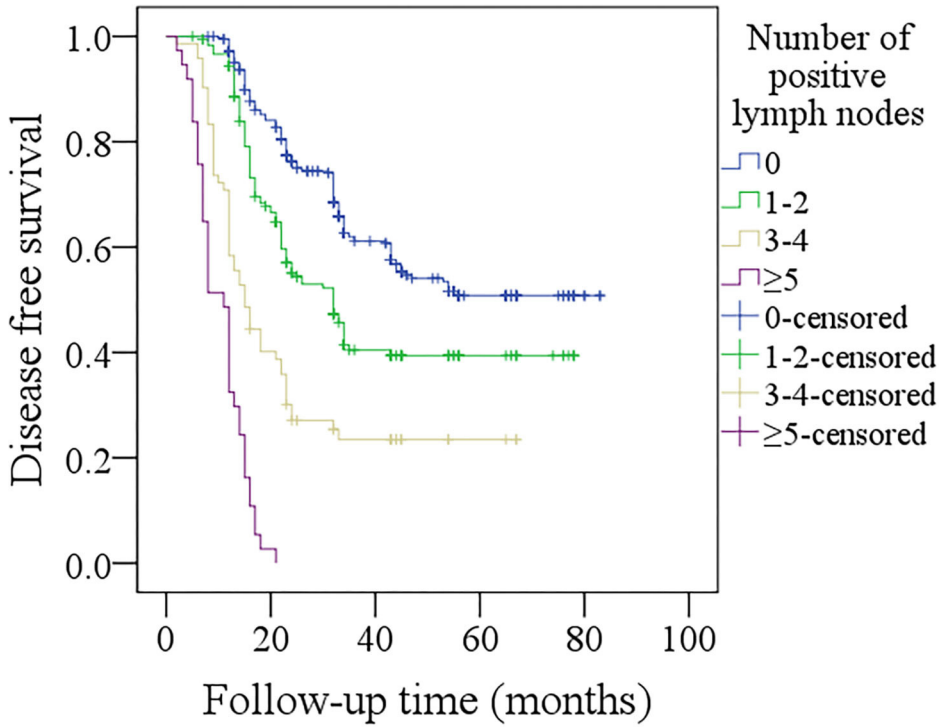

FIGURE 1 | Comparison of disease-free survival among patients with different numbers of positive lymph nodes ($p < 0.001$).

RESULTS

Demography and Pathologic Data

A total of 674 patients were enrolled for analysis, there were 517 (76.7%) male and 157 (23.3%) female, the mean age was 57.5 years with a range from 32 years to 78 years. Smoker and drinker were noted in 519 (77.0%) patients and 387 (57.4%) patients, respectively.

Primary sites were characterized as tongue in 248 (36.8%) patients, buccal in 177 (26.3%) patients, gingiva in 132 (19.6%) patients, and the floor of the mouth in 117 (17.4%) patients. Pathologic tumor stages were distributed as T1 in 118 (17.5%) patients, T2 in 267 (39.6%) patients, T3 in 189 (28.0%) patients, and T4 in 100 (14.8%) patients. The mean pathologic DOI was 9.8 mm with a range from 1.4 to 24.5 mm. Tumor differentiation was distributed as well in 266 (39.5%) patients, moderate in 300 (44.5%) patients, and poor in 108 (16.0%) patients. PNI and LVI was presented in 273 (40.5%) patients and 234 (34.7%) patients, respectively. Positive margin occurred in 35 (5.2%) patients.

The mean number of lymph nodes examined was 21.5 with a range from 12 to 47. Positive cervical disease occurred in 289 (42.9%) patients, and pathologic neck lymph node stages were distributed as N0 in 385 (57.1%) patients, N1 in 103 (15.3%) patients, N2 in 107 (15.9%) patients, and N3 in 79 (11.7%) patients. In the 289 patients with cervical nodal metastasis, 130 (45.0%) patients had one positive lymph node, 50 (17.3%) patients had two positive lymph nodes, 40 (13.8%) patients had three positive lymph nodes, 32 (11.1%) patients had four positive lymph nodes, 20 (6.9%) patients had five positive lymph nodes, and 17 (5.9%) patients had more than 5 positive lymph nodes. ECS occurred in 79 (11.7%) patients (**Table 1**).

HPV and p16 Test

HPV show positivity in 69 patients with a rate of 10.2%, in whom 30 (43.5%) patients had a tumor arising from the tongue, 15 (21.7%) cases from the buccal, 10 (14.5%) cases from the gingiva, and 14 (20.2%) cases from the floor of the mouth. 7 (10.1%) of the 69 patients also showed p16 positivity.

p16 showed positivity in 85 patients with a rate of 12.6%, in whom 50 (61.0%) patients had a tumor arising from the tongue, 8 (9.4%) cases from the buccal, 7 (8.2%) cases from the gingiva, and 20 (23.5%) cases from the floor of the mouth. 8 (9.4%) of the 85 patients also showed HPV positivity.

Comparison Among the Four Groups

The four groups had similar distribution regarding age ($p = 0.235$), drinker status ($p = 0.893$), and HPV positivity ($p = 0.946$). There was significant difference of distribution of gender ($p = 0.013$), smoker status ($p < 0.001$), primary site ($p < 0.001$), pathologic tumor stage ($p < 0.001$), tumor differentiation ($p < 0.001$), PNI ($p < 0.001$), LVI ($p < 0.001$), ECS ($p < 0.001$), positive margin ($p < 0.001$), neck lymph node stage ($p < 0.001$), and p16 positivity($p = 0.024$) (**Table 2**). Patients with greater number of positive lymph nodes tended to be a smoking man with SCC arising from the tongue or the floor of the mouth. Adverse pathologic characteristics including high tumor stage, presence of PNI, LVI, and ECS, and cervical nodal disease were more frequent in patients having more than 5 positive lymph

TABLE 3 | Univariate and Cox model analyses of the disease-free survival in the 674 patients.

Variables	Univariate	Cox model	
	p	*p*	HR [95% CI]
Age (<40 vs. ≥40)	0.156		
Sex (Male vs. female)	0.342		
Smoker	<0.001	<0.001	1.461 [1.197–1.998]
Drinker	0.471		
Primary site			
Tongue + The mouth floor vs. others	<0.001	<0.001	2.476 [1.227–3.471]
Pathologic tumor stage			
T3+T4 vs. T1+T2	<0.001	<0.001	3.446 [1.385–6.331]
Tumor differentiation			
Moderate + poor vs. well	<0.001	<0.001	1.998 [1.264–3.558]
Perineural invasion	<0.001	<0.001	2.363 [1.277–4.338]
Lymphovascular invasion	<0.001	<0.001	2.255 [1.304–4.264]
Neck lymph node stage	<0.001		
N0			
N1		<0.001	1.685 [1.125–2.138]
N2		<0.001	2.453 [1.773–3.467]
N3		<0.001	3.007 [2.162–6.487]
Number of lymph node examined			
<22 vs. ≥22	0.267		
HPV positivity	0.993		
p16 positivity	<0.001	<0.001	1.565 [1.183–2.021]
Positive margin	<0.001	<0.001	1.996 [1.317–2.778]
Number of positive lymph nodes	<0.001		
0			
1–2		<0.001	1.981 [1.241–2.525]
3–4		<0.001	3.126 [2.612–4.178]
≥5		<0.001	5.453 [4.431–8.465]

nodes. Additionally, p16 positivity was associated with greater number of positive lymph nodes.

During our follow-up, with a mean time of 40.0 months, a total of 463 patients received adjuvant treatment, of which 286 patients received radiotherapy, 177 patients received chemoradiotherapy. Recurrence occurred in 340 patients: 252 patients had locoregional recurrence, and 88 patients had concurrent locoregional recurrence and distant metastasis. Hundred patients received salvage surgical treatment, and the rest received palliative chemotherapy. Two hundred and sixty seven patients died of the disease. The overall 5-year DFS and DSS rates were 41 and 41%, respectively.

In patients with no positive lymph nodes, the 5-year DFS rate was 49%, in patients with 1–2 positive lymph nodes, the 5-year DFS rate was 39%, in patients with 3–4 positive lymph nodes, the 5-year DFS rate was 23%, in patients with ≥5 positive lymph nodes, all patients developed recurrence within 2 years after operation. The difference was significant (**Figure 1**, $p < 0.001$). In further Cox model analysis, the factors of smoker, the number of positive lymph nodes, primary site, pathologic tumor stage, tumor differentiation, PNI, LVI, neck lymph node stage, and p16 were significantly associated with the DFS (**Table 3**). The Harrell's C-concordance index for number of positive lymph nodes system and the 8th AJCC neck lymph node classification was 0.7312 and 0.7299.

In patients with no positive lymph nodes, the 5-year DSS rate was 57%, in patients with 1–2 positive lymph nodes, the 5-year DSS rate was 39%, in patients with 3–4 positive lymph nodes, the 5-year DSS rate was 17%, in patients with ≥5 positive lymph nodes, all patients died of the disease within 4 years after operation. The difference was significant (**Figure 2**, $p < 0.001$). In further Cox model analysis, the factors of smoker status, the number of positive lymph nodes, primary site, pathologic tumor stage, tumor differentiation, PNI, LVI, neck lymph node stage, and p16 were significantly associated with the DSS (**Table 4**). The Harrell's C-concordance index for number of positive lymph nodes system and the 8th AJCC neck lymph node classification was 0.7200 and 0.7186.

In further sub-group analysis of patients with p16 negativity, in patients with no positive lymph nodes, the 5-year DFS rate was 52%, in patients with 1–2 positive lymph nodes, the 5-year DFS rate was 39%, in patients with 3–4 positive lymph nodes, the 5-year DFS rate was 21%, in patients with ≥5 positive lymph nodes, all patients developed recurrence within 2 years after operation. The difference was significant (**Figure 3**, $p < 0.001$). In patients with no positive lymph nodes, the 5-year DSS rate was 60%, in patients with 1–2 positive lymph nodes, the 5-year DSS rate was 38%, in patients with 3–4 positive lymph nodes, the 5-year DSS rate was 18%, in patients with ≥5 positive lymph nodes, all patients died within 4 years after operation. The difference was significant (**Figure 4**, $p < 0.001$).

In further sub-group analysis of patients with p16 positivity, its sample size was relatively small, therefore, we divided them into two groups based on the number of positive lymph nodes (0–2 vs. ≥3). In patients with 0–2 positive lymph nodes, the 3-year DFS rate was 41%, in patients with ≥3 positive lymph nodes, the 3-year DFS rate was 17%, the difference was significant (**Figure 5**, $p < 0.001$). In patients with 0–2 positive lymph nodes, the 3-year DSS rate was 84%, in patients with ≥3 positive lymph nodes, the 3-year DSS rate was 46%, the difference was significant (**Figure 6**, $p < 0.001$).

DISCUSSION

The most important finding in the current study was that we confirmed the prognostic significance of the number of positive lymph nodes in oral SCC, and the effect was unaffected by p16 status. Additionally, the number of positive lymph nodes system was superior to the 8th AJCC neck lymph node classification. It provided a reliable method to instruct adjuvant treatment and a better tool for doctor-to-patient communication.

Cervical node status was the most important prognostic factor in oral SCC, the newest version of AJCC classification

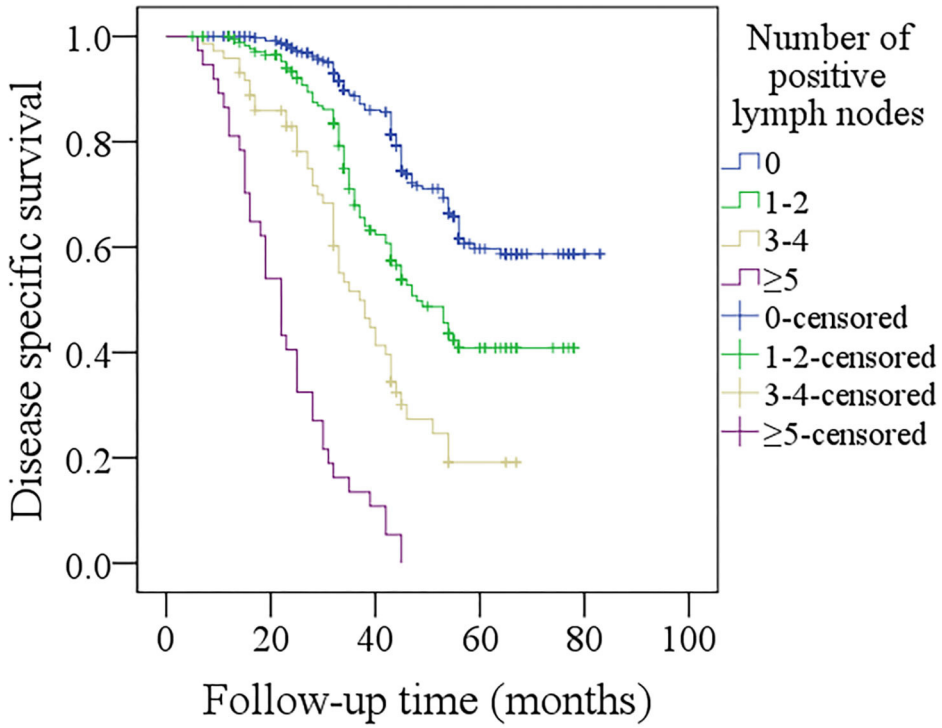

FIGURE 2 | Comparison of disease-specific survival among patients with different numbers of positive lymph nodes ($p < 0.001$).

was made based on analyzing the pooled database from two famous medical centers (6, 19), taking the size, number, ECS, and laterality of positive lymph nodes into consideration, although there was significant improvement in neck staging (20), apparent deficiency could not be neglected. It was previously believed that contralateral or bilateral cervical disease was associated with aggressive biologic behavior, but current evidence showed the uncommon performance tended to be contributed by unpredictable lymphatic drainage patterns rather than aggressive biology (8, 11, 21, 22).

Then some researchers introduced a revision version of neck lymph node status based on positive lymph node number. Roberts et al. (9) divided 12,437 patients with head and neck SCC into 4 groups based on the number of positive lymph nodes (0 vs. 1 vs. 2–5 vs. >5), and found patients with >5 positive lymph nodes had the worst prognosis, and the association remained independent in multivariate analysis with a lower Akaike information criterion than that in AJCC N stage. Ho et al. (23) identified 8,351 laryngohypopharyngeal patients from the National Cancer Database, in whom 56.4% had neck metastatic disease, in the survival analysis, the authors reported as number of positive lymph nodes increased, mortality risk escalated continuously without plateau, and the hazard per node was the most pronounced up to 5 metastatic lymph nodes, moreover, when accounting for positive lymph node number, the factors of the size of positive lymph nodes and contralaterality in standard nodal system had no prognostic value. The same research team selected 14,554 oral SCC patients from the National Cancer Database, and found in univariate analysis the 5-year overall survival rates were 65.3, 49.9, 41.1, 29.7, 27.5, 18.5, and 9.7% for those with 0, 1, 2, 3, 4 to 6, 7 to 9, and 10 or more positive lymph nodes, respectively, and there was still a strong relationship between the number of positive lymph nodes and overall survival after adjusting for important confounding factors (11). However, all those authors did not evaluate the effect of positive lymph node number on the DSS which was not affected by general body status. Additionally, racial difference played a significant role on cancer survival. Our study was the first to confirm the prognostic significance of positive lymph node number in DSS in oral SCC patients in China, and the model showed superiority to the AJCC N stage with higher Harrell's C-concordance index. A similar finding was also reported by Rajappa et al. (10) and Ebrahimi et al. (8).

p16 was usually used as a surrogate marker of HPV infection owing to the significant association between them in oropharynx SCC, but it was not like that in oral SCC. Harris et al. (24) noted 44% of the 25 tongue SCC patients showed p16 positivity, but none had HPV16 positivity by PCR analysis. Similarly, Poling et al. (25) found HPV positivity was only detected in 1 of the 9 cases with p16 positivity from 78 tongue SCC patients. Our finding would be consistent with these reports. The prognostic role of p16 in oral SCC was not frequently analyzed, and the existing literature showed conflicted effect results. Almangush et al. (26) previously performed a meta-analysis consisting of

TABLE 4 | Univariate and Cox model analyses of the disease-specific survival in the 674 patients.

Variables	Univariate	Cox model	
	p	*p*	HR[95% CI]
Age (<40 vs. ≥40)	0.231		
Sex (Male vs. female)	0.156		
Smoker	<0.001	<0.001	1.336 [1.075–1.748]
Drinker	0.374		
Primary site			
Tongue + The mouth floor vs. others	<0.001	<0.001	2.132 [1.426–3.164]
Pathologic tumor stage			
T3+T4 vs. T1+T2	<0.001	<0.001	3.128 [1.476–5.129]
Tumor differentiation			
Moderate + poor vs. well	<0.001	<0.001	2.006 [1.387–3.814]
Perineural invasion	<0.001	<0.001	2.061 [1.337–3.994]
Lymphovascular invasion	<0.001	<0.001	2.116 [1.452–3.860]
Neck lymph node stage	<0.001		
N0			
N1		<0.001	1.456 [1.027–1.999]
N2		<0.001	2.375 [1.564–3.555]
N3		<0.001	3.467 [2.622–5.932]
Number of lymph node examined			
<22 vs. ≥22	0.513		
HPV positivity	0.673		
p16 positivity	0.024	<0.001	1.321 [1.048–1.733]
Positive margin	<0.001	<0.001	3.776 [1.671–5.997]
Number of positive lymph nodes	<0.001		
0			
1-2		<0.001	1.862 [1.122–2.442]
3-4		<0.001	3.189 [2.611–4.554]
≥5		<0.001	6.316 [4.673–10.227]

174 studies and found there was no sufficient evidence to support the prognostic role of p16 in tongue SCC. Lai et al. (27) enrolled 143 patients with oral or oropharynx SCC, and determined the functional HPV presence by analyzing HPV *in situ* hybridization and p16 immunohistochemistry, in the survival analysis, the authors reported there was no significant difference of overall survival and DFS between patients with or without p16 positivity. A similar finding was also reported by Fakhry et al. (28). But Chung et al. (29) noted 62 (19.3%) of the 322 non-oropharynx head and neck SCC showed p16 positivity, and p16 over expression carried a protective effect on progression-free survival and overall survival. On the contrary, Larque et al. (30) and Dediol et al. (16) concluded p16 expression was related to worse survival in oral SCC. Our finding would also support this viewpoint. A possible explanation might be that p16 positivity meant higher number of positive lymph nodes induced by aggressive tumor behavior. More importantly, we were the first to evaluate the interaction effect of the number of positive lymph nodes and p16 positivity and note that the prognostic significance of the positive lymph node number did not alter with p16 status. The finding was novel, and provided the first possibility and feasibility of the revision nodal staging system based on the number of positive lymph nodes without considering p16 status. In a previous study by Divi et al. (31), the authors also reported the prognostic effect of the number of lymph nodes examined was not associated with p16 positivity.

Another attractive variable was the lymph node yield (LNY), which was the number of lymph nodes retrieved after neck dissection. Lemieux et al. (32) selected 4,341 patients with pN0 oral SCC, and found the mean LNY increased with tumor stage from T1 to T3, the cut-off of 22 nodes removed indicated a significant predictor of overall survival, and each additional lymph node excised was related to improved survival, and the effect maintained until 43 nodes removed. Pou et al. (33) reported that in 118 patients with cN0 head and neck SCC, metastatic disease was present in 23.73% of cases. Positive lymph node was the most likely to be detected in patients with LNY >35, and the rate was comparable in patients with LNY 26 to 35. And in patients with <18 lymph nodes, the detected rate was the lowest, then the authors concluded that the minimum for LNY was 18 for an adequate level I–III neck dissection. Kuo et al. (34) used the SEER database and found there was significant survival benefit related to ≥16 lymph nodes removed compared with lower LNY in 3097 cN0 patients, and there was survival benefit related to ≥26 lymph nodes removed compared with lower LNY in 1,268 cN+ patients. Similar findings were also reported by Divi et al. (35) and El Asmar et al. (36), but we failed to note the prognostic significance of LNY if cN0 and cN+ patients were analyzed together. There were some aspects must be considered when comprehending this finding: LNY was mainly based on the surgeon's ability of dissecting lymph nodes, the pathologist's ability of identifying the lymph nodes, and the level dissected. Treatment in academic medical center was also responsible for LNY (36). The relationship between survival and LNY was an association but not a causality, and this effect was easily affected by the neck status.

The concept of lymph node ratio (LNR), which was defined as the ratio of the number of positive lymph nodes to the number of lymph nodes examined, became more and more attentive. Hua et al. (17) enrolled 81 hypopharyngeal SCC patients, and divided these patients into three groups based on the metastatic nodes ratio (0 vs. <10% vs. >10%), and found patients with high LNR had worse prognosis in both univariate and multivariate analyses. Similar findings were also reported by Huang et al. (37) and Ding et al. (38). However, LNR was very vulnerable because of variable LNY. LNY was significantly different and increased with the number of neck levels dissected, and even in the same type neck dissection, LNY might not be the same (39), then this would lead patients with the same number of positive lymph nodes but different LNY to different neck stage. The inferiority of LNR had been verified by Ho et al. (23) and Roberts et al. (9).

Limitations in the current study must be acknowledged: firstly, the retrospective design had inherent bias; secondly, the sample size and follow-up time of patients with p16

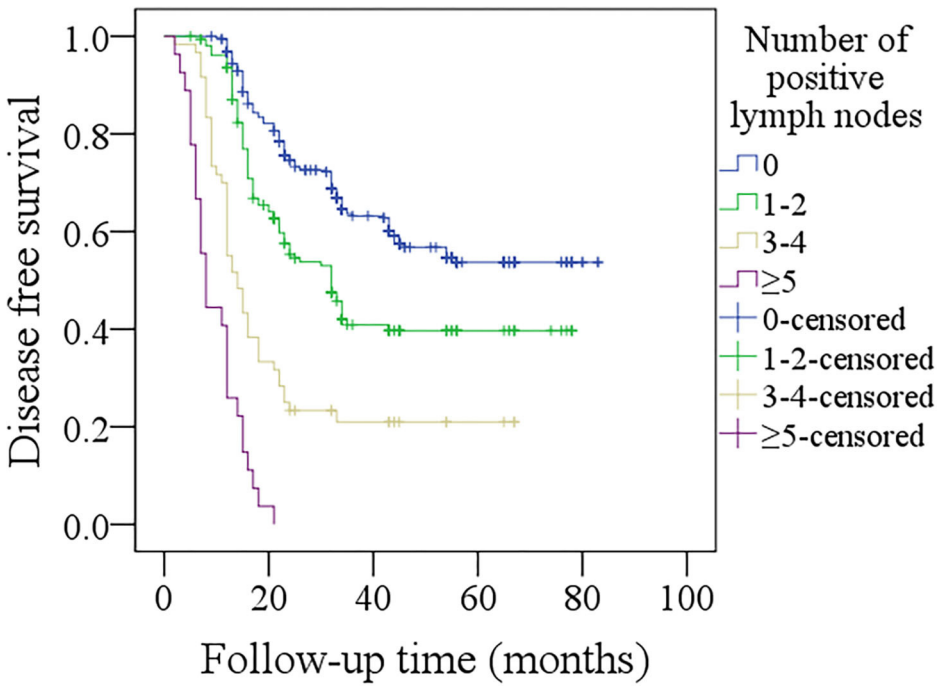

FIGURE 3 | Comparison of disease-free survival among p16 negative patients with different numbers of positive lymph nodes ($p < 0.001$).

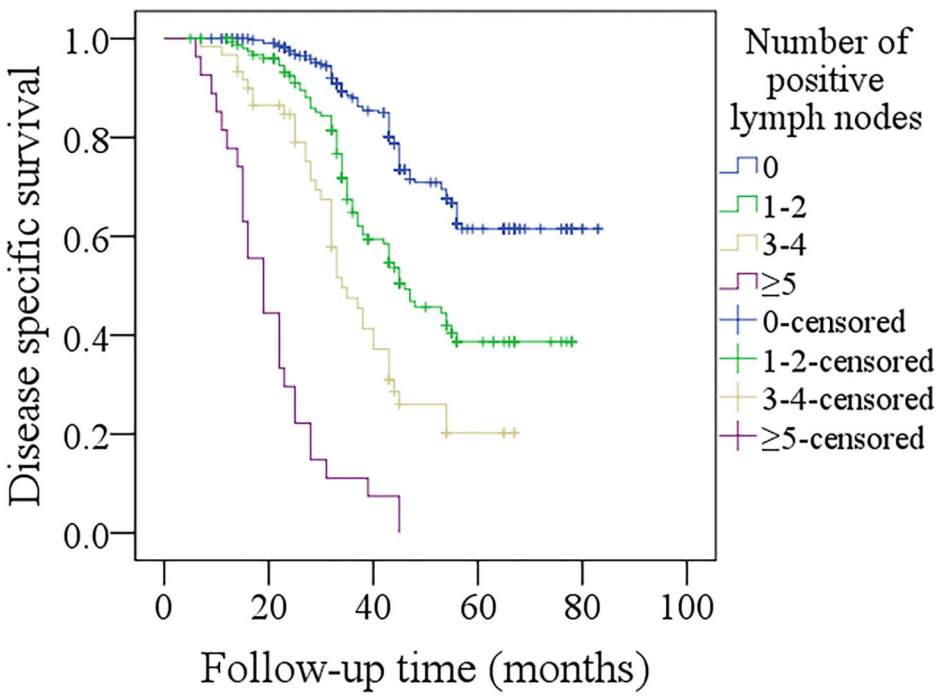

FIGURE 4 | Comparison of disease-specific survival among p16 negative patients with different numbers of positive lymph nodes ($p < 0.001$).

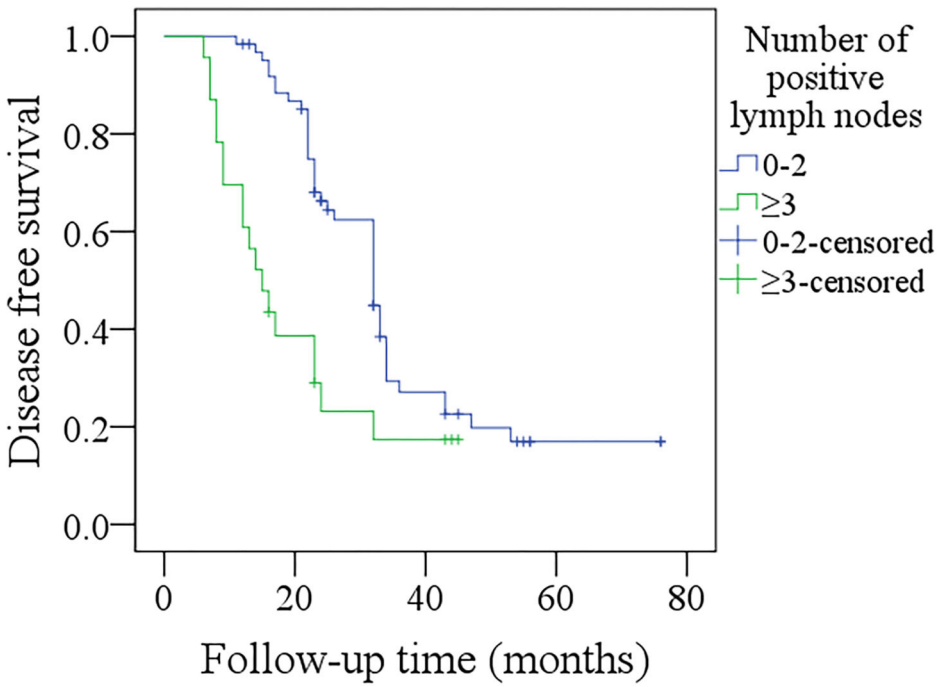

FIGURE 5 | Comparison of disease-free survival among p16 positivity patients with different numbers of positive lymph nodes ($p < 0.001$).

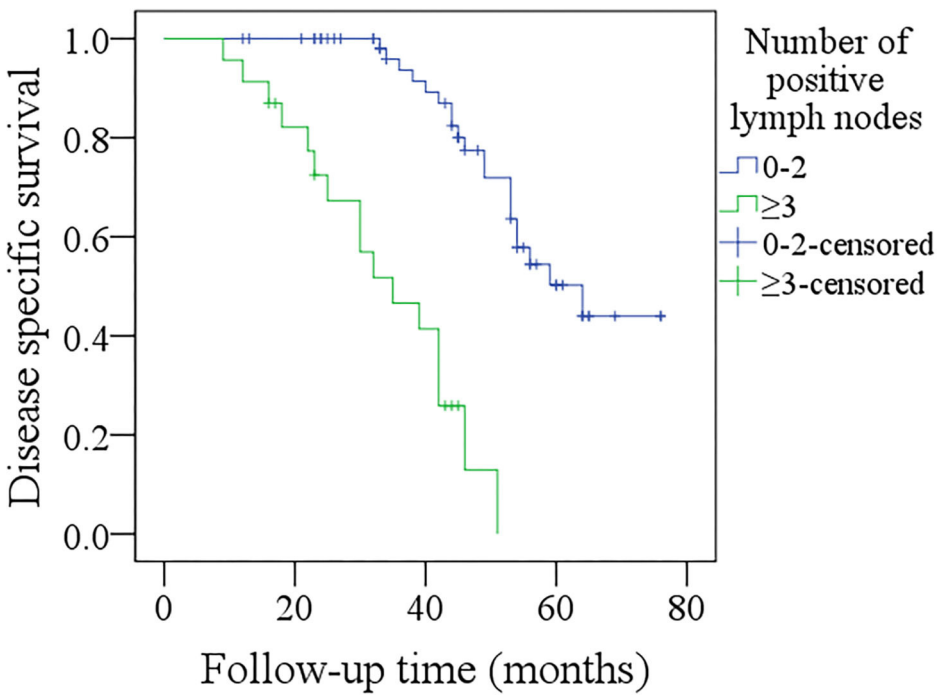

FIGURE 6 | Comparison of disease-free survival among p16 positivity patients with different numbers of positive lymph nodes ($p < 0.001$).

positivity was limited, higher quality studies are needed to clarify these questions.

In conclusion, the number of positive lymph nodes are significantly associated with survival in oral SCC, and it shows superiority to AJCC N stage in predicting the prognosis. Its survival effect is not affected by p16 status.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Henan Cancer Hospital institutional research committee approved our study, and all participants signed an informed consent agreement.

AUTHOR CONTRIBUTIONS

All the authors made the contribution in study design, manuscript writing, studies selecting, data analysis, study quality evaluating, manuscript revising, and read and approved the final manuscript.

REFERENCES

1. Zhang B, Du W, Gan K, Fang Q, Zhang X. Significance of the neutrophil-to-lymphocyte ratio in young patients with oral squamous cell carcinoma. *Cancer Manag Res.* (2019) 11:7597–603. doi: 10.2147/CMAR.S211847
2. Du W, Fang Q, Wu Y, Wu J, Zhang X. Oncologic outcome of marginal mandibulectomy in squamous cell carcinoma of the lower gingiva. *BMC Cancer.* (2019) 19:775. doi: 10.1186/s12885-019-5999-0
3. Fang Q, Li P, Qi J, Luo R, Chen D, Zhang X. Value of lingual lymph node metastasis in patients with squamous cell carcinoma of the tongue. *Laryngoscope.* (2019) 129:2527–30. doi: 10.1002/lary.27927
4. Cui M, Du W, Fang Q, Dai L, Qi J, Luo R. Prognostic value of a family history of oral tongue squamous cell carcinoma: a matched-pair study. *Laryngoscope.* (2019) 130:E605–10. doi: 10.1002/lary.28471
5. Cerezo L, Millán I, Torre A, Aragón G, Otero J. Prognostic factors for survival and tumor control in cervical lymph node metastases from head and neck cancer. A multivariate study of 492 cases. *Cancer.* (1992) 69:1224–34. doi: 10.1002/cncr.2820690526
6. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin.* (2017) 67:122–37. doi: 10.3322/caac.21389
7. Ebrahimi A, Gil Z, Amit M, Yen TC, Liao CT, Chatturvedi P, et al. Comparison of the American Joint Committee on Cancer N1 versus N2a nodal categories for predicting survival and recurrence in patients with oral cancer: Time to acknowledge an arbitrary distinction and modify the system. *Head Neck.* (2016) 38:135–9. doi: 10.1002/hed.23871
8. Ebrahimi A, Gil Z, Amit M, Yen TC, Liao CT, Chaturvedi P, et al. The prognosis of N2b and N2c lymph node disease in oral squamous cell carcinoma is determined by the number of metastatic lymph nodes rather than laterality: evidence to support a revision of the American Joint Committee on Cancer staging system. *Cancer.* (2014) 120:1968–74. doi: 10.1002/cncr.28686
9. Roberts TJ, Colevas AD, Hara W, Holsinger FC, Oakley-Girvan I, Divi V. Number of positive nodes is superior to the lymph node ratio and American Joint Committee on Cancer N staging for the prognosis of surgically treated head and neck squamous cell carcinomas. *Cancer.* (2016) 122:1388–97. doi: 10.1002/cncr.29932
10. Rajappa SK, Maheshwari U, Jaipuria J, Singh AK, Goyal S, Batra U, et al. Number of positive nodes - current relevance in determining prognosis of oral cavity cancer after the recent AJCC staging update. *Oral Oncol.* (2019) 90:1–5. doi: 10.1016/j.oraloncology.2019.01.001
11. Ho AS, Kim S, Tighiouart M, Gudino C, Mita A, Scher KS, et al. Metastatic lymph node burden and survival in oral cavity cancer. *J Clin Oncol.* (2017) 35:3601–9. doi: 10.1200/JCO.2016.71.1176
12. Solomon B, Young RJ, Rischin D. Head and neck squamous cell carcinoma: genomics and emerging biomarkers for immunomodulatory cancer treatments. *Semin Cancer Biol.* (2018) 52(Pt. 2):228–40. doi: 10.1016/j.semcancer.2018.01.008
13. Jiang S, Dong Y. Human papillomavirus and oral squamous cell carcinoma: a review of HPV-positive oral squamous cell carcinoma and possible strategies for future. *Curr Probl Cancer.* (2017) 41:323–7. doi: 10.1016/j.currproblcancer.2017.02.006
14. Dai L, Fang Q, Li P, Wu J, Zhang X. Secondary squamous cell carcinoma of the oral cavity after nasopharyngeal carcinoma. *Cancer Res Treat.* (2020) 52:109–16. doi: 10.4143/crt.2019.202
15. Mermod M, Tolstonog G, Simon C, Monnier Y. Extracapsular spread in head and neck squamous cell carcinoma: a systematic review and meta-analysis. *Oral Oncol.* (2016) 62:60–71. doi: 10.1016/j.oraloncology.2016.10.003
16. Dediol E, Sabol I, Virag M, Grce M, Muller D, Manojlović S. HPV prevalence and p16INKa overexpression in non-smoking non-drinking oral cavity cancer patients. *Oral Dis.* (2016) 22:517–22. doi: 10.1111/odi.12476
17. Hua YH, Hu QY, Piao YF, Tang Q, Fu ZF. Effect of number and ratio of positive lymph nodes in hypopharyngeal cancer. *Head Neck.* (2015) 37:111–6. doi: 10.1002/hed.23574
18. Subramaniam N, Balasubramanian D, Kumar N, Murthy S, Vijayan SN, Nambiar A, et al. Lymph node staging systems in oral squamous cell carcinoma: a comparative analysis. *Oral Oncol.* (2019) 97:92–8. doi: 10.1016/j.oraloncology.2019.08.002
19. Amin MB, Edge S, Greene F. *AJCC Cancer Staging Manual*. 8th ed. New York, NY:Springer (2017).
20. Huang SH, O'Sullivan B. Overview of the 8th Edition TNM classification for head and neck cancer. *Curr Treat Options Oncol.* (2017) 18:40. doi: 10.1007/s11864-017-0484-y
21. Hall SF, Groome PA, Dixon PF. Does N stage predict survival?. *J Otolaryngol.* (1996) 25:296–9.
22. Hall SF, Groome PA, Rothwell D, Dixon PF. Using TNM staging to predict survival in patients with squamous cell carcinoma of head & neck. *Head Neck.* (1999) 21:30–8.
23. Ho AS, Kim S, Tighiouart M, Gudino C, Mita A, Scher KS, et al. Association of quantitative metastatic lymph node burden with survival in hypopharyngeal and laryngeal cancer. *JAMA Oncol.* (2018) 4:985–9. doi: 10.1001/jamaoncol.2017.3852
24. Harris SL, Thorne LB, Seaman WT, Hayes DN, Couch ME, Kimple RJ. Association of p16(INK4a) overexpression with improved outcomes in young patients with squamous cell cancers of the oral tongue. *Head Neck.* (2011) 33:1622–7. doi: 10.1002/hed.21650
25. Poling JS, Ma XJ, Bui S, Luo Y, Li R, Koch WM, et al. Human papillomavirus (HPV) status of non-tobacco related squamous cell carcinomas of the lateral tongue. *Oral Oncol.* (2014) 50:306–10. doi: 10.1016/j.oraloncology.2014.01.006
26. Almangush A, Heikkinen I, Mäkitie AA, Coletta RD, Läärä E, Leivo I, et al. Prognostic biomarkers for oral tongue squamous cell carcinoma: a systematic review and meta-analysis. *Br J Cancer.* (2017) 117:856–66. doi: 10.1038/bjc.2017.244
27. Lai K, Killingsworth M, Matthews S, Caixeiro N, Evangelista C, Wu X, et al. Differences in survival outcome between oropharyngeal and oral cavity squamous cell carcinoma in relation to HPV status. *J Oral Pathol Med.* (2017) 46:574–82. doi: 10.1111/jop.12535

28. Fakhry C, Westra WH, Wang SJ, van Zante A, Zhang Y, Rettig E, et al. The prognostic role of sex, race, and human papillomavirus in oropharyngeal and nonoropharyngeal head and neck squamous cell cancer. *Cancer.* (2017) 123:1566–75. doi: 10.1002/cncr.30353
29. Chung CH, Zhang Q, Kong CS, Harris J, Fertig EJ, Harari PM, et al. p16 protein expression and human papillomavirus status as prognostic biomarkers of nonoropharyngeal head and neck squamous cell carcinoma. *J Clin Oncol.* (2014) 32:3930–8. doi: 10.1200/JCO.2013.54.5228
30. Larque AB, Conde L, Hakim S, Alos L, Jares P, Vilaseca I, et al. P16(INK4a) overexpression is associated with CDKN2A mutation and worse prognosis in HPV-negative laryngeal squamous cell carcinomas. *Virchows Arch.* (2015) 466:375–82. doi: 10.1007/s00428-015-1725-8
31. Divi V, Harris J, Harari PM, Cooper JS, McHugh J, Bell D, et al. Establishing quality indicators for neck dissection: correlating the number of lymph nodes with oncologic outcomes (NRG Oncology RTOG 9501 and RTOG 0234). *Cancer.* (2016) 122:3464–71. doi: 10.1002/cncr.30204
32. Lemieux A, Kedarisetty S, Raju S, Orosco R, Coffey C. Lymph node yield as a predictor of survival in pathologically node negative oral cavity carcinoma. *Otolaryngol Head Neck Surg.* (2016) 154:465–72. doi: 10.1177/0194599815622409
33. Pou JD, Barton BM, Lawlor CM, Frederick CH, Moore BA, Hasney CP. Minimum lymph node yield in elective level I-III neck dissection. *Laryngoscope.* (2017) 127:2070–3. doi: 10.1002/lary.26545
34. Kuo P, Mehra S, Sosa JA, Roman SA, Husain ZA, Burtness BA, et al. Proposing prognostic thresholds for lymph node yield in clinically lymph node-negative and lymph node-positive cancers of the oral cavity. *Cancer.* (2016) 122:3624–31. doi: 10.1002/cncr.30227
35. Divi V, Chen MM, Nussenbaum B, Rhoads KF, Sirjani DB, Holsinger FC, et al. Lymph node count from neck dissection predicts mortality in head and neck cancer. *J Clin Oncol.* (2016) 34:3892–7. doi: 10.1200/JCO.2016.67.3863
36. El Asmar M, Tsai HL, Fakhry C, Maroun CA, Mandal R, Pardoll DM, et al. The prognostic impact of pathologic lymph nodes in HPV-positive oropharyngeal cancers. *Oral Oncol.* (2019) 89:23–9. doi: 10.1016/j.oraloncology.2018.12.005
37. Huang TH, Li KY, Choi WS. Lymph node ratio as prognostic variable in oral squamous cell carcinomas: systematic review and meta-analysis. *Oral Oncol.* (2019) 89:133–43. doi: 10.1016/j.oraloncology.2018.12.032
38. Ding D, Stokes W, Eguchi M, Hararah M, Sumner W, Amini A, et al. Association between lymph node ratio and recurrence and survival outcomes in patients with oral cavity cancer. *JAMA Otolaryngol Head Neck Surg.* (2019) 145:53–61. doi: 10.1001/jamaoto.2018.2974
39. Marres CC, de Ridder M, Hegger I, van Velthuysen ML, Hauptmann M, Navran A, et al. The influence of nodal yield in neck dissections on lymph node ratio in head and neck cancer. *Oral Oncol.* (2014) 50:59–64. doi: 10.1016/j.oraloncology.2013.09.014

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Histological Severity Risk Factors Identification in Juvenile-Onset Recurrent Respiratory Papillomatosis: How Immunohistochemistry and AI Algorithms Can Help?

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Juvenile-onset recurrent respiratory papillomatosis (JoRRP) is a condition characterized by the repeated growth of benign exophytic papilloma in the respiratory tract. The course of the disease remains unpredictable: some children experience minor symptoms, while others require multiple interventions due to florid growth. Our study aimed to identify histologic severity risk factors in patients with JoRRP. Forty-eight children from two French pediatric centers were included retrospectively. Criteria for a severe disease were: annual rate of surgical endoscopy $\geq$ 5, spread to the lung, carcinomatous transformation or death. We conducted a multi-stage study with image analysis. First, with Hematoxylin and eosin (HE) digital slides of papilloma, we searched for morphological patterns associated with a severe JoRRP using a deep-learning algorithm. Then, immunohistochemistry with antibody against p53 and p63 was performed on sections of FFPE samples of laryngeal papilloma obtained between 2008 and 2018. Immunostainings were quantified according to the staining intensity through two automated workflows: one using machine learning, the other using deep learning. Twenty-four patients had severe disease. For the HE analysis, no significative results were obtained with cross-validation. For immunostaining with anti-p63 antibody, we found similar results between the two image analysis methods. Using machine learning, we found 23.98% of stained nuclei for medium intensity for mild JoRRP vs. 36.1% for severe JoRRP ($p = 0.041$); and for medium and strong intensity together, 24.14% for mild JoRRP vs. 36.9% for severe JoRRP ($p = 0.048$). Using deep learning, we found 58.32% for mild JoRRP vs. 67.45% for severe JoRRP ($p = 0.045$) for medium and

strong intensity together. Regarding p53, we did not find any significant difference in the number of nuclei stained between the two groups of patients. In conclusion, we highlighted that immunochemistry with the anti-p63 antibody is a potential biomarker to predict the severity of the JoRRP.

Keywords: juvenile onset recurrent respiratory papillomatosis, machine learning, deep learning, p53, p63, HPV, immunohistochemistry

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is characterized by the repeated growth of benign exophytic papilloma in the respiratory tract (1, 2), primarily in the larynx (1). The age distribution of RRP in Europe is trimodal with a peak in children at a median age of 7 years and two other peaks in adults at a median age of 35 and 64 years old (3). This rare condition is referred to as Juvenile-onset Recurrent Respiratory Papillomatosis (JoRRP) when it occurs in children. Epidemiologic data vary depending on the country. In France, there are no available data. In Denmark, between 1969 and 1984, the incidence was 3.6 cases per year per 100,000 children (4). In Canada, based on a national database, the incidence and prevalence from 1994 to 2007 were respectively 0.24 per 100 000 children and 1.11 per children, median age at diagnosis was 4.4 years with a sex ratio close to 1:1 (5). In the United States, data are similar (6), however incidence and prevalence seem correlated to the socioeconomic status (7). JoRRP is caused by an HPV infection, mostly by genotypes 6 and 11 (8). These epidemiological data may change in countries with a strong HPV vaccination policy: an Australian study shows a decrease in the incidence of RRP in children under 14 years of age after the introduction of the national HPV vaccination program in 2007. The incidence decreased from 0.16 cases per 100,000 children in 2012 to 0.02 cases per 100,000 in 2016 ($p = 0.034$) (9). Three modes of transmission are suggested: vertical transmission at birth [HPV type concordance between mother and newborn in different studies are however contradictory (10–12)], vertical transmission in utero (13) and horizontal transmission via the child's environment (10). Whatever the transmission mode, several studies have demonstrated that maternal condyloma at the time of delivery was a major risk factor of developing JoRRP (14, 15). While the prevalence of HPV 6 and 11 infection in pregnant women is around 2%, the prevalence of JoRRP is surprisingly low. Thus, HPV infection alone does not explain the development of the disease and strong arguments suggest that JoRRP is tied to immunity defects and genetic susceptibilities. Patients with RRP are associated with HLA DRB1*0102/0301, DQB1*0201/0202 (16, 17) and present a lack of KIR genes 3DS1 et 2DS1 (18). Moreover, their immune response presents a Th2 polarization (19) which is not suitable for viral infection control. The management of this disease is challenging because its evolution remains unpredictable: some children experience minor symptoms with spontaneous remission, while others undergo multiple interventions due to florid growth. For the most severe cases, JoRRP may lead to airway compromise, and malignant transformation to carcinoma can occur, although it is extremely rare [most often over pulmonary spread (20, 21)]. The standard treatment of JoRRP is a surgical excision (SE) with cold instruments or microdebriders. Multiple endolaryngeal procedures can lead to glottis synechia and irreversible damage to the vocal cords as well as impaired social life (22). To improve the surgical outcome and extend symptom-free periods, numerous adjuvant treatments have been tried: interferon α (23), celecoxib (24), bevacizumab (25), cidofovir (26, 27), PD-1/PD-L1 immunotherapy (28, 29), and the quadrivalent HPV vaccine (30). At the time of writing, none of these treatments have been recommended for routine use by the International Pediatric Otolaryngology Group (31). The most promising ones are the quadrivalent HPV vaccine, bevacizumab and PD-1/PD-L1 immunotherapies which appear to decrease relapses (28, 29, 32, 33).

In light of the multiplication of neo-adjuvant treatments and the impossibility to predict the evolution of the disease, we have sought to identify severity risk factors in order to improve the handling of these children. Although many studies have focused on clinical severity risk factors, the only one identified to date is the early age of onset of the disease (34, 35). To our knowledge only one article investigated in JoRRP histological criteria related to disease severity (such as the presence of mitosis above the basal cell layer) but without significant results (36). Several studies have looked for histological criteria with the help of immunohistochemistry. Ahn et al. (37) studied the density of cells expressing CD8, CD4, FoxP3, PD-1, or PD-L1 in papilloma samples in a cohort of 39 patients. Only CD8+ cells density was inversely correlated with disease severity ($p = 0.01$). Another study on papilloma samples involving 12 patients found a trend between a greater number of cells marked by the anti-p53 antibody and greater disease activity (defined by more than 3 SE per year); however this association was not statistically significant ($p = 0.1$) (38). As a reminder, TP53 is a tumor suppressor gene, so its loss of function leads to tumor development. The p53 protein acts as a transcription factor regulating the expression of a large number of genes involved in the cell cycle, apoptosis, cell differentiation, DNA repair, cell metabolism, migration and angiogenesis (39). p53 immunohistochemistry is used as a prognostic factor (40, 41). It is also used to distinguish dysplastic epithelium (overexpressing p53) from epithelium with reactive changes (presenting a wild-type staining) (42). The p63 protein is a transcription factor belonging to the same family as the p53 protein. p63 protein appears to play an important role in the development of squamous epithelium (43). Given the scarcity of data in the literature on histological

criteria associated with JoRRP severity, we decided to conduct this multi-stage study assisted by computerized image analysis. From Hematoxylin and eosin (HE) digital slides of papilloma, we first focused on morphological patterns associated with severe JoRRP. Finding morphological predictive patterns on HE slides could help optimize patient management. To our knowledge, no study has yet been able to find such criteria; and no computerized analysis was performed to determine such morphological criteria in this pathology. Thus, we extended our queries about potential morphological discriminative patterns using artificial intelligence. Indeed, artificial intelligence has an increasing impact on digital pathology as a help for decision-making that could usher in an acceleration of clinical workflows: several models showed a capability to recapitulate patterns that experts had already recognized (44). Some previous works even succeeded in predicting gene mutation on HE slides using deep-learning algorithms (45). In parallel, we explored p53 and p63 expressions with immunohistochemistry as potential markers of JoRRP severity, and compared quantitative results with two automated workflows: one based on machine learning, a second one based on deep learning. Machine learning refers to mathematical models that are designed to learn from experience, in order to make predictions or decisions without being explicitly programmed to do so. A machine-learning algorithm might require extraction of intermediate handcrafted features, for example typical cell size, or staining intensity histogram for a given object. The algorithm would base its prediction on these selected features. Deep learning is a subtype of machine learning that goes even beyond: the model learns and builds by itself relevant features to make a final prediction, making it more generalizable and unbiased in the way features are extracted. Our step toward a deep-learning-based approach was supported by the overwhelming majority of state-of-the-art architectures that now rely on deep learning in every computer vision task. We relied on both approaches to strengthen our conclusion and ensure a high confidence in our final quantitative results.

MATERIALS AND METHODS

Population

This retrospective study was approved by an ethical committee (notice number: CPP2019-02'-019a/2019-00352-55/19.02.05.67237) and by the "Commission Nationale Informatique et Libertés" (application number: 919150). Patients were selected from two pediatric University Hospital Centers (CHU) treating JoRRP: Necker-Enfants Malades Hospital and Robert Debré Hospital (both in Paris). Patients were selected by querying each hospital database via the laboratory management software Diamic for samples taken between 2008 and 2017 with the following diagnoses: juvenile papillomatosis, viral papilloma, squamous papilloma, and papillomatosis. The single most recent sample per patient was selected, thus allowing for the best possible slide quality to be obtained for immunohistochemistry. The inclusion criteria were:

- A positive HPV "low risk" DNA *in situ* hybridization test or a positive PCR targeting HPV 6 and/or 11.
- Recurrence after diagnosis.

Clinical data were collected retrospectively in March 2018, and gathered the following information: gender, exact age at diagnosis, dates of each SE performed in the two University Hospitals, number of SE, number of Cidofovir injections received, potential tracheostomy in relation to the disease, presence of surgical sequelae (defined as the appearance of synechia of the glottis or even stenosis), location of papilloma lesions, presence of lung involvement (proven by at least one chest CT scan), presence of a lesion at the last flexible endoscopy, notion of carcinomatous transformation, potential death related to the disease.

From the dates of the SE, an average interval in days between each SE was calculated. The number of SE per year was calculated by dividing the total number of SE by the number of years between the first and last SE.

HPV Typing

When the HPV type was not already known, FFPE papilloma samples from the patient were sent to the Georges Pompidou European Hospital's Virology Department, where PCR were performed with the INNO-LiPA® kit from Innogenetics©, targeting 28 HPV genotypes including 6 and 11.

Immunohistochemistry and Staining

Immunohistochemistry was performed on sections of FFPE tissue samples of laryngeal papilloma with anti-p53 (Dako, DO-7 clone, 1/50 dilution) and anti-p63 antibodies (Roche, 4A4 clone, 1/50 dilution) carried out on a Leica™ Bond III® automat according to the protocols routinely used in the pathology department of the Necker-Enfants Malades hospital.

For each patient, we also collected an HE slide of the same laryngeal papilloma used for immunohistochemistry. Each HE slide contained at least one and up to six levels.

Image Analysis

Each p53 and p63 immunohistochemistry was scanned with a Vectra Polaris® slide scanner from Akoya Biosciences™ with a magnification corresponding to a 10x objective. Each HE slide was scanned with a NanoZoomer® from Hamamatsu® with a magnification corresponding to a 40× objective.

Prediction of Disease Severity Using Solely HE With a Deep Neural Network

We decided to apply a deep neural architecture to classify HE slides into mild or severe JoRRP, and potentially unveil what was learned by the model to highlight specific tissue regions that activated the decision.

We designed a deep-learning architecture relying on CHOWDER (46), an end-to-end framework that extended WELDON (47) for Whole Slide Images (WSI) classification: the goal of such network is to classify WSI into classes of interest (mild and severe JoRRP). Due to the size of WSI (typically 100,000 × 100,000 pixels), it is not possible to pass an entire digitized slide as is through a neural network due to memory limitations. To overcome these, tissue regions are located with

Otsu thresholding (48), and are then cut out into tiles (of size 224 × 224 pixels). A score is attributed to each of these tiles by a convolutional neural network, then aggregated through a fully connected network to make a final decision. The full architecture is described in **Figure 1**. We also worked on unveiling which specific tiles activated the final decision. For each evaluation slide, we extracted the tiles to which the model was paying the most attention and highlighted them via heatmaps, as shown in **Figure 2**.

We validated our implementation by collecting 1,580 non-Small Lung Carcinoma (NSLC) H&E slides, made publicly available by The Cancer Genome Atlas (TCGA). The details of the validation steps of our model and the heatmaps are described in the **Supplementary Data**.

Using such a large cohort allowed us to validate our implementation with an overall AUC of 0.966 to predict cancer types, thus reaching high classification performance on a task already managed by pathologists. However, our JoRRP cohort was small by the standards of such WSI classification task in machine-learning community ($n = 48$), so we tried different approaches described below, to synthetically increase the dataset size and to regularize the model. We used a four-fold cross-validation procedure in all our experiments, to confirm that our method could be generalized over an independent dataset, and flag problems such as overfitting or selection bias. Thus, for each experiment, the JoRRP cohort was splitted into four subsets, and four models were trained: each one was trained with three subsets and evaluated on the remaining one. The performance is then reported as the average of the four models performances. To address data scarcity, we tested different ways to augment and regularize our training set: basic data augmentation on tiles (flip, rotations), increasing training set size by considering different neighboring slices as independent cases, using bags dropout (49) by randomly sending a subset of the input tiles in the network (90, 80, and 70% were tested out), using a pretrained ResNet-50 feature extractor (on ImageNet and on TCGA-lung). Additionally, we experimented with different magnification levels for tiling (20X, 10X, and 5X), to ensure we scanned all potentially relevant morphological structures.

Machine-Learning Approach for p53 and p63 Immunohistochemistry

p53 and p63 quantitative analysis was performed with the Inform® 2.3 software from Akoya Biosciences™, which enables users to fine-tune built-in quantification algorithms. The analysis is a two-stage procedure: nuclei segmentation and nuclei phenotyping. Nuclei segmentation was performed by the software based on the DAB algorithm provided by the manufacturer. Then, for nuclei phenotyping, the model, which was based on multinomial logistic regression, needed to be trained to perform phenotyping. We thus selected 13 regions of interest from virtual immunohistochemistry slides of p53 (9 ROI) and p63 (4 ROI) antibodies, and had them annotated by a pathologist. Each region of interest came from a different patient, to foster staining expression and morphological heterogeneity within the training set. We gathered a training set of 500 annotated nuclei in these fields, with five labels as described in **Figure 3** [weak (1+), medium (2+) and strong staining (3+), unstained and irrelevant for non-nuclei objects]. We manually labeled nuclei until the automatized recognition by the Inform® software was concordant with visual count on the training set. Once trained, we selected at least 8 regions per p53 and p63 virtual slide to run a full quantitative analysis. The size of a region of interest was 0.47 mm × 0.35 mm. Fields of interest were selected to contain only the entire surface of the papilloma epithelium with as little connective tissue as possible. They were then analyzed by the Inform® software trained algorithm and each region of interest analyzed was visually verified. Viray et al. (50) found high accuracy between the software results and manual analysis by pathologists, yet we quantitatively assessed the algorithm performance by comparing its predictions to a pathologist annotations. We randomly selected six regions of interest from two different patients, three ROI from p53 staining and three ROI from p63 staining, containing approximately a total of 4,000 nuclei. Results show a global positive predictive value of 0.83 and a global sensitivity of 0.95. In details, positive predictive value/sensitivity results per class are: unstained (0.92/0.95), weak staining (0.87/0.95), medium staining (0.94/0.98), strong staining (0.97/0.87), and irrelevant (0.80/0.90). At the end, data of each ROI were extracted with R software.

Deep-Learning Approach for p53 and p63 Immunohistochemistry

For this approach, we selected a Faster R-CNN architecture (51) to perform cell localization and classification. This is a two-stage architecture that first tells the model where to look (with the Region Proposal Network), and then classifies the proposed objects among classes of interest. The model was trained on 10 regions of interest of size 0.512 mm × 0.512 mm, coming from five different slides (three p63 and two p53 slides). Each region was fully annotated by a pathologist with point annotations for each nuclei. Five classes were predefined: stroma, unstained (0), weakly stained (1+), moderately stained (2+), and strongly stained (3+). We chose to add a dedicated class for stroma (although this is not taken into account in staining level expression) to enforce the network to learn the distinction between stroma cells and unmarked epithelial cells despite their staining intensity similarities. By adding an extra class for stroma cells, we regularized the network and fostered morphological context learning to distinguish epithelium from stroma. The model was trained during 10,000 iterations with a weighted cross-entropy (weights equal to the inverse of the class frequency in the training set), a learning rate of 10–3, and Adam optimizer (52). As for the machine learning analysis, we randomly selected 6 regions of interest (size of 0.256 mm × 0.256 mm) from the same two patients, containing approximately a total of 3,000 nuclei. We reported a global positive predictive value of 0.90 and a global sensitivity of 0.91. In details, positive predictive value/sensitivity results per class are: unstained (0.98/0.83), weak staining (0.84/0.88), medium staining (0.92/0.98), strong staining (0.97/0.92), and stroma (0.84/0.91).

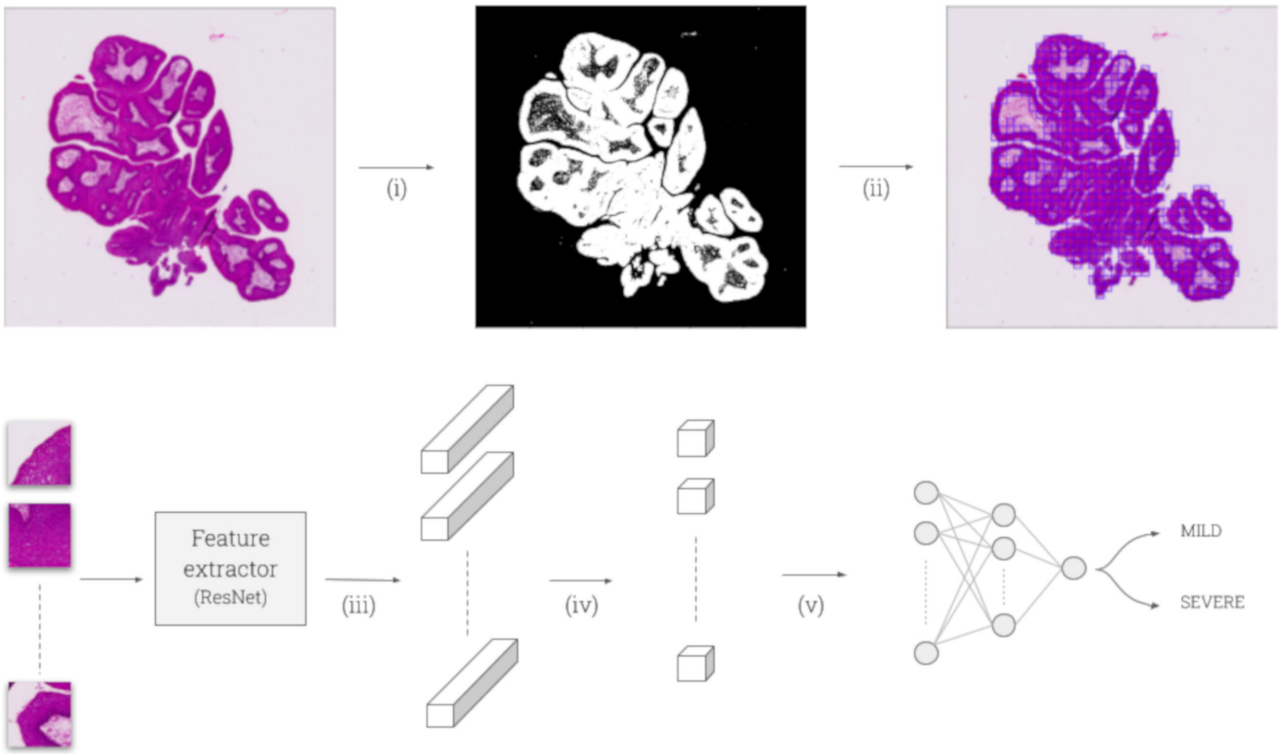

FIGURE 1 | (i) WSI is thresholded with Otsu to separate foreground (tissue) from background. (ii) Selected foreground is cut out into tiles of size 224 × 224 pixels. (iii) Tiles are fed into a convolutional feature extractor—here ResNet-50, pretrained on ImageNet—leading to a 2,048 feature vector for each tile, after the 5th ResNet block and mean pooling. (iv) A 1-by-1 convolution is applied to get a single value per feature vector. (v) Tiles scores are sorted: *R* maximal scores and *R* minimal scores are selected to go through a final two-layer perceptron (200 and 100 hidden units) to make a final softmax prediction of the class: either "mild" or "severe." We set $R = 5$ as suggested by CHOWDER.

Statistical Analysis

Statistical analyses were carried out using R software. For p53 and p63 immunohistochemistry, we calculated for each patient a percentage of nuclei stained by level of intensity from raw data, by dividing the number of nuclei in each category by the total number of nuclei, on all regions of interest. For the deep-learning approach, the nuclei in the stroma were not taken into account. Qualitative variables were analyzed with a Chi2 or Fisher test depending on sample size. Univariate analyses with quantitative data were performed using a non-parametric Mann–Whitney test. Finally, all tests were bilateral and a $p < 0.05$ was considered significant.

Outcome

Patients were classified into two groups: severe and mild. Severity was defined by at least one of the following criteria: a number of SE per year ≥5, death related to disease, pulmonary location of JoRRP proven at least by a chest CT scan, carcinomatous transformation of an JoRRP localization. One of the aims of this work was to identify histological criteria associated with a severe JoRRP. As there are, to our knowledge, no existing morphological JoRRP severity criteria, we tried a hypothesis-agnostic approach by using a deep-learning algorithm to classify patients in each of the two groups according to the HE alone. If validated, such algorithm could be used to extract tissue areas on which the algorithm particularly relied to make its decision, thus potentially highlighting discriminating histological criteria. Given the small size of our dataset, hence limiting the potential of such algorithm, we also planned to stain slides with anti-p53 and anti-p63 antibodies. We compared the percentage of nuclei stained by these two antibodies between the two groups.

RESULTS

Population

Forty-eight children were included, 22 boys and 26 girls. The average age at diagnosis was 3.8 years with a median age of 2 (age range: 0.5–13 years). Twenty-seven percent of patients had HPV 11 infection, 65% had HPV 6 infection, and 6% had co-infection with HPV 6 and 11. It was not possible to perform HPV typing in one patient due to sample depletion. All patients had glottic involvement. 73% of patients had supraglottic tumors, 68.7% had subglottic ones and 25% and 8% had respectively tracheal and pulmonary involvement. Patients had a median rate of 4.8 SE per year. Regarding adjuvant treatment, 73% of patients received at least one injection of Cidofovir. Patients received an average of 7.1 injections of Cidofovir with a median of 3.5 injections. Six patients (12.5%) received Cidofovir during an SE prior to

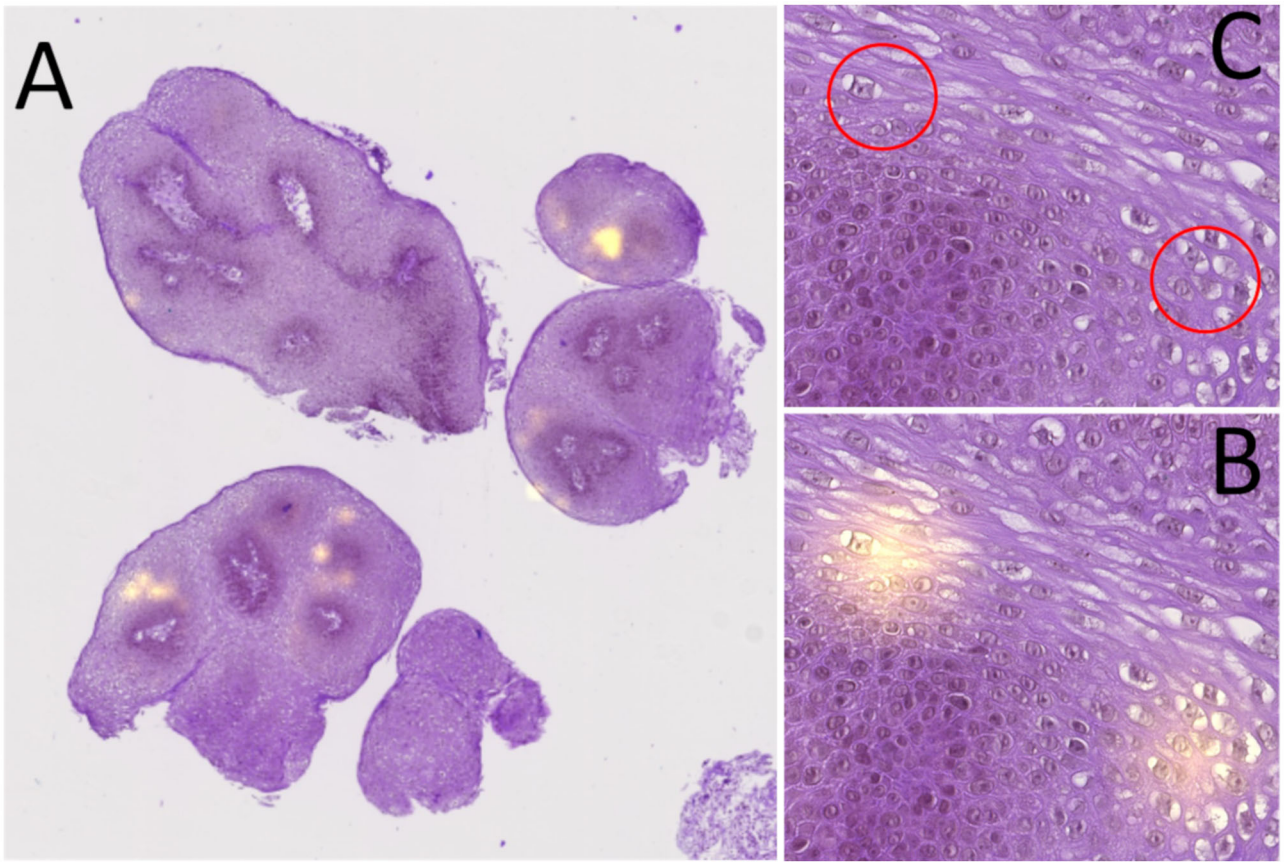

FIGURE 2 | Heatmap analysis process: **(A)** global view of an HE slide with its heatmap; the yellow zones represent the areas that have impacted the classifier (hotspots). **(B)** View of two heatmap hotspots. **(C)** HE area corresponding to the two hotspots, allowing to see the presence of viral cytopathogenic effect (red circle corresponding to the hotspots).

the study specimen. The delay between the first and last SE was on average 3.6 years and the median was 2 years. Moreover, 71% of patients had a lesion at the last check-up. Additionally, a young patient in our cohort died at the age of 18 from the malignant transformation of a pulmonary localization of her JoRRP into bronchopulmonary squamous cell carcinoma. Her JoRRP progressed for 17 years: 132 SE were performed, with a mean interval between each endoscopy of 47 days. She also received 67 injections of Cidofovir. According to our severity criteria, 24 patients had a severe disease and 24 had a mild disease. Characteristics of the two populations are summarized in **Table 1**. The two populations were comparable: there were no statistically significant differences in the gender of the patients, the type of HPV, the age at diagnosis, the total number of SE, the number of SE in the first year, the total number of injections of Cidofovir, or post-surgical morbidity. Patients with severe disease had a significantly shorter mean interval between each SE compared with patients with mild disease (median 51 days vs. 213 days, $p < 0.0001$). Patients with severe JoRRP had a shorter delay between first and last SE (1.0 year vs. 2.7 years, $p = 0.001$); and had significantly more tracheostomies than patients with mild JoRRP ($p = 0.048$).

Prediction of Disease Severity Using Solely HE With a Deep Neural Network

We tested different approaches (as described in our "Methods" section) to face data scarcity, which is an obstacle for such multiple instance learning tasks. Given the small evaluation set size for a given training (corresponding to 11–12 slides), running a cross-validation was compulsory to properly validate a method. Here, we systematically carried out a four-fold cross-validation. If one configuration sometimes gave good results on specific sets (we reached 0.83 AUC on a set with a single slice per patient, all tiles being used at each training iteration), we never reached significant results on cross-validation (mean AUC of 0.57 with a non-statistically significant *p*-value). Beyond evaluation metrics, we strove to understand whether the algorithm took into account histological criteria visible to a pathologist. To find potential histological criteria that would allow mild/severe stratification solely with HE slides, we randomly compared five heatmaps of patients with severe JoRRP with five heatmaps of patients with mild disease that had been accurately classified by the model. For each heatmap, we noted the different locations of the hotspots (in the three thirds of the epithelium and in the conjunctivo-vascular axis).

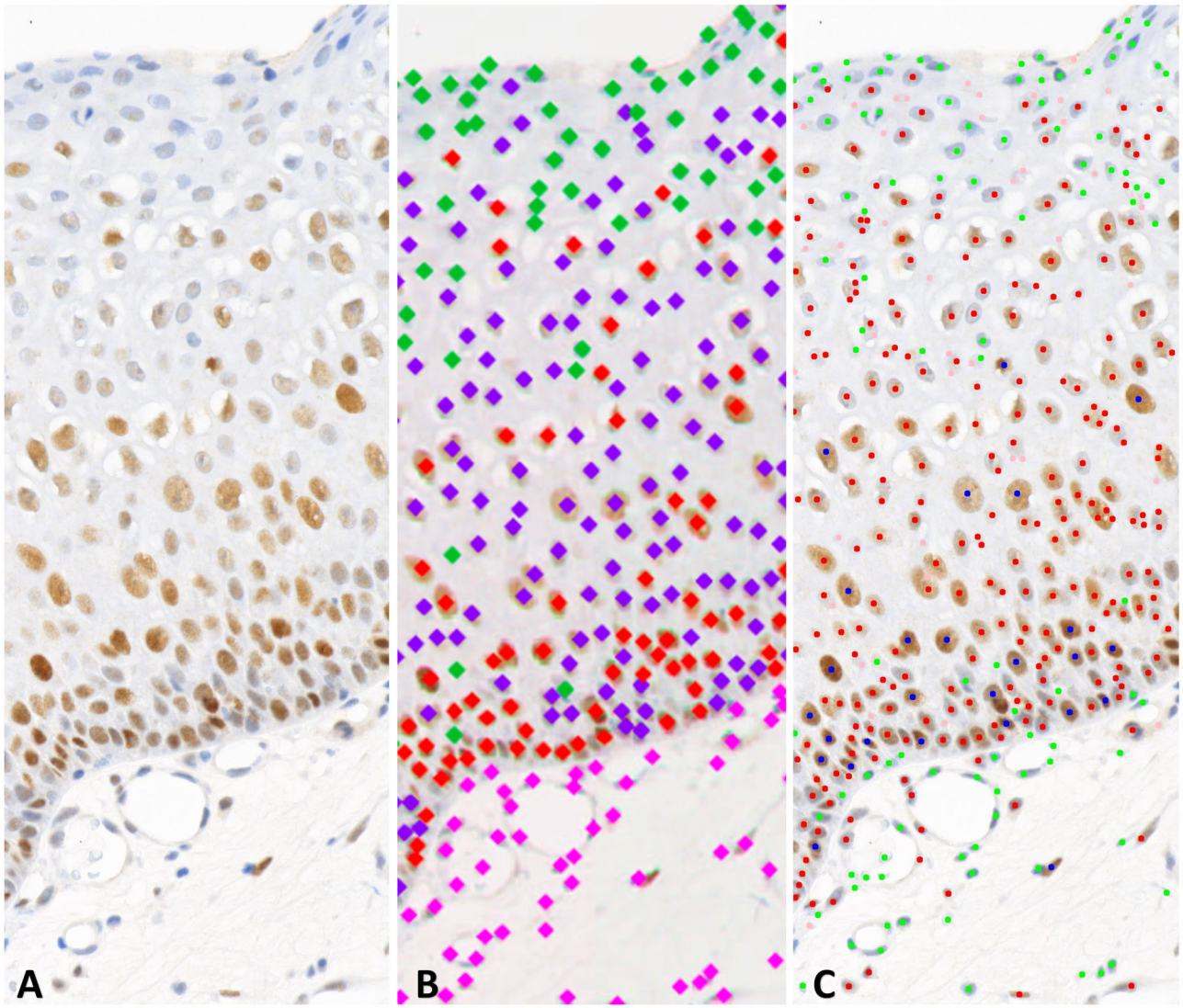

FIGURE 3 | Examples of the machine-learning and deep-learning phenotyping. **(A)** area of an ROI from a p53 slide. **(B)** Deep-learning approach, labeling of the colors: pink, stroma; green, unstained; purple, low intensity staining; red, medium intensity staining. **(C)** Machine-learning approach, labeling of the colors: pink, irrelevant; green, unstained; red, low intensity staining; blue, medium intensity staining.

We also collected the presence of visible histological signs in the hotspot area (presence of lymphocytes, neutrophil polynuclear cells, viral cytopathogenic effect, prominent nucleoli, nuclear hyperchromatism, and mitosis). The results are summarized in **Supplementary Table 1**. Briefly, according to the heatmaps analyzed, there was an average of 11 hotspots per patient. There is a slightly different distribution of hotspots depending on the severity of the disease, with more hotspots in the basal third and in the stroma for patients with severe disease and more hotspots in the middle third for patients with mild disease. We found 19 out of 27 hotspots with histological criteria. Some features are only found for patients with a mild JoRRP, such as a prominent nucleoli and mitosis. Neutrophils are only found for patients with a severe JoRRP.

Image Analysis of p53 and p63 Immunohistochemistry

Given the small size of our dataset limiting the outcome of a WSI classification task, we also planned to stain slides with anti-p53 and anti-p63 antibodies. An example of the nuclei phenotyping results with each approach is shown in **Figure 3**.

Machine-Learning Approach

Concerning the machine-learning approach, results are summarized in **Table 2**. Patients with severe disease had statistically significant higher numbers of stained nuclei with anti-p53 antibody for strong intensity compared with patients with mild disease (0.14 vs. 0.08, $p = 0.015$). There was no significant difference for the other intensity groups. With the p63

TABLE 1 | Clinical characteristics of patients with mild and severe JoRRP.

		Mild disease: 24 (%)	Severe disease: 24 (%)	*p*
Gender	Boys	11 (46%)	11 (46%)	1
	Girls	13 (54%)	13 (54%)	1
HPV type*	HPV6 and 11	3 (12%)	0 (0%)	0.234
	HPV11	4 (17%)	9 (38%)	0.194
	HPV6	17 (71%)	14 (58%)	0.546
Tracheostomy		1 (4%)	7 (29%)	**0.048**
Sub-glottic involvement		14 (58%)	19 (79%)	0.119
Tracheal involvement		5 (21%)	7 (29%)	0.505
Postoperative morbidity		5 (21%)	5 (21%)	1
Lesion at last check-up		15 (63%)	19 (79%)	0.204
Pulmonary involvement		0	4 (17%)	
Death		0	1 (4%)	
Malignant transformation		0	1 (4%)	
Median age at diagnosis (year)		3	2	0.180
Median time between 1st and last SE (years)		2.7	1	**0.001**
Median total number of SE		8	9.5	0.193
Median number of SE first year after diagnosis		3.5	5	0.054
Median average interval between each SE (days)		213	51	**<0.0001**
Median total number of Cidofovir injections		3	5	0.311

*One patient could not have HPV typing due to sample depletion.
Bold value indicate statistically significant (<0.05).

TABLE 2 | Comparison of the percentage of nuclei stained by antibody against p53 and p63 between patients with mild and severe JoRRP with the machine-learning approach.

	Staining intensity	Mild disease (24)	Severe disease (24)	*p*
% of nuclei stained by p53 antibody (median)	+	61.08	62.68	0.564
	++	3.2	4.67	0.073
	+++	0.08	0.14	**0.015**
	All of the 3	65.38	69.46	0.266
	++ and +++	3.36	4.91	0.063
% of nuclei stained by p63 antibody (median)	+	55.7	49.56	0.108
	++	23.98	36.1	**0.041**
	+++	0.14	0.74	0.122
	All of the 3	82.02	86.07	0.055
	++ and +++	24.14	36.9	**0.048**

Bold value indicate statistically significant (<0.05).

TABLE 3 | Comparison of the percentage of nuclei stained by antibody against p53 and p63 between patients with mild and severe JoRRP with the deep-learning approach.

	Staining intensity	Mild disease (24)	Severe disease (24)	p
% of nuclei stained by p53 antibody (median)	+	49.51	47.97	0.951
	++	14.42	16.51	0.483
	+++	0.08	0.18	0.085
	All of the 3	64.93	71.82	0.303
	++ and +++	14.59	16.94	0.483
% of nuclei stained by p63 antibody (median)	+	25.4	19.8	0.303
	++	53.85	57.65	0.201
	+++	0.84	3.94	0.066
	All of the 3	84.64	87.55	**0.023**
	++ and +++	58.32	67.45	**0.045**

Bold value indicate statistically significant (<0.05).

antibody, patients with severe disease had statistically significant higher numbers of stained nuclei compared with patients with mild disease for medium intensity (36.1 vs. 23.98%, $p = 0.041$) and medium and strong intensity together (36.9 vs. 24.14%, $p = 0.048$).

Deep-Learning Approach

Concerning the deep-learning approach, results are summarized in **Table 3**. With the p63 antibody, patients with severe disease had statistically significant higher numbers of stained nuclei compared to patients with mild disease for the three intensities together (87.55 vs. 84.64%, $p = 0.023$) and medium and strong intensity together (67.45 vs. 58.32%, $p = 0.045$). There was no significant difference between the two populations regarding the number of nuclei stained by the p53 antibody.

DISCUSSION

Population

Juvenile recurrent respiratory papillomatosis is a rare disease and studies often involve small cohorts, which severely limits their scope. In order to improve the management of these patients, it is necessary to carry out studies to find new severity risk factors. To our knowledge, our cohort of JoRRP is the largest ever studied in Europe. National databases in the U.S. and Canada have been established, covering 603 and 243 children with JoRRP (5, 53); our population has characteristics comparable to these two cohorts. We found a median rate of SE per year of 4.8 comparable to the U.S. cohort's, which was of 4.3, higher than the Canadian one of 1.5. Our median age at diagnosis was slightly lower, 2 years old vs. 3 years old in the U.S. cohort and 4 years old in the Canadian one. These data are also similar with a more recent publication on an international cohort of juvenile and adult RRP (35). Interestingly, the percentage of patients treated with Cidofovir was much higher in our cohort than in the Canadian cohort (respectively 73 vs. 4.7%). The differences in terms of Cidofovir treatment could be explained by variability in local practices. Regarding the distribution of HPV types, our data are comparable to the literature. We found a low proportion

of co-infection with HPV6 and 11 (6%) and a predominance of HPV6 (65%), as described elsewhere (54, 55). One of the main difficulties in our study was to define disease severity. Currently, no consensual definition exists in the literature. Some authors use composite scores incorporating criteria for disease localization, such as the Derkay–Wiatrak score, and intervention-related criteria, such as the number of SE per year (5, 35, 56). Others use only intervention-related criteria. A total number of SE greater than or equal to 10 or a number of SE/year > 3 or 4 is frequently found as a criterion of severity (34). We were unable to use Derkay–Wiatrak score as one of the two centers involved was not used to performing it systematically. We chose the criteria mainly representing the symptomatology of these two groups of patients. Our cut-off value for the number of SE per year seems relevant for our cohort, since patients classified as severe presented more severe items of disease activity than patients classified as mild. Thus, the median mean interval between each endoscopy was 51 days for severe JoRRP and 213 days for mild JoRRP ($p < 0.001$). Additionally, patients with severe disease had statistically significantly more tracheostomies than those with mild disease ($p = 0.048$). It should be noted that as 71% of patients had a lesion at the last check-up it may be possible that the number of SE/year would have changed until remission.

Prediction of Disease Severity Using Solely HE With a Deep Neural Network

The principal aim of this study was to identify histological criteria associated with disease severity. We first aimed to determine whether we could predict JoRRP severity solely relying on HE slides. The difficulty was twofold: the cohort was small for WSI classification tasks with respect to machine learning community standards, and this was a discovery task, meaning that there are no known predictive morphological discriminative patterns that distinguish severe from mild JoRRP for pathologists. Despite our efforts to address data scarcity, we did not find a configuration capable of performing well on all cross-validation sets. We concluded that our dataset did not make it possible to extract from HE slides the information relevant to predict JoRRP severity with our multiple instance learning approach. It shows that such architecture was not able to extract extra information as for a pathologist, at least on such small dataset. We acknowledge that it does not imply that no such morphological pattern in HE could be useful to predict JoRRP severity; yet, we think that highlighting what did not work is still an informative milestone for the community to design future projects. A larger transnational cohort would facilitate research and statistically strengthen the approach, given the difficulty of such discovery tasks. The classification model for JoRRP was not sufficiently effective to allow complete heatmaps analysis. However, it is very easy for a pathologist to analyze the areas used by the algorithm to classify a case. This may prove to be time-saving for the analysis of a cohort and helpful in identifying histological items potentially associated with the severity of the disease. Indeed, by simply exploring 5 cases, we found a slightly different distribution of the hotspots on the slides between the two groups and some differences in histological criteria found below the hotspots between mild and severe JoRRP. Even though it was not possible to draw conclusions from these data, this kind of analysis with secondary morphological analysis of area of interest seems promising for pathologists.

Image Analysis of p53 and p63 Immunohistochemistry

Considering the lack of significant results on HE, we also explored p63 and p53 immunostainings. Based on Rabah et al. (38) results, we set out to explore the expression of p53 in these tumors, and by extension, of p63. We decided to compare percentages of stained nuclei rather than density of labeled cells because machine-learning analysis tends to segment large nuclei in half, artificially increasing the number of cells in the ROI. The contribution of automated image analysis in this study considerably helped us save time and strengthened the robustness of such quantitative task. For p53, we did not find any difference in number of nuclei stained between the two groups of patients, except for the machine-learning approach concerning strong intensity. However, there is little difference between the percentage of stained nuclei of the two groups (0.08% for mild JoRRP vs. 0.14% for severe JoRRP) and it is questionable whether this discrepancy with the deep-learning approach is related to the fact that some stromal cells were taken into account in the analysis with machine learning (as exposed in **Figure 3**). The inability to detect these stromal areas in the machine-learning analysis and to exclude them may induce a bias in the counting of stained nuclei. This is why we opted for two distinct approaches for image analysis, deep learning allowing a finer analysis by taking into account the tumor cells exclusively, not the stromal cells. Indeed, our analysis with Inform® software did not allow distinction between these two types of cells. Moreover, these results are consistent with other studies that have looked at the expression of p53 in RRP. Stern et al. (57) found an higher percentage of p53 positive cells in patient that underwent malignant transformation than in tumors with benign course (68.3 vs. 14.2%, $p < 0.05$), however only 4 had malignant transformation over the 35 patients included and no correlation with other aggressiveness disease criteria was found. Perdana et al. (58) also reported no correlation between severity and the expression of p53. With the p63 antibody, the stromal cells are not stained but are counted as unstained cells by the algorithm. We found similar results between the two image analysis methods for anti-p63 antibody for medium and high intensities together, with a greater number of nuclei stained with these intensities in patients with severe disease. For each approach, we found around 10% differences in labeled cells between severe JoRRP and mild JoRRP (37 vs. 24% for machine learning and 67 vs. 58% for deep learning). These gross percentage differences between machine-learning and deep-learning approaches could be explained by the detection of stromal cells, which were detected as unstained nuclei with machine-learning approach, artificially biasing results. There may also have been a slight variability in the pathologist's annotation of the different classes for each approach, since his eyes were the only judge of the intensity of the staining. Nevertheless, the same pathologist

made the annotations for both approaches, limiting variability. On the other hand, the deep-learning approach seemed more reliable since the model was trained to differentiate stromal from epithelial cells based on morphological context regardless of staining intensity. Obtention of similar results with the two image analysis methods strengthened the reliability of these results. Additionally, the positive predictive values and sensitivity of both models are very good. The fact that patients with severe disease had a higher percentage of cells labeled with p63 for medium and high intensities than patients with mild disease is a first step toward using p63 as a predictor of disease severity. A possible confounding factor in our study is the blend of patients treated or not treated with Cidofovir. Indeed, it is described in the literature that in HPV-induced cancer cell lines, Cidofovir causes an accumulation of p53 (59). However, these data concern high-grade HPV, and the low-risk HPV proteins involved here in JoRRP do not share the same properties. The E6 protein has a lower affinity for p53, which does not induce p53 degradation but retains an inhibitory activity to the p53 transcriptional activity necessary for viral genome replication (60). It is thus difficult to extrapolate the role of Cidofovir on the expression of p53 in JoRRP. We also analyzed our cohort in subgroups to take into account Cidofovir treatment, results are summarized in **Supplementary Tables 2**, **3**. However, these results are difficult to interpret given that the groups are very disproportionate in size, as 73% of patients received at least one injection of Cidofovir. Even if Cidofovir has an impact on p53 expression in JoRRP, in our cohort the patient groups with a mild or a severe disease are well balanced with 29 and 25% of untreated patients respectively (7 patients out of 24 vs. 6 patients out of 24, $p = 0.745$). To confirm our results, a national prospective cohort with a larger number of patients will have to be set up with samples before and after injection of Cidofovir to study the impact of the latter on the expression of our markers.

CONCLUSION

In conclusion, we highlighted that patients with a severe JoRRP presented a higher percentage of cells stained by the anti-p63 antibody for medium and strong intensities compared to patients with mild JoRRP. This was not found with the anti-p53 antibody. Use of a biomarker to predict an aggressive disease could allow to implement adjuvant treatment at the early stage of the disease. It could also be an opportunity to better inform patients and their parents of the potential course of the disease. We also presented an innovative approach in digital pathology, which consists in analyzing an area taken into account by a deep-learning algorithm for its predictions in an attempt to discover new histological criteria of severity in this disease. These analyses were possible thanks to close collaboration between pathologists and data scientists, and this should inspire us in the future development of our profession as pathologists. These data are a first step toward a better prediction of severe cases and better management tailored to the severity of JoRRP.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP2019-02'-019a/2019-00352-55/19.02.05.67237. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization: LG and CB; methodology: CB, CL, LG, SB, and PK; validation: CB, CL, LG, MM, and PK; formal analysis: CL, TV, and PK; investigation: CL, PK, and MM; resources: LG, DB, NT, NL, and FP; data curation: CL LT, and MC; writing — original draft preparation: CL and PK; writing — review and editing: LG, NT, CB, SO-G, ET, SB, TM, PK, and CL; visualization: CL; supervision: CB, LG, and SB; project administration: CB and CL; funding acquisition: CB, CL, LG, NL. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.596499/full#supplementary-material

REFERENCES

1. Kashima H, Mounts P, Leventhal B, Hruban RH. Sites of predilection in recurrent respiratory papillomatosis. *Ann Otol Rhinol Laryngol.* (1993) 102(8 Pt 1):580–3. doi: 10.1177/000348949310200802
2. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ, editors. *WHO Classification of Head and Neck Tumours, 4th edn*. Lyon: International Agency for Research on Cancer; World Health Organization classification of tumours (2017) 347 p.
3. San Giorgi MR, van den Heuvel ER, Tjon Pian Gi RE, Brunings JW, Chirila M, Friedrich G, et al. Age of onset of recurrent respiratory papillomatosis: a distribution analysis. *Clin Otolaryngol.* (2016) 41:448–53. doi: 10.1111/coa.12565
4. Lindeberg H, Elbrønd O. Laryngeal papillomas: the epidemiology in a Danish subpopulation 1965-1984. *Clin Otolaryngol Allied Sci.* (1990) 15:125–31. doi: 10.1111/j.1365-2273.1990.tb00444.x
5. Campisi P, Hawkes M, Simpson K, Canadian Juvenile Onset Recurrent Respiratory Papillomatosis Working Group. The epidemiology of juvenile onset recurrent respiratory papillomatosis derived from a population level national database. *Laryngoscope*. (2010). 120:1233–45. doi: 10.1002/lary.20901
6. Armstrong LR, Preston EJ, Reichert M, Phillips DL, Nisenbaum R, Todd NW, et al. Incidence and prevalence of recurrent respiratory papillomatosis among children in Atlanta and Seattle. *Clin Infect Dis.* (2000) 31:107–9. doi: 10.1086/313914
7. Marsico M, Mehta V, Chastek B, Liaw K-L, Derkay C. Estimating the incidence and prevalence of juvenile-onset recurrent respiratory papillomatosis in publicly and privately insured claims databases in the United States. *Sex Transm Dis.* (2014) 41:300–5. doi: 10.1097/OLQ.0000000000000115
8. Donne AJ, Hampson L, Homer JJ, Hampson IN. The role of HPV type in recurrent respiratory papillomatosis. *Int J Pediatr Otorhinolaryngol.* (2010) 74:7–14. doi: 10.1016/j.ijporl.2009.09.004
9. Novakovic D, Cheng ATL, Zurynski Y, Booy R, Walker PJ, Berkowitz R, et al. A prospective study of the incidence of juvenile-onset recurrent respiratory papillomatosis after implementation of a national HPV vaccination program. *J Infect Dis.* (2018) 217:208–12. doi: 10.1093/infdis/jix498
10. Castellsagué X, Drudis T, Cañadas MP, Goncé A, Ros R, Pérez JM, et al. Human Papillomavirus (HPV) infection in pregnant women and mother-to-child transmission of genital HPV genotypes: a prospective study in Spain. *BMC Infect Dis.* (2009) 9:74. doi: 10.1186/1471-2334-9-74
11. Smith EM, Parker MA, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Evidence for vertical transmission of HPV from mothers to infants. *Infect Dis Obstet Gynecol.* (2010) 2010:326369. doi: 10.1155/2010/326369
12. Puranen M, Yliskoski M, Saarikoski S, Syrjänen K, Syrjänen S. Vertical transmission of human papillomavirus from infected mothers to their newborn babies and persistence of the virus in childhood. *Am J Obstet Gynecol.* (1996) 174:694–9. doi: 10.1016/S0002-9378(96)70452-0
13. Zouridis A, Kalampokas T, Panoulis K, Salakos N, Deligeoroglou E. Intrauterine HPV transmission: a systematic review of the literature. *Arch Gynecol Obstet.* (2018) 298:35–44. doi: 10.1007/s00404-018-4787-4
14. Silverberg MJ, Thorsen P, Lindeberg H, Grant LA, Shah KV. Condyloma in pregnancy is strongly predictive of juvenile-onset recurrent respiratory papillomatosis. *Obstet Gynecol.* (2003) 101:645–52. doi: 10.1097/00006250-200304000-00007
15. Quick CA, Krzyzek RA, Watts SL, Faras AJ. Relationship between condylomata and laryngeal papillomata: clinical and molecular virological evidence. *Ann Otol Rhinol Laryngol.* (1980) 89:467–71. doi: 10.1177/000348948008900521
16. Bonagura VR, Vambutas A, DeVoti JA, Rosenthal DW, Steinberg BM, Abramson AL, et al. HLA alleles, IFN-gamma responses to HPV-11 E6, and disease severity in patients with recurrent respiratory papillomatosis. *Hum Immunol.* (2004) 65:773–82. doi: 10.1016/j.humimm.2004.05.014
17. Gelder CM, Williams OM, Hart KW, Wall S, Williams G, Ingrams D, et al. HLA Class II polymorphisms and susceptibility to recurrent respiratory papillomatosis. *J Virol.* (2003) 77:1927–39. doi: 10.1128/JVI.77.3.1927-1939.2003
18. Bonagura VR, Du Z, Ashouri E, Luo L, Hatam LJ, DeVoti JA, et al. Activating killer cell immunoglobulin-like receptors 3DS1 and 2DS1 protect against developing the severe form of recurrent respiratory papillomatosis. *Hum Immunol.* (2010) 71:212–9. doi: 10.1016/j.humimm.2009.10.009
19. Bonagura VR, Hatam L, DeVoti J, Zeng F, Steinberg BM. Recurrent respiratory papillomatosis: altered CD8(+) T-cell subsets and T(H)1/T(H)2 cytokine imbalance. *Clin Immunol.* (1999) 93:302–11. doi: 10.1006/clim.1999.4784
20. Go C, Schwartz MR, Donovan DT. Molecular transformation of recurrent respiratory papillomatosis: viral typing and P53 overexpression. *Ann Otol Rhinol Laryngol.* (2003) 112:298–302. doi: 10.1177/000348940311200402
21. Gélinas J-F, Manoukian J, Côté A. Lung involvement in juvenile onset recurrent respiratory papillomatosis: a systematic review of the literature. *Int J Pediatr Otorhinolaryngol.* (2008) 72:433–52. doi: 10.1016/j.ijporl.2007.12.003
22. Montaño-Velázquez BB, Nolasco-Renero J, Parada-Bañuelos JE, Garcia-Vázquez F, Flores-Medina S, García-Romero CS, et al. Quality of life of young patients with recurrent respiratory papillomatosis. *J Laryngol Otol.* (2017) 131:425–8. doi: 10.1017/S0022215117000354
23. Healy GB, Gelber RD, Trowbridge AL, Grundfast KM, Ruben RJ, Price KN. Treatment of recurrent respiratory papillomatosis with human leukocyte interferon. Results of a multicenter randomized clinical trial. *N Engl J Med.* (1988) 319:401–7. doi: 10.1056/NEJM198808183190704
24. Limsukon A, Susanto I, Soo Hoo GW, Dubinett SM, Batra RK. Regression of recurrent respiratory papillomatosis with celecoxib and erlotinib combination therapy. *Chest.* (2009) 136:924–6. doi: 10.1378/chest.08-2639
25. Rogers DJ, Ojha S, Maurer R, Hartnick CJ. Use of adjuvant intralesional bevacizumab for aggressive respiratory papillomatosis in children. *JAMA Otolaryngol Head Neck Surg.* (2013) 139:496–501. doi: 10.1001/jamaoto.2013.1810
26. Naiman AN, Ayari S, Nicollas R, Landry G, Colombeau B, Froehlich P. Intermediate-term and long-term results after treatment by cidofovir and excision in juvenile laryngeal papillomatosis. *Ann Otol Rhinol Laryngol.* (2006) 115:667–72. doi: 10.1177/000348940611500903
27. McMurray JS, Connor N, Ford CN. Cidofovir efficacy in recurrent respiratory papillomatosis: a randomized, double-blind, placebo-controlled study. *Ann Otol Rhinol Laryngol.* (2008) 117:477–83. doi: 10.1177/000348940811700702
28. Creelan BC, Ahmad MU, Kaszuba FJ, Khalil FK, Welsh AW, Ozdemirli M, et al. Clinical activity of nivolumab for human papilloma virus-related juvenile-onset recurrent respiratory papillomatosis. *Oncologist*. (2019) 24:829–35. doi: 10.1634/theoncologist.2018-0505
29. Allen CT, Lee S, Norberg SM, Kovalovsky D, Ye H, Clavijo PE, et al. Safety and clinical activity of PD-L1 blockade in patients with aggressive recurrent respiratory papillomatosis. *J Immunother Cancer.* (2019) 7:119. doi: 10.1186/s40425-019-0603-3
30. Chirilă M, Bolboacă SD. Clinical efficiency of quadrivalent HPV (types 6/11/16/18) vaccine in patients with recurrent respiratory papillomatosis. *Eur Arch Otorhinolaryngol.* (2014) 271:1135–42. doi: 10.1007/s00405-013-2755-y
31. Lawlor C, Balakrishnan K, Bottero S, Boudewyns A, Campisi P, Carter J, et al. International Pediatric Otolaryngology Group (IPOG): Juvenile-onset recurrent respiratory papillomatosis consensus recommendations. *Int J Pediatr Otorhinolaryngol.* (2020) 128:109697. doi: 10.1016/j.ijporl.2019.109697
32. Rosenberg T, Philipsen BB, Mehlum CS, Dyrvig A-K, Wehberg S, Chirilă M, et al. Therapeutic use of the human papillomavirus vaccine on recurrent respiratory papillomatosis: a systematic review and meta-analysis. *J Infect Dis.* (2019) 219:1016–25. doi: 10.1093/infdis/jiy616
33. Matsuzaki H, Makiyama K, Hirai R, Suzuki H, Asai R, Oshima T. Multi-year effect of human papillomavirus vaccination on recurrent respiratory papillomatosis. *Laryngoscope.* (2020) 130:442–7. doi: 10.1002/lary.27993
34. Niyibizi J, Rodier C, Wassef M, Trottier H. Risk factors for the development and severity of juvenile-onset recurrent respiratory papillomatosis: a systematic review. *Int J Pediatr Otorhinolaryngol.* (2014) 78:186–97. doi: 10.1016/j.ijporl.2013.11.036
35. Buchinsky FJ, Valentino WL, Ruszkay N, Powell E, Derkay CS, Seedat RY, et al. Age at diagnosis, but not HPV type, is strongly associated with clinical course in recurrent respiratory papillomatosis. *PLoS One.* (2019) 14:e0216697. doi: 10.1371/journal.pone.0216697
36. Achkar VNRE, Duarte A, Carlos R, León JE, Ribeiro-Silva A, Pignatari SSN, et al. Histopathological features of juvenile-onset laryngeal papillomatosis related to severity. *Head Neck.* (2019) 41:1412–7. doi: 10.1002/hed.25602

37. Ahn J, Bishop JA, Roden RBS, Allen CT, Best SRA. The PD-1 and PD-L1 pathway in recurrent respiratory papillomatosis. *Laryngoscope.* (2018) 128:E27–32. doi: 10.1002/lary.26847
38. Rabah R, Sakr W, Thomas R, Lancaster WD, Gregoire L. Human papillomavirus type, proliferative activity, and p53. *Arch Pathol Lab Med.* (2000) 124:721–4. doi: 10.5858/2000-124-0721-HPTPAA
39. Pflaum J, Schlosser S, Müller M. p53 family and cellular stress responses in cancer. *Front Oncol.* (2014) 4:285. doi: 10.3389/fonc.2014.00285
40. Fu H-L, Shao L, Wang Q, Jia T, Li M, Yang D-P. A systematic review of p53 as a biomarker of survival in patients with osteosarcoma. *Tumour Biol.* (2013) 34:3817–21. doi: 10.1007/s13277-013-0966-x
41. Yao W, Qin X, Qi B, Lu J, Guo L, Liu F, et al. Association of p53 expression with prognosis in patients with esophageal squamous cell carcinoma. *Int J Clin Exp Pathol.* (2014) 7:7158–63. doi: 10.31838/srp.2020.9.28
42. McKenney JK, Desai S, Cohen C, Amin MB. Discriminatory immunohistochemical staining of urothelial carcinoma in situ and non-neoplastic urothelium: an analysis of cytokeratin 20, p53, and CD44 antigens. *Am J Surg Pathol.* (2001) 25:1074–8. doi: 10.1097/00000478-200108000-00013
43. Crum CP, McKeon FD. p63 in epithelial survival, germ cell surveillance, and neoplasia. *Annu Rev Pathol.* (2010) 5:349–71. doi: 10.1146/annurev-pathol-121808-102117
44. Ehteshami Bejnordi B, Mullooly M, Pfeiffer RM, Fan S, Vacek PM, Weaver DL, et al. Using deep convolutional neural networks to identify and classify tumor-associated stroma in diagnostic breast biopsies. *Mod Pathol.* (2018) 31:1502–12. doi: 10.1038/s41379-018-0073-z
45. Coudray N, Ocampo PS, Sakellaropoulos T, Narula N, Snuderl M, Fenyö D, et al. Classification and mutation prediction from non-small cell lung cancer histopathology images using deep learning. *Nat Med.* (2018) 24:1559–67. doi: 10.1038/s41591-018-0177-5
46. Courtiol P, Tramel EW, Sanselme M, Wainrib G. Classification and disease localization in histopathology using only global labels: a weakly-supervised approach. *arXiv:180202212.* (2020).
47. Durand T, Thome N, Cord M. WELDON: weakly supervised learning of deep convolutional neural networks. In: *2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR).* Las Vegas, NV (2016). p. 4743–52.
48. Otsu N. A threshold selection method from gray-level histograms. *IEEE Trans Syst Man Cybernet.* (1979) 9:62–6. doi: 10.1109/TSMC.1979.4310076
49. Singh KK, Lee YJ. Hide-and-seek: forcing a network to be meticulous for weakly-supervised object and action localization. *2017 IEEE International Conference on Computer Vision (ICCV).* (2017) 3544–3553. *arXiv:170404232.*
50. Viray H, Coulter M, Li K, Lane K, Madan A, Mitchell K, et al. Automated objective determination of percentage of malignant nuclei for mutation testing. *Appl Immunohistochem Mol Morphol.* (2014) 22:363–71. doi: 10.1097/PAI.0b013e318299a1f6
51. Ren S, He K, Girshick R, Sun J. Faster R-CNN: towards real-time object detection with region proposal networks. IEEE Transactions on Pattern Analysis and Machine Intelligence. (2015) 39, 1137–1149. *arXiv:150601497.*
52. Kingma D, Ba J. Adam: a method for stochastic optimization. In: *International Conference on Learning Representations.* San diego, CA (2014).
53. Reeves WC, Ruparelia SS, Swanson KI, Derkay CS, Marcus A, Unger ER. National registry for juvenile-onset recurrent respiratory papillomatosis. *Arch Otolaryngol Head Neck Surg.* (2003) 129:976–82. doi: 10.1001/archotol.129.9.976
54. Wiatrak BJ, Wiatrak DW, Broker TR, Lewis L. Recurrent respiratory papillomatosis: a longitudinal study comparing severity associated with human papilloma viral types 6 and 11 and other risk factors in a large pediatric population. *Laryngoscope.* (2004) 114(11 Pt 2 Suppl 104):1–23. doi: 10.1097/01.mlg.000148224.83491.0f
55. Draganov P, Todorov S, Todorov I, Karchev T, Kalvatchev Z. Identification of HPV DNA in patients with juvenile-onset recurrent respiratory papillomatosis using SYBR Green real-time PCR. *Int J Pediatr Otorhinolaryngol.* (2006) 70:469–73. doi: 10.1016/j.ijporl.2005.07.018
56. Derkay CS, Malis DJ, Zalzal G, Wiatrak BJ, Kashima HK, Coltrera MD. A staging system for assessing severity of disease and response to therapy in recurrent respiratory papillomatosis. *Laryngoscope.* (1998) 108:935–7. doi: 10.1097/00005537-199806000-00026
57. Stern Y, Heffelfinger SC, Walner DL, Cotton RT. Expression of Ki-67, tumor suppressor proteins, growth factor, and growth factor receptor in juvenile respiratory papillomatosis: Ki-67 and p53 as predictors of aggressive disease. *Otolaryngol Head Neck Surg.* (2000) 122:378–86. doi: 10.1016/S0194-5998(00)70053-2
58. Perdana RF, Herawati S, Yusuf M, Kristyono I, Nugroho PS. Correlation between P53 and KI67 with aggressiveness factor in recurrent respiratory papillomatosis. *Syst Rev Pharm.* (2020) 11:163–8.
59. Abdulkarim B, Sabri S, Deutsch E, Chagraoui H, Maggiorella L, Thierry J, et al. Antiviral agent Cidofovir restores p53 function and enhances the radiosensitivity in HPV-associated cancers. *Oncogene.* (2002) 21:2334–46. doi: 10.1038/sj.onc.1205006
60. Klingelhutz AJ, Roman A. Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. *Virology.* (2012) 424:77–98. doi: 10.1016/j.virol.2011.12.018

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Nonsmoking and Nondrinking Oral Squamous Cell Carcinoma Patients: A Different Entity

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Objective: Our goal was to analyze the demographic and pathologic characteristics as well as prognosis in nonsmoking and nondrinking (NSND) oral squamous cell carcinoma (SCC) patients compared with typical oral SCC patients.

Patients and Methods: A total of 353 patients were retrospectively enrolled and divided into two groups: the NSND group and the current smoking/current drinking (CSCD) group. Demographic, pathologic, and molecular data were compared between the two groups. The main research endpoints were locoregional control (LRC) and disease-specific survival (DSS).

Results: In the NSND group, 16.3%, 41.9%, and 53.5% of patients were aged no more than 40 years, were female, and had an educational background of high school or above compared to 3.7%, 6.0%, and 38.2% of patients in the CSCD group, respectively. A total of 15.1% of the NSND patients had SCC of the lower gingiva and floor of the mouth, which was lower than the 35.6% of patients in the CSCD group. CSCD patients were likely to have an advanced disease stage (48.7% vs 32.5%, p=0.042) and poorly differentiated cancer (26.6% vs 16.3%, p=0.042). The NSND patients had a mean Ki-67 index of 24.5%, which was lower than the mean of 35.7% in the CSCD patients. The two groups had no HPV infection and similar p16 expression (4.7% vs 10.1%, p=0.132), but there was higher expression of p53 (38.6% vs 17.4%, p<0.001) and p63 (59.9% vs 29.1%, p<0.001) in the CSCD group. The 5-year LRC rates for NSND patients and CSCD patients were 48% and 38%, respectively, and the difference was significant (p=0.048). The 5-year DSS rates for NSND patients and CSCD patients were 56% and 39%, respectively, and the difference was significant (p=0.047). Further, a Cox model confirmed the independence of smoking and drinking status for affecting LRC and DSS.

Conclusion: NSND oral SCC patients are a different entity. HPV infection has a limited role in carcinogenesis in NSND patients, and p16 expression is associated with worse locoregional control.

Keywords: nonsmoking, nondrinking, HPV, head and neck squamous cell carcinoma, p16

INTRODUCTION

Oral squamous cell carcinoma (SCC) is the most common malignancy in cancers of the head and neck (1), and it significantly threatens people's lives and quality of life. The latest epidemiologic data in 2011 showed that in China, the age-standardized incidence and mortality rates of oral SCC were 2.22 per 100,000 and 0.9 per 100,000, respectively (2). Tobacco smoking and alcohol consumption are considered to be the main risk factors and are responsible for at least 80% of oral SCC patients (3–5). There are 50 potential carcinogens including polycyclic aromatic hydrocarbons and nitrosamines in tobacco, and they can result in mutations of some important genes such as the tumor suppressor gene p53 that disturb modulation of the immune system and cell cycle regulation (6). The carcinogenic mechanism of alcohol is complex and might be involved in the genotoxic effects of acetaldehyde, genetic polymorphisms, cytochrome P450 2E1-mediated generation of reactive oxygen species, aberrant metabolism of folate and retinoids, and increased estrogen (7).

Although there has been increased knowledge regarding giving up smoking and drinking, the incidence of oral SCC has not decreased significantly (8, 9), and even nonsmoking and nondrinking (NSND) oral SCC patients are increasingly common. A number of previous researchers have tried to determine the difference regarding etiology, pathologic characteristics, and molecular expression as well as prognosis between nonsmoking patients and typical patients (10–14), but unfortunately, there is great controversy. Some authors have depicted that there is no significant survival difference between these two groups (10–12), some have reported that nonsmoking patients have a better prognosis (13), and some have described that there is worse survival in young nonsmoking patients (14). The majority of these studies did not limit their patients to NSND patients, and this minor designation flaw may not completely eliminate their potential confounding effects (1). On the other hand, literature on the molecular expression of NSND patients remains scarce, even though the reported rates of HPV16 infection, p16 expression, and p53 expression vary greatly (15–19). Therefore, in the current study, we aimed to analyze the demographic and pathologic characteristics as well as prognosis in NSND oral SCC patients compared with typical oral SCC patients.

PATIENTS AND METHODS

Ethnic Consideration

Our Hospital institutional research committee approved our study, and all participants signed an informed consent agreement. All methods were performed in accordance with the relevant guidelines and regulations. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patient Selection

From January 2014 to December 2018, the medical records of 654 patients with surgically treated oral SCC were retrospectively reviewed. Oral SCC referred to SCC arising from the tongue; buccal, lower and upper gingiva, and the floor of the mouth. The included patients met the following criteria: the disease was primary; there was no history of other cancers; there was no habit of betel-nut chewing; the patient was classified as a NSND or a current smoker or current drinker (CSCD); and there was enough paraffin-embedded tissue available for HPV detection. Patients without sufficient demographic, pathologic, or follow-up data were excluded from the analysis. Information regarding age, sex, smoking, alcohol consumption, educational background, family cancer history, pathologic TNM stage (8^{th} AJCC system), pathologic reports, treatment, and follow-up was extracted and analyzed.

Important Variable Definition

A NSND patient was defined as a patient who had smoked no more than 100 cigarettes and had simultaneously drank wine no more than once every two weeks in their lifetime (20–22). A CSCD patient was defined as a patient who had smoked at least 20 cigarettes per day for at least 10 years or had drank wine at least once per day for at least 10 years (14, 15, 19). All pathological sections were re-reviewed by at least two pathologists in a double-blind manner. Perineural invasion (PNI) was considered to be present if tumor cells were identified within the perineural space and/or nerve bundle; lymphovascular infiltration (LVI) was positive if tumor cells were noted within the lymphovascular channels (3, 23). Similar to our previous research (23), data on the family cancer history were obtained at initial treatment. During the preparation of this article, a questionnaire was sent to the patients or their family by email, postal letter, or WeChat if the information was not recorded clearly. The family members in the current study only consisted of first-degree relatives, and the patients were categorized as having a family cancer history if any of those relatives had any cancer other than nonmelanoma skin cancer. Otherwise, the patient was recorded as not having a family cancer history (23). The pathologic depth of invasion (DOI) was measured from the level of the adjacent normal mucosa to the deepest point of tumor infiltration, regardless of the presence or absence of ulceration (24).

Immunohistochemical (IHC) Analysis

From July 2013, routine immunohistochemical analysis of Ki-67, p16, p53, and p63 was performed for every head and neck SCC patient. The level of positivity of p16 overexpression was consistent with previous studies (17, 19): 0-+, defined as less than 25% tumor staining; ++, defined as 25-50% tumor stating; +++, defined as 50-75% tumor staining; and ++++: defined as more than 75% tumor staining. Tumors with levels of +++ and ++++ were classified as having p16 positivity. Similar standards were used for p53 and p63. The Ki-67 score (0-100%) was calculated by the ratio of the number of immunostained nuclei to the total number of nuclei in tumor cells. The counting

was performed in three randomly selected fields at ×400 magnification. The cut-off value of the Ki-67 score in the current study was defined as the median value (25, 26).

HPV Assessment

From July 2013, HPV detection was selectively performed in fresh tumor tissue from oral SCC patients in our cancer center. DNA was extracted using the TIANcombi DNA Lyse&Det PCR Kit (TIANGEN Cooperation, Beijing, China) and was then subjected to real-time PCR with the INNO-LIPA HPV Genotyping Extra System® kit (Innogenetics), which can detect 7 low-risk HPV types (6, 11, 40, 43, 44, 54, 70), 3 indeterminate-risk types (69, 71, 74), and 18 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82). For paraffin-embedded tissue, at least five 10-μm thick slices were used for DNA extraction with the TIANcombi DNA Lyse&Det PCR Kit (TIANGEN Cooperation, Beijing, China) according to the instructions. The following procedures were similar to those described above.

Surgical Principle

In our cancer center, systemic ultrasound, CT, MRI and/or PET-CT examinations were routinely performed for every patient. All oral SCC operations were performed under general anesthesia. The primary tumor was completely excised with at least a 1 cm margin; if necessary, a pedicled flap or free flap was used to close the defect. Neck dissection was usually performed except for tumors with very small sizes in the upper gingiva; levels of I to III were manipulated for a cN0 neck, and levels of I to IV or V were manipulated for a cN+ neck. Adjuvant treatment was suggested if T3/4 disease, cervical nodal metastasis, PNI, LVI, or positive margins were present.

Statistical Analysis

Student's *t* test was used to compare the continuous variables between the two groups, and the Chi-square test was used to compare the categorical variables between the two groups. The main study points were locoregional control (LRC) and disease-specific survival (DSS). The survival time of LRC was calculated from the date of surgery to the date of local, regional or locoregional recurrence or to the last follow-up, and the survival time of DSS was calculated from the date of surgery to the date of cancer-related death or the last follow-up. The Kaplan-Meier method (log-rank test) was used to calculate the LRC and DSS rates. The factors that were significant in univariate analysis were then analyzed in the Cox proportional risk regression model to determine the independent prognostic factors. All reported p values were two-sided, and a value of $p<0.05$ was considered significant. All statistical analyses were performed with SPSS 20.0.

RESULTS

Demographic Characteristics

A total of 353 patients (301 males and 52 females) were enrolled for analysis. The NSND group consisted of 86 patients with a mean age of 50.6 (range: 30-68) years; 14 (16.3%) patients were aged ≤40 years, and there were 50 (58.1%) males and 36 (41.9%) females. Forty-six (53.5%) patients had an educational background of high school or above. Six (7.0%) patients had a family cancer history: esophageal cancer was noted in 4 (66.7%) families, and lung cancer was noted in the remaining two families (33.3%). The CSCD group consisted of 267 patients with a mean age of 62.5 (range: 38-76) years; 10 (3.7%) patients were aged ≤40 years, and there were 251 (94.0%) males and 16 (6.0%) females. A total of 102 (38.2%) patients had an educational background of high school or above. Twenty-nine (10.9%) patients had a family cancer history: esophageal cancer was noted in 13 (44.8%) families, lung cancer was noted in 7 (24.1%) families, breast cancer was noted in 4 (13.8%) families, liver cancer was noted in 3 (10.3%) families, and colorectal cancer was noted in 2 (6.9%) families. Patients in the NSND group were more likely to be female ($p<0.001$), have a younger age ($p<0.001$) and have a higher educational background ($p=0.012$) than those in the CSCD group. There were no apparent differences regarding family cancer history between the two groups ($p=0.294$) (**Table 1**).

Operation and Pathologic Characteristics

In the NSND group, 15 (17.4%) patients underwent free flap reconstruction: 10 with radial forearm flaps, 3 with anterolateral flaps, and 2 with fibular flaps. Tongue SCC was present in 37 (43.0%) patients, buccal SCC was present in 20 (23.3%) patients, and SCC of the upper and lower gingiva was present in 16 (18.6%) and 7 (8.1%) patients, respectively. SCC in the floor of the mouth was present in 6 (7.0%) patients. The median DOI was 8.2 mm, with a range from 2.0 mm to 23.5 mm. The pathologic tumor stages were distributed as T1 in 19 (22.1%) patients, T2 in 39 (45.3%) patients, T3 in 18 (20.9%) patients, and T4 in 10 (11.6%) patients. Tumor differentiations of well, moderate, and poor were reported in 37 (43.0%), 35 (40.7%), and 14 (16.3%) patients, respectively. PNI and LVI were reported in 13 (15.1%) and 12 (14.0%) patients, respectively. Negative margins were achieved in 80 (93.0%) patients. Neck dissection was performed in 76 patients, and the pathologic neck lymph node stages were distributed as N0 in 45 (59.2%) patients, N1 in 20 (26.3%) patients, and N2 in 11 (14.5%) patients.

In the CSCD group, 61 (22.8%) patients underwent free flap reconstruction: 37 with radial forearm flaps, 9 with anterolateral flaps, and 15 with fibular flaps. Twenty (7.5%) patients underwent submental island flap reconstruction. Tongue SCC was present in 89 (33.3%) patients, buccal SCC was present in 57 (21.3%) patients, and SCC of the upper and lower gingiva was present in 26 (9.7%) and 53 (19.9%) patients, respectively. SCC in the floor of the mouth was present in 42 (15.7%) patients. The median DOI was 9.9 mm, with a range from 2.0 mm to 27.1 mm. The pathologic tumor stages were distributed as T1 in 55 (20.6%) patients, T2 in 82 (30.7%) patients, T3 in 80 (30.0%) patients, and T4 in 50 (18.7%) patients. Tumor differentiations of well, moderate, and poor were reported in 80 (30.0%), 116 (43.4%), and 71 (26.6%) patients, respectively. PNI and LVI were reported in 65 (24.3%) and 57 (21.3%) patients, respectively. Negative margins were achieved in 240 (89.9%) patients. Neck dissection was performed in 252 patients, and the pathologic neck lymph

TABLE 1 | Comparison of demographic, pathologic, and molecular information between the non-smoker and non-drinker group (NSND) and the current-smoker/current-drinker (CSCD) group.

Variables	NSND group (n = 86)	CSCD group (n = 267)	p
Age			
≤40	14 (16.3%)	10 (3.7%)	
40-60	52 (60.4%)	95 (35.5%)	
≥60	20 (23.3%)	165 (61.8%)	<0.001
Sex			
Male	50 (58.1%)	251 (94.0%)	
Female	36 (41.9%)	16 (6.0%)	<0.001
Education background			
High school or above	46 (53.5%)	102 (38.2%)	
Under high school	40 (46.5%)	165 (61.8%)	0.012
A family cancer history			
Yes	6 (7.0%)	29 (10.9%)	
No	80 (93.0%)	238 (89.1%)	0.294
Primary tumor site			
Tongue	37 (43.0%)	89 (33.3%)	
Buccal	20 (23.3%)	57 (21.3%)	
Upper gingiva	16 (18.6%)	26 (9.7%)	
Lower gingiva	7 (8.1%%)	53 (19.9%)	
Floor of the mouth	6 (7.0%)	42 (15.7%)	0.005
Depth of invasion (mm)	8.2 (2.0-23.5)	9.9 (2.0-27.1)	<0.001
Pathologic tumor stage			
T1	19 (22.1%)	55 (20.6%)	
T2	39 (45.3%)	82 (30.7%)	
T3	18 (20.9%)	80 (30.0%)	
T4	10 (11.6%)	50 (18.7%)	0.042
Tumor differentiation			
Well	37 (43.0%)	80 (30.0%)	
Moderate	35 (40.7%)	116 (43.4%)	
Poor	14 (16.3%)	71 (26.6%)	0.042
Perineural invasion			
Positive	13 (15.1%)	65 (24.3%)	
Negative	73 (84.9%)	202 (75.7%)	0.073
Lymphovascular invasion			
Positive	12 (14.0%)	57 (21.3%)	
Negative	74 (86.0%)	210 (78.7%)	0.133
Pathologic neck stage*			
N0	45 (59.2%)	116 (46.0%)	
N1	20 (26.3%)	86 (34.1%)	
N2	11 (14.5%)	50 (19.8%)	0.130
Margin status			
Positive	6 (7.0%)	27 (10.1%)	
Negative	80 (93.0%)	240 (89.9%)	0.385
p16			
Positive	4 (4.7%)	27 (10.1%)	
Negative	82 (95.3%)	240 (89.9%)	0.132
p53			
Positive	15 (17.4%)	103 (38.6%)	
Negative	71 (82.6%)	164 (61.4%)	<0.001
p63			
Positive	25 (29.1%)	160 (59.9%)	
Negative	61 (70.9%)	107 (40.1%)	<0.001
Ki-67	24.5% (3.0%-78.5%)	35.7% (5.5%-93.0%)	<0.001

**Only patients who underwent neck dissection were analyzed.*

node stages were distributed as N0 in 116 (46.0%) patients, N1 in 86 (34.1%) patients, and N2 in 50 (19.8%) patients.

The two groups had significant differences regarding the primary tumor site (p=0.005), pathologic DOI (p<0.001), pathologic tumor stage (p=0.042), and tumor differentiation (p=0.042). Additionally, the two groups had a similar distribution of pathologic neck lymph node stage (p=0.130), PNI (p=0.073), and LVI (p=0.133) (**Table 1**).

HPV Infection, p16, p53, p63, and Ki-67

In the NSND group, no patients had HPV infection. Positivity of p53, p63, and p16 was reported in 15 (17.4%), 25 (29.1%), and 4

(4.7%) patients, respectively. The mean Ki-67 proliferation index was 24.5% (range: 3.0%-78.5%).

In the CSCD group, no patients had HPV infection. Positivity of p53, p63, and p16 was reported in 103 (38.6%), 160 (59.9%), and 27 (10.1%) patients, respectively. The mean Ki-67 proliferation index was 35.7% (range: 5.5%-93.0%).

Compared to the CSCD patients, the NSND patients had a significantly lower Ki-67 index (p<0.001). However, the CSCD patients had higher expression of p53 (p<0.001) and p63 (p<0.001). The two groups had similar distributions of p16 expression (p=0.132).

Survival Analysis

During our follow-up with a median time of 34 months, in the NSND group, 45 patients received adjuvant radiotherapy, and 19 patients underwent adjuvant chemotherapy. A total of 37 patients suffered from disease recurrence: 34 cases locoregionally and 3 cases distantly. Only 10 patients were successfully salvaged by radical surgery. Nineteen patients died of the disease.

In the CSCD group, 162 patients received adjuvant radiotherapy, and 81 patients underwent adjuvant chemotherapy. A total of 150 patients suffered from disease recurrence: 141 cases locoregionally and 9 cases distantly. Only 40 patients were successfully salvaged by radical surgery. A total of 100 patients died of the disease.

The 5-year LRC rates for NSND patients and CSCD patients were 48% and 38%, respectively, and the difference was significant (**Figure 1**, p=0.048). Further, the Cox model confirmed the independence of smoking and drinking status for affecting LRC (p=0.022, **Table 2**).

The median DSS time for NSND patients and CSCD patients was 59.3 months and 54.0 months, respectively. The 5-year DSS rates for NSND patients and CSCD patients were 56% and 39%, respectively, and the difference was significant (**Figure 2**, p=0.047). Further, the Cox model confirmed the independence of smoking and drinking status for affecting DSS (p=0.015, **Table 3**).

DISCUSSION

The most significant finding in the current study was that compared to typical oral SCC patients, NSND patients had significantly different epidemiological, pathologic, and molecular features and better prognosis, suggesting that NSND patients might be a different entity. This finding prompts more personalized cancer treatment for traditional and NSND oral SCC patients and more high-quality studies to clearly clarify the etiology of NSND patients.

In the beginning of preparing this research, one of the most important factors was to identify a clear definition of NSND and CSCD patients, which would improve the reliability of this study. Different definitions of never/current smokers and never/current drinkers have been described by previous authors (1, 11–15, 17–22), and it was noted that in most of those studies, an affirmative

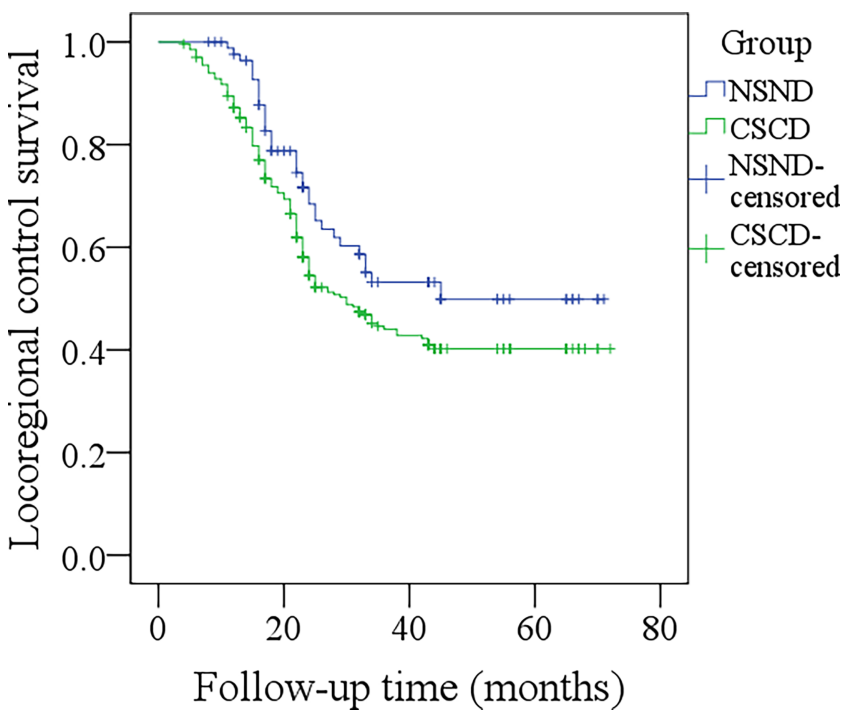

FIGURE 1 | Comparison of locoregional control survival between the non-smoker and non-drinker group and the current-smoker or current-drinker (CSCD) group (p = 0.048).

TABLE 2 | Univariate analysis and Cox model analysis of risk factors for locoregional recurrence in oral squamous cell carcinoma.

Variables	Univariate		Cox model	
	p	HR	95% CI	p
Age (≤40 *vs* >40)	0.036	2.465	0.337-7.543	0.754
Sex	0.224			
Education background	0.463			
Family cancer history	0.044	0.576	0.257-0.832	0.032
Tumor stage (T1+T2 *vs* T3+T4)	<0.001	6.563	2.341-18.427	<0.001
Tumor differentiation	<0.001			
Well				
Moderate		2.867	1.632-6.778	0.006
Poor		4.876	2.559-16.142	<0.001
Neck stage (N0 *vs* N+)	<0.002	5.337	1.863-19.226	<0.001
Perineural invasion	0.004	3.206	1.332-6.786	0.003
Lymphovascular invasion	0.012	5.789	0.116-30.321	0.554
Margin status	<0.001	5.216	1.632-18.331	<0.001
Status of smoking and drinking (NSND *vs* CSCD)	0.048	2.442	1.278-6.442	0.022
p16	0.036	2.335	1.327-7.002	0.019
p53	0.543			
p63	0.478			
Ki-67 (≤32.5% *vs* >32.5%)	<0.001	3.547	1.542-8.673	0.001
Adjuvant treatment	0.669			

never drinker even had one drink once a week. Current evidence distinctly proves that alcohol consumption apparently increases the risk of oral SCC (27). More importantly, the association of alcohol consumption with the relative risk for developing cancer tends to be dose-dependent (14); therefore, we should make a stricter standard for NSND patients, such as the definition used in this research. On the other hand, a typical oral SCC patient is usually associated with heavy tobacco and alcohol use for 10 years or more (28), and a similar viewpoint has also been reported by Brennan et al. (6), Koch et al. (10), Farshadpour

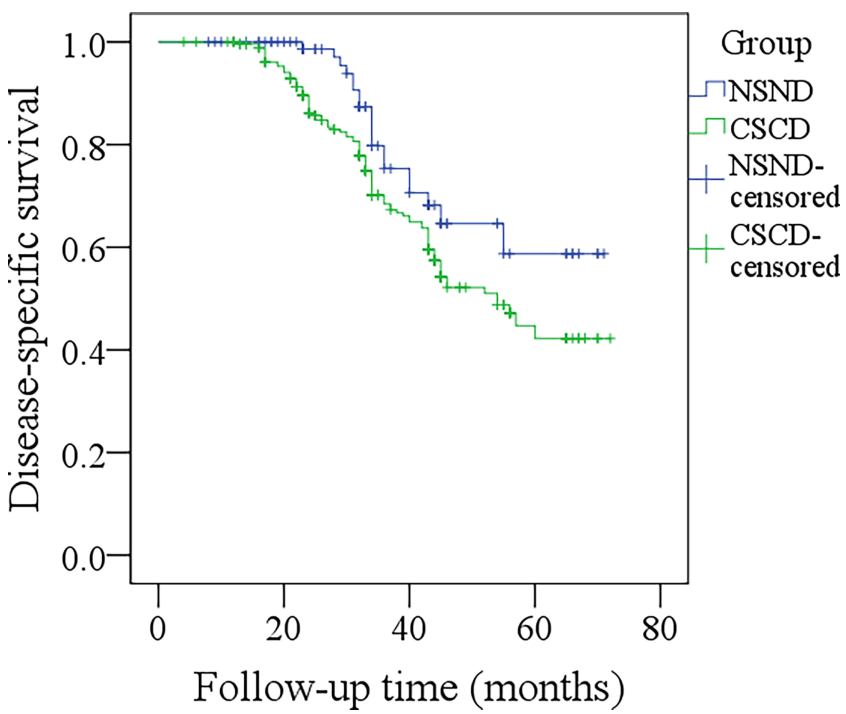

FIGURE 2 | Comparison of disease-specific survival between the non-smoker and non-drinker group and the current-smoker or current-drinker (CSCD) group (p = 0.047).

TABLE 3 | Univariate analysis and Cox model analysis of risk factors for cancer-caused death in oral squamous cell carcinoma.

Variables	Univariate		Cox model	
	p	HR	95% CI	p
Age (≤40 *vs* >40)	0.089			
Sex	0.546			
Education background	0.882			
Family cancer history	0.034	0.694	0.221-0.829	0.016
Tumor stage (T1+T2 *vs* T3+T4)	<0.001	7.322	2.005-21.563	<0.001
Tumor differentiation	<0.001			
Well				
Moderate		3.097	1.547-7.355	0.004
Poor		6.863	2.444-19.337	<0.001
Neck stage (N0 *vs* N+)	<0.001	5.442	1.476-13.356	<0.001
Perineural invasion	0.032	3.206	0.832-6.786	0.324
Lymphovascular invasion	0.031	4.761	0.976-30.321	0.067
Margin status	<0.001	4.224	1.355-13.217	<0.001
Status of smoking and drinking (NSND vs CSCD)	0.047	2.665	1.443-7.614	0.015
p16	0.077			
p53	0.431			
p63	0.785			
Ki-67 (≤32.5% *vs* >32.5%)	<0.001	2.632	0.775-9.435	0.101
Adjuvant treatment	0.338			

et al. (11), and Harris et al. (12). Therefore, to clearly determine the difference between NSND and CSCD groups and eliminate the influence of confounding factors, we identified a stricter standard for CSCD patients.

It was noted that there was a younger age in the NSND group, and a similar finding was also described by previous authors (9–11). However, literature regarding age distribution is scarce. There were significantly more patients aged less than 40 years in the NSND group. On the other hand, there was a male predominance in both groups but a significantly higher proportion of women in the NSND group in the current study; a similar finding was also noted by Bachar et al. (14) and Durr et al. (20). These two demographic findings might vaguely suggest that there are unknown factors explaining the occurrence of SCC in NSND patients; however, the influence caused by environmental tobacco cannot be ignored. Tan et al. (29) found that exposure to environmental tobacco in the home was always reported by elderly women with head and neck SCC, and men usually had a higher possibility of second-hand smoke exposure owing to their occupational nature (19).

Tumor site specificity has been demonstrated by a number of researchers (21, 30). Compared to CSCD patients, NSND patients had a lower possibility of developing SCC of the floor of the mouth and the lower gingiva but a higher possibility of developing SCC in the upper gingiva. It has been proposed that because of gravity dependence, pooling saliva containing alcohol/tobacco-derived carcinogens leads to an increased prevalence of cancer in the lower location of the oral cavity. A greater presence of adverse pathologic characteristics, including PNI, LVI, poor tumor differentiation, and advanced disease stage, has also been reported by previous authors (13, 14, 22), and similar findings were also noted by us. However, it is difficult to attribute this phenomenon to internal differences between the two groups because long-term alcohol and tobacco use can accelerate the development of cancer and change the biological behavior of disease (12).

The clarification of molecular expression variation was one of our main goals, as it would provide the strongest evidence for answering whether NSND patients are a different entity. Very few authors have performed similar analyses (17–19). Considerable attention has been given to the HPV virus owing to its possible etiological mechanism in head and neck SCC occurrence (28). Western researchers have even described HPV as being responsible for at least 70% of newly diagnosed cases of oropharynx SCC (31), but the role of HPV in inducing oral SCC remains unclear. Dediol et al. (17) reported that 27% of their NSND patients were HPV positive, but HPV detected by PCR did not distinguish whether HPV had been activated, and this finding did not support the causal relationship of HPV infection with tumorigenesis. Recent evidence by de Abreu et al. (32) showed that the frequency of high-risk HPV types in oral cavity SCC was very low and was less than 4%, and the authors concluded that HPV was not involved in the genesis of oral cavity SCC. Our study would also support this viewpoint, as no HPV infection occurred in either groups.

Furthermore, p16 is usually evaluated together with HPV. For oropharynx SCC, there is a reliable association between HPV infection and p16 overexpression, and p16-IHC is usually regarded as a surrogate marker of HPV infection. However, in the current study, we noted that approximately 5% of the NSND patients showed p16 positivity, although no HPV infection was detected by PCR. In a previous report by Harris et al. (12), 40% of young oral tongue SCC patients had p16 positivity, but no HPV was found in any of the tumor samples. Similar findings were also noted by Poling et al. (33): 9 of the 78 patients had p16 positivity, but only 1 patient had HPV E6/E7 mRNA transcripts. Moreover, our two groups had similar distributions of p16 expression. These findings suggest that p16 is not suitable for assessing the etiology associated with HPV infection in oral SCC.

In addition, p53 and p63 have been widely analyzed in head and neck SCC, but only a few authors have analyzed their expression in NSND patients. Heaton et al. (18) reported that a total of 16 tumors had strong p53 expression with a prevalence of 31.4%, and a previous review depicted that the overall rate of p53 positivity in head and neck SCC varied from 20% to 90% (33), which was slightly higher than that (17.4%) in our NSND patients but was consistent with that in our CSCD patients. The variation was attributed to the fact that both tobacco and alcohol could lead to mutations in the TP 53 gene. p63 was rarely assessed in NSND patients, and we might be the first to report that 29.1% of NSND patients show strong expression of p63. Previous studies have shown that the expression of p63 in SCC tissue is significantly higher than that in epithelial dysplasia and normal tissues (34). Together with our findings, these results suggest a role for p63 expression in carcinogenesis, and the effect might be enhanced by tobacco and alcohol. Ki-67 is an indicator of cancer cell proliferation, and a greater Ki-67 index might indicate more aggressive and poorer disease survival (26). We might be the first to report that the mean Ki-67 proliferation index was 24.5% for NSND patients, which was significantly lower than that in typical patients. This finding again provides evidence that NSND patients might be a different entity.

Survival differences between NSND patients and CSCD patients have been frequently compared, and conflicting results have been reported. Bachar et al. (14) divided 291 patients into two groups based on the status of tobacco smoking and alcohol abuse, and the two groups had similar local and regional control rates as well as overall survival rates. However, Durr et al. (20) described that compared to former or current smoking patients, never smoking patients tended to have decreased overall survival. In our opinion, long-term exposure to tobacco and alcohol is linked to a higher risk of peripheral vascular disease, chronic obstructive pulmonary disease, and coronary artery disease. Therefore, the index of overall survival might not be reliable enough for detecting the survival difference between the two groups. Pytynia et al. (13) found that after being matched to 50 ever smokers according to important variables, never smokers had a greater DSS and recurrence-free survival, and a further Cox model confirmed its independence. Our previous study also suggested that smoking was associated with an approximately 2-fold increase in the risk for recurrence and a 5-fold increase in the risk for disease-related death (22). In the current study, we noted that compared to CSCD patients, NSND patients had significantly better LRC and DSS in both univariate and multivariate analyses. A similar finding was also reported by Farshadpour et al. (11). Thus, NSND oral SCC patients might be a different entity.

It was interesting to find the negative prognostic significance of p16 expression in oral SCC. As usual, p16 expression was related to better survival in oropharynx SCC, but the exact opposite result was found in oral SCC. In a recent publication by Dediol et al. (17), the authors also reported that p16 expression carried a negative prognosis in oral SCC patients. However, in a recent meta-analysis, Almangush et al. (35) noted that there was no sufficient evidence to support p53, Ki-67 and p16 as prognostic biomarkers for oral SCC. The prognostic significance of p63 in oral SCC remains unknown, and our study failed to report a significant relationship between p63 expression and survival. However, Xu-Monette et al. (36) described the protective effect of p63 expression in high-risk diffuse large B-cell lymphoma. Therefore, more high-quality studies are needed to clarify these questions.

The limitations of the current study must be stated: there was inherent bias within this retrospective study, which may have decreased our statistical power; some other potential risk factors including chronic periodontitis, oral hygiene and economic status were not taken into consideration; and our strict standard may have artificially widened the difference between the two groups.

CONCLUSIONS

In summary, NSND oral SCC patients are a different entity compared with typical patients. HPV infection has a limited role in carcinogenesis in NSND, and p16 expression is associated with worse locoregional control.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article; the primary data can be obtained from the corresponding authors.

ETHICS STATEMENT

The Zhengzhou University institutional research committee approved our study, and all participants signed an informed consent agreement.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Chen F, Yan L, Lin L, Liu F, Qiu Y, Liu F, et al. Independent and Joint Effects of Tea and Milk Consumption on Oral Cancer Among Non-Smokers and Non-Drinkers: A Case-Control Study in China. *Oncotarget* (2017) 8:50091–7. doi: 10.18632/oncotarget.15096
2. Zhang SK, Zheng R, Chen Q, Zhang S, Sun X, Chen W. Oral Cancer Incidence and Mortality in China, 2011. *Chin J Cancer Res* (2015) 27:44–51.

3. Dai L, Fang Q, Li P, Liu F, Zhang X. Oncologic Outcomes of Patients With Sarcomatoid Carcinoma of the Hypopharynx. *Front Oncol* (2019) 9:950. doi: 10.3389/fonc.2019.00950
4. Du W, Fang Q, Wu Y, Wu J, Zhang X. Oncologic Outcome of Marginal Mandibulectomy in Squamous Cell Carcinoma of the Lower Gingiva. *BMC Cancer* (2019) 19:775. doi: 10.1186/s12885-019-5999-0
5. Fang Q, Li P, Qi J, Luo R, Chen D, Zhang X. Value of Lingual Lymph Node Metastasis in Patients With Squamous Cell Carcinoma of the Tongue. *Laryngoscope* (2019) 129:2527–30. doi: 10.1002/lary.27927
6. Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, et al. Association Between Cigarette Smoking and Mutation of the p53 Gene in Squamous-Cell Carcinoma of the Head and Neck. *N Engl J Med* (1995) 332:712–7. doi: 10.1056/NEJM199503163321104
7. Ratna A, Mandrekar P. Alcohol and Cancer: Mechanisms and Therapies. *Biomolecules* (2017) 7:61. doi: 10.3390/biom7030061
8. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer Statistics in China, 2015. *CA Cancer J Clin* (2016) 66:115–32. doi: 10.3322/caac.21338
9. Llewellyn CD, Johnson NW, Warnakulasuriya KA. Risk Factors for Squamous Cell Carcinoma of the Oral Cavity in Young People–a Comprehensive Literature Review. *Oral Oncol* (2001) 37:401–18. doi: 10.1016/S1368-8375(00)00135-4
10. Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and Neck Cancer in Nonsmokers: A Distinct Clinical and Molecular Entity. *Laryngoscope* (1999) 109:1544–51. doi: 10.1097/00005537-199910000-00002
11. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-Smoking and Non-Drinking Patients With Head and Neck Squamous Cell Carcinoma: A Distinct Population. *Oral Dis* (2007) 13:239–43. doi: 10.1111/j.1601-0825.2006.01274.x
12. Harris SL, Kimple RJ, Hayes DN, Couch ME, Rosenman JG. Never-Smokers, Never-Drinkers: Unique Clinical Subgroup of Young Patients With Head and Neck Squamous Cell Cancers. *Head Neck* (2010) 32:499–503. doi: 10.1002/hed.21220
13. Pytynia KB, Grant JR, Etzel CJ, Roberts DB, Wei Q, Sturgis EM. Matched-Pair Analysis of Survival of Never Smokers and Ever Smokers With Squamous Cell Carcinoma of the Head and Neck. *J Clin Oncol* (2004) 22:3981–8. doi: 10.1200/JCO.2004.02.133
14. Bachar G, Hod R, Goldstein DP, Irish JC, Gullane PJ, Brown D, et al. Outcome of Oral Tongue Squamous Cell Carcinoma in Patients With and Without Known Risk Factors. *Oral Oncol* (2011) 47:45–50. doi: 10.1016/j.oraloncology.2010.11.003
15. Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal Carcinoma in Non-Smokers and non-Drinkers: A Role for HPV. *Oral Oncol* (2009) 45:486–91. doi: 10.1016/j.oraloncology.2008.07.008
16. Mirghani H, Amen F, Moreau F, Lacau St Guily J. Do High-Risk Human Papillomaviruses Cause Oral Cavity Squamous Cell Carcinoma? *Oral Oncol* (2015) 51:229–36. doi: 10.1016/j.oraloncology.2014.11.011
17. Dediol E, Sabol I, Virag M, Grce M, Muller D, Manojlović S. HPV Prevalence and p16INKa Overexpression in Non-Smoking Non-Drinking Oral Cavity Cancer Patients. *Oral Dis* (2016) 22:517–22. doi: 10.1111/odi.12476
18. Heaton CM, Durr ML, Tetsu O, van Zante A, Wang SJ. TP53 and CDKN2a Mutations in Never-Smoker Oral Tongue Squamous Cell Carcinoma. *Laryngoscope* (2014) 124:E267–73. doi: 10.1002/lary.24595
19. Dahlstrom KR, Little JA, Zafereo ME, Lung M, Wei Q, Sturgis EM. Squamous Cell Carcinoma of the Head and Neck in Never Smoker-Never Drinkers: A Descriptive Epidemiologic Study. *Head Neck* (2008) 30:75–84. doi: 10.1002/hed.20664
20. Durr ML, van Zante A, Li D, Kezirian EJ, Wang SJ. Oral Tongue Squamous Cell Carcinoma in Never-Smokers: Analysis of Clinicopathologic Characteristics and Survival. *Otolaryngol Head Neck Surg* (2013) 149:89–96. doi: 10.1177/0194599813482876
21. Durr ML, Li D, Wang SJ. Oral Cavity Squamous Cell Carcinoma in Never Smokers: Analysis of Clinicopathologic Characteristics and Survival. *Am J Otolaryngol* (2013) 34:388–93. doi: 10.1016/j.amjoto.2013.01.017
22. Fang QG, Shi S, Liu FY, Sun CF. Squamous Cell Carcinoma of the Oral Cavity in Ever Smokers: A Matched-Pair Analysis of Survival. *J Craniofac Surg* (2014) 25:934–7. doi: 10.1097/SCS.0000000000000710
23. Cui M, Du W, Fang Q, Dai L, Qi J, Luo R. Prognostic Value of a Family History of Oral Tongue Squamous Cell Carcinoma: A Matched-Pair Study. *Laryngoscope* (2020) 130:E605–10. doi: 10.1002/lary.28471
24. Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, et al. Head and Neck Cancers-Major Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual. *CA Cancer J Clin* (2017) 67:122–37. doi: 10.3322/caac.21389
25. Thorstad W, Gay H, Daly M, Jackson R, Rich J, Paniello R, et al. Correlation of Ki-67 Proliferative Antigen Expression and Tumor Response to Induction Chemotherapy Containing Cell Cycle-Specific Agents in Head and Neck Squamous Cell Carcinoma. *Head Neck Pathol* (2017) 11:338–45. doi: 10.1007/s12105-016-0775-9
26. Gioacchini FM, Alicandri-Ciufelli M, Magliulo G, Rubini C, Presutti L, Re M. The Clinical Relevance of Ki-67 Expression in Laryngeal Squamous Cell Carcinoma. *Eur Arch Otorhinolaryngol* (2015) 272:1569–76. doi: 10.1007/s00405-014-3117-0
27. Ghantous Y, Schussel JL, Brait M. Tobacco and Alcohol-Induced Epigenetic Changes in Oral Carcinoma. *Curr Opin Oncol* (2018) 30:152–8. doi: 10.1097/CCO.0000000000000444
28. Lingen M, Sturgis EM, Kies MS. Squamous Cell Carcinoma of the Head and Neck in Nonsmokers: Clinical and Biologic Characteristics and Implications for Management. *Curr Opin Oncol* (2001) 13:176–82. doi: 10.1097/00001622-200105000-00008
29. Tan EH, Adelstein DJ, Droughton ML, Van Kirk MA, Lavertu P. Squamous Cell Head and Neck Cancer in Nonsmokers. *Am J Clin Oncol* (1997) 20:146–50. doi: 10.1097/00000421-199704000-00008
30. Schmidt BL, Dierks EJ, Homer L, Potter B. Tobacco Smoking History and Presentation of Oral Squamous Cell Carcinoma. *J Oral Maxillofac Surg* (2004) 62:1055–8. doi: 10.1016/j.joms.2004.03.010
31. Wirth LJ, Burtness B, Nathan CO, Grégoire V, Richmon J. Point/Counterpoint: Do We De-Escalate Treatment of HPV-Associated Oropharynx Cancer Now? And How? *Am Soc Clin Oncol Educ Book* (2019) 39:364–72. doi: 10.1200/EDBK_238315
32. de Abreu PM, Có ACG, Azevedo PL, do Valle IB, de Oliveira KG, Gouvea SA, et al. Frequency of HPV in Oral Cavity Squamous Cell Carcinoma. *BMC Cancer* (2018) 18:324. doi: 10.1186/s12885-018-4247-3
33. Solomon B, Young RJ, Rischin D. Head and Neck Squamous Cell Carcinoma: Genomics and Emerging Biomarkers for Immunomodulatory Cancer Treatments. *Semin Cancer Biol* (2018) 52:228–40. doi: 10.1016/j.semcancer.2018.01.008
34. Sniezek JC, Matheny KE, Westfall MD, Pietenpol JA. Dominant Negative p63 Isoform Expression in Head and Neck Squamous Cell Carcinoma. *Laryngoscope* (2004) 114:2063–72. doi: 10.1097/01.mlg.0000149437.35855.4b
35. Almangush A, Heikkinen I, Mäkitie AA, Coletta RD, Läärä E, Leivo I, et al. Prognostic Biomarkers for Oral Tongue Squamous Cell Carcinoma: A Systematic Review and Meta-Analysis. *Br J Cancer* (2017) 117:856–66. doi: 10.1038/bjc.2017.244
36. Xu-Monette ZY, Zhang S, Li X, Manyam GC, Wang XX, Xia Y, et al. p63 Expression Confers Significantly Better Survival Outcomes in High-Risk Diffuse Large B-Cell Lymphoma and Demonstrates p53-Like and p53-Independent Tumor Suppressor Function. *Aging (Albany NY)* (2016) 8:345–65. doi: 10.18632/aging.100898

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Blocking of EGFR Signaling Is a Latent Strategy for the Improvement of Prognosis of HPV-Induced Cancer

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Human papillomavirus (HPV) is a double-stranded DNA (dsDNA) virus, and its high-risk subtypes increase cancer risks. However, the mechanism of HPV infection and pathogenesis still remain unclear. Therefore, understanding the molecular mechanisms and the pathogenesis of HPV are crucial in the prevention of HPV-related cancers. In this study, we analyzed cervix squamous cell carcinoma (CESC) and head and neck carcinoma (HNSC) combined data to investigate various HPV-induced cancer common features. We showed that epidermal growth factor receptor (EGFR) was downregulated in HPV-positive (HPV+) cancer, and that HPV+ cancer patients exhibited better prognosis than HPV-negative (HPV–) cancer patients. Our study also showed that TP53 mutation rate is lower in HPV+ cancer than in HPV– cancer and that TP53 can be modulated by HPV E7 protein. However, there was no significant difference in the expression of wildtype TP53 in both groups. Subsequently, we constructed HPV-human interaction network and found that EGFR is a critical factor. From the network, we also noticed that EGFR is regulated by HPV E7 protein and hsa-miR-944. Moreover, while phosphorylated EGFR is associated with a worse prognosis, EGFR total express level is not significantly correlated with prognosis. This indicates that EGFR activation will induce a worse outcome in HPV+ cancer patients. Further enrichment analysis showed that EGFR downstream pathway and cancer relative pathway are diversely activated in HPV+ cancer and HPV– cancer. In summary, HPV E7 protein downregulates EGFR that downregulates phosphorylated EGFR and inhibit EGFR-related pathways which in turn and consequently induce better prognosis.

Keywords: EGFR, hNSC, head and neck squamous cell carcinoma, cesC, human papillomavirus, E7

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1 INTRODUCTION

Tumor can be caused by several factors (1). A virus is a small pathogen that often causes pathological changes or diseases in the target host (2). Some viral infections have been linked to be essential factors that induce numerous forms of cancer such as liver cancer and nasopharyngeal carcinoma (3). Virus lifecycle requires intracellular environment owing to its simple structure (4).

It hijacks the cell's complex protein and nucleic acid synthesis system for self-proliferation and also controls the functional protein of cells to modulate the normal cell signaling pathway (5).

Human papillomavirus (HPV) is a nonenveloped double-stranded DNA (dsDNA) tumor virus. Almost all cervical squamous cell carcinoma and about 40% of head and neck cancers are consequences of HPV infection (6, 7). HPV preferably infect the mucosal layer, and no evidence shows that HPV has the ability to infect other cells except basal cells of the epithelia. Basal cell is the high differential ability cell of the epithelia. Hence, host cell development and differentiation ability are probably required for HPV infection (8). The carcinogenesis of squamous cell carcinoma is often accompanied by changes in development-related functions (9). Therefore, epithelia development-regulated protein may be the key target of HPV infection and oncogenesis. HPV genome encodes seven early-phase proteins (E1 to E7) and two late-phase proteins (L1 and L2) for its proliferation. E6 and E7 proteins can modulate p53 and Rb through downregulation or inhibition, which is the basic mechanism of HPV+ cancer genesis (7). Therefore, E6 and E7 can be regarded as the most essential HPV oncogenic proteins (10). Due to its small genome and limited virus-encoded protein, virus proteins require high efficiency and multifunctionality for complicated manipulation. For example, evidences showed that E6 and E7 proteins can interact with many human proteins and participate in a lot of biological processes (11). Likewise, HPV capsid protein L1 and L2 have been reported to interact with human proteins (12).

Many studies have examined HPV infection features in several types of cancers and HPV infection induced cancers have also been sufficiently investigated. For example, over 90% of the occurrence of cervix squamous cell carcinoma (CESC) is attributed to HPV infection (13). Also, head and neck carcinoma (HNSC) highly linked to HPV infection (14). Epidermal growth factor receptor (EGFR) is a cancer-related gene and it also has been reported to be a potential biomarker of HPV infection (15–17). There are also studies that have demonstrated that some subtypes of HNSC exhibit higher HPV infection rate than other subtypes, and that HPV copy number is lower in HPV infected subtypes (18). Furthermore, EGFR is associated with HPV-related cancer prognosis. It was reported that EGFR and pEGFR (phosphorylated epidermal growth factor receptor) are potential biomarkers of prognosis for oropharyngeal cancer (19). In cervical cancer, EGFR signaling can be affected by Hippo/YAP pathway and eventually influence cancer progression (20). Some reports suggested that EGFR expression can be regulated by HPV E5 protein (17), while contradicting reports showed that E5 protein does not regulate the expression of EGFR and cancer prognosis (21). Other reports showed that EGFR can be possibly regulated by miRNA. For example, in HPV-infected patients, smoking-induced control of miR-133a-3p regulates the expression of EGFR and human antigen R (HuR) (22). Hence, EGFR expression in HPV-infected cancer may be regulated by multiple factors such as existing complex mechanisms and HPV viral protein. However, no study has completely established the HPV protein is the key modulator of EGFR.

Since most of the previous studies focused on comparing single cancer type or HPV+ groups with normal group, there are limited studies that focused on multiple-cancer types or compared HPV+ cancer with HPV− cancer. Therefore, it is noteworthy to investigate EGFR regulating mechanisms in a multiple-cancer type. Owing to existing EGFR-targeted drugs, EGFR would be a potential target for HPV-induced cancer prognosis improvement.

Our study analyzed CESC and HNSC combined data at multiple levels including mRNA, miRNA, SNV, and protein expression level. We also constructed a global network with HPV proteins, HPV differentially expressed genes, and miRNAs in HPV+ cancers versus HPV− cancers. Through the network, our study showed that EGFR is regulated by HPV E7 protein and downregulated by miR-944. Furthermore, our findings showed that pEGFR and its up- and downstream protein activation are negatively correlated with HPV+ cancer survival. These findings are evidences that EGFR is regulated in a complex mechanism and that E7 is the HPV protein that regulates EGFR expression in HPV-induced cancer.

2 RESULTS

2.1 HPV-Positive Cancer Patients Are Significantly Different From HPV-Negative Cancer Patients in Gene Expression and They Show Higher Survival Possibility

In order to understand the relationships between HPV regulation and cancer, we selected the two most common HPV-related cancers which are CESC and HNSC for combined analysis. For RNA-Seq read counts matrix from ICGC database, principal component analysis (PCA) showed sample distribution of HPV + and HPV− samples (**Figure 1A**). After removing the outlier at the lower right area, PCA plot was redrawn as displayed in **Figure 1B**, in which, HPV+ samples showed different distribution patterns against HPV− samples. The different distribution pattern shows that HPV+ cancer is distinct from HPV− cancer in gene expression. Further differentially expressed gene analysis was carried out by grouping samples by their HPV infection status. Eight hundred thirty-four differentially expressed genes (DEGs) were screened, and these genes basically distinguished HPV+ from HPV− samples (**Figure 1C**). Survival analysis based on clinical data from FireBrowse database showed that HPV+ patients had better prognosis compared with HPV− patients (**Figure 1D**). It implies that different survival rates are attributed to DEGs to some degree.

The miRNA-Seq read counts data were also analyzed using PCA and differential expression analysis. PCA distribution showed no obvious difference between HPV+ and HPV− samples (**Figure 1E**), which indicates that there is no clear difference in miRNA expression level between HPV+ cancer and HPV− cancer. Differentially express analysis further showed that only five miRNAs were significantly differentially expressed. They were hsa-miR-944, hsa-miR-196, hsa-miR-206, hsa-miR-

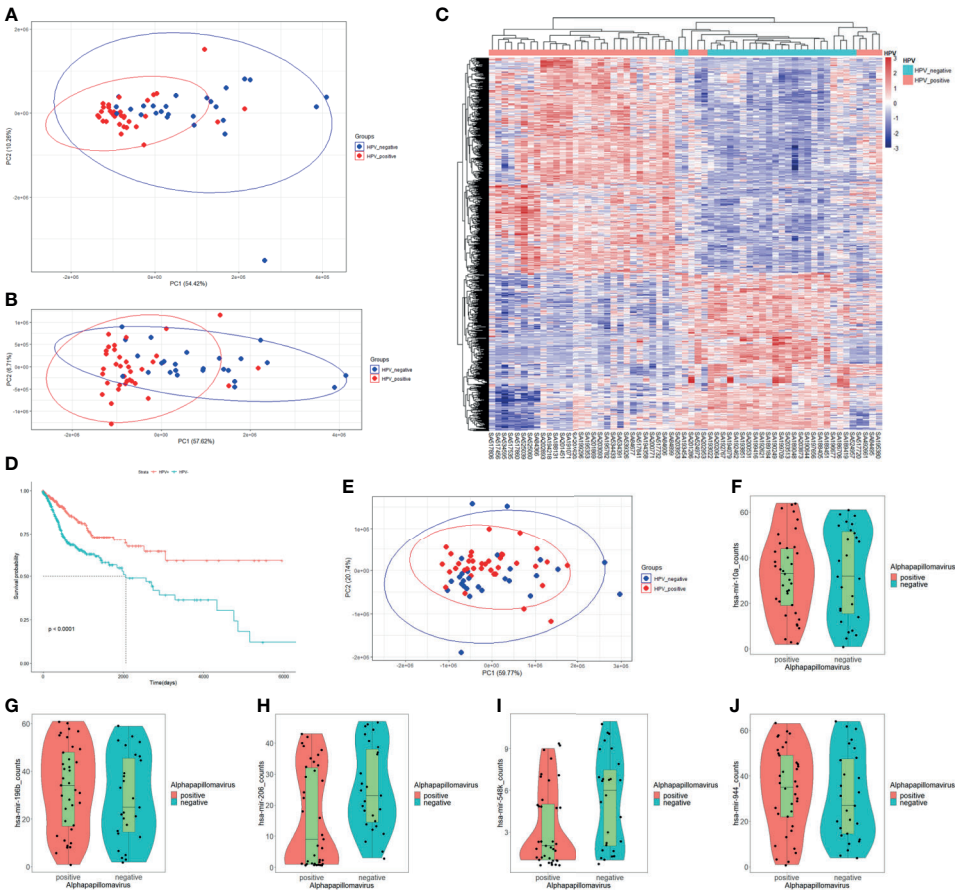

FIGURE 1 | mRNA expression, miRNA expression, and prognosis differences in HPV+ cancers against HPV– cancers. **(A)** Principal component analysis (PCA) plot of the RNA-Seq data of CESC and HNSC samples within PCAWG program of ICGC database. HPV+ samples are marked in red; HPV– samples are marked in blue. **(B)** Redrawn PCA plot after outlier was removed. HPV+ samples are marked in red, and HPV– samples are marked in blue. **(C)** Differentially expressed genes (DEGs) heatmap of HPV+ group vs. HPV– group. Gene FPKMs were scaled with z-score by samples. Pearson's correlation coefficients were taken for samples and gene clustering. **(D)** KM-plot of HPV+ patients and HPV– patient survival status from TCGA CESC and HNSC project. **(E)** PCA plot of miRNA-Seq data of CESC and HNSC sample within the PCAWG program of ICGC database. HPV+ samples are marked in red, HPV– samples are marked in blue. **(F–J)** hsa-miR-10a, hsa-miR-196b, hsa-miR-206, hsa-miR-548k, and hsa-miR-944 expression status in HPV+ samples and HPV– samples respectively.

10a, and hsa-miR-548k (**Figures 1F–J**). Further screening of the target in intersection of differentially expressed miRNAs showed only hsa-miR-944, hsa-miR-206, and hsa-miR-548k DEG targets, which signifies that hsa-miR-196 and hsa-miR-10a probably do not participate in DEG-related functions although they were differentially expressed. Both hsa-miR-196 and hsa-miR-10a were upregulated in HPV+ cancers. The differential expression may be related to HPV proliferation. Small miRNA expressing differences between HPV+ cancer and HPV– cancer shows that only few miRNAs participate in HPV infection-specific regulation and most of them are only cancer related or are steadily expressed in both situations.

2.2 TP53 Mutation Proportion Is Lower in HPV+ Cancer Than in HPV– Cancer

Single nucleotide variation (SNV) analysis was done for CESC and HNSC combined data. Comparing HPV+ and HPV– groups, the number of samples was almost the same (**Figure 2A**). In determining the nucleotide variation type, we showed that HPV+ sample variation types and rates differ from that of HPV– samples. It showed that C>G mutation rates of HPV+ samples are higher when compared with C>A mutation, while they are almost the same in HPV– samples (**Figures 2B, C**). At gene level for all samples, TP53 ranked at the 2nd place for single nucleotide mutation for all the genes. Moreover, TP53 mutation takes up 37% samples of the total mutation and it got the first place of all matched genes (**Figure 2D**). These results suggest that TP53 mutation plays a critical role in CESC and HNSC.

Furthermore, we selected top 30 genes with the highest mutation frequency and used oncoplot to show their mutation rates in each of the samples. The result showed that the number of TP53 mutation is significantly higher in HPV– cancer than in HPV+ cancer. Whereas most of the genes with high mutation

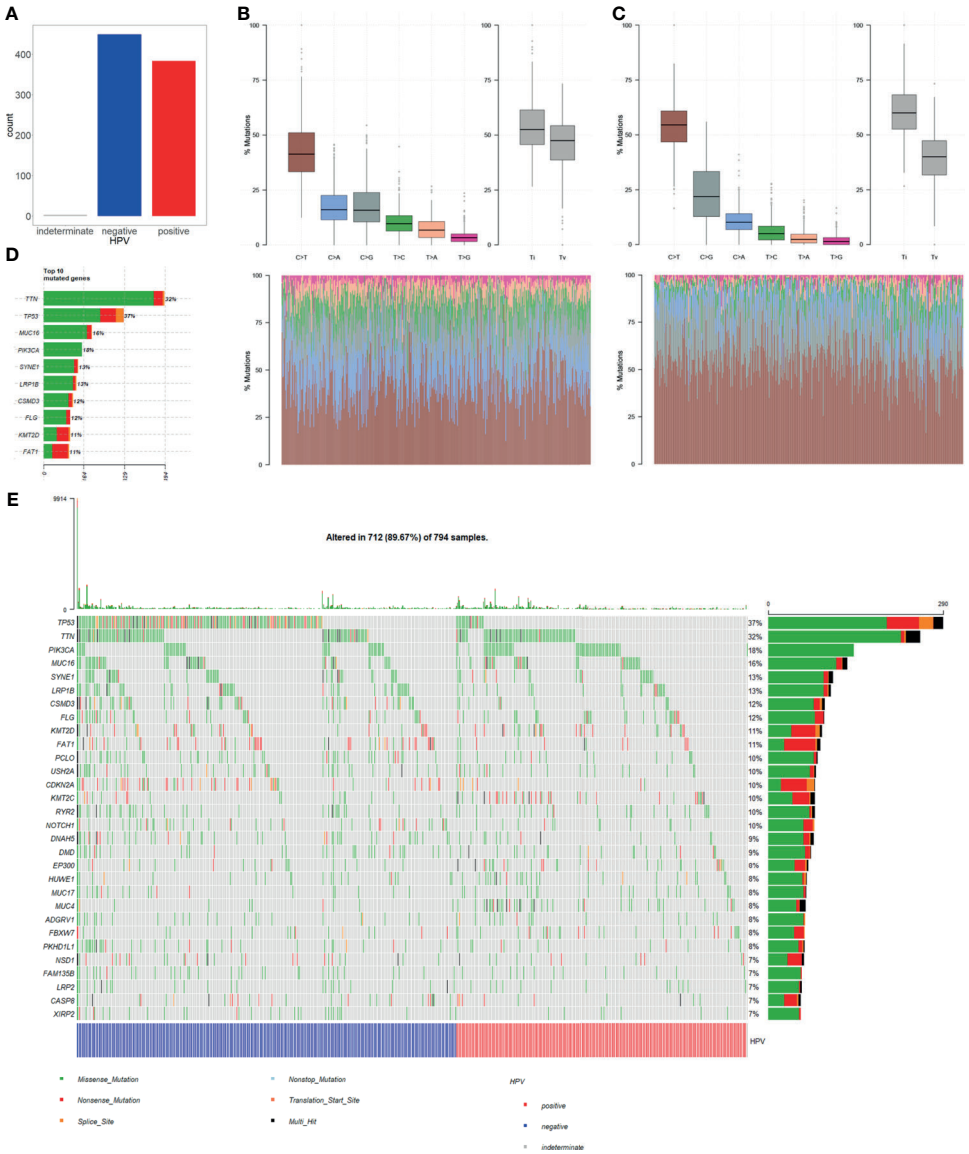

FIGURE 2 | Single nucleotide variation (SNV) in HPV+ cancers and HPV– cancers. **(A)** Total numbers of HPV+ samples and HPV– samples in SNV data of TCGA CESC and HNSC project. MUSE software processed SNV data was used in our study. **(B)** HPV+ samples single nucleotide mutation-type proportions. **(C)** HPV– samples single nucleotide mutation-type proportions. **(D)** Top 10 highly mutation rates and mutated sample counts of mutated genes. **(E)** Oncoplot of the top 30 highly mutated genes; samples were grouped by HPV infection status.

rates showed no significant difference in both groups (**Figure 2E**). This result suggests that TP53 mutation is an important mechanism for the occurrence of HPV–. However, HPV+ cancer shows no relative involvement with TP53 mutation, and cancer occurrence may be involved in other mechanisms.

2.3 HPV Protein Regulation of Human Protein Is an Important Mechanism for the Occurrence of HPV+ Cancer

With virus protein-human protein, mRNA-mRNA (differentially expressed), and miRNA-mRNA interaction pairs, we constructed a miRNA-mRNA-protein interaction network. The result showed that a considerable number of human genes are regulated by HPV proteins. Thus, the genes that are modulated by HPV viral protein may possibly be the key factors that induce tumor occurrence (**Figure 3A**). Overall, most of the genes directly regulated by HPV are not differentially expressed gene. This suggests that HPV-regulated genes have similar expression pattern in HPV– tumors.

We further extracted a subnetwork that contains only HPV protein and its regulated genes to investigate the manipulation details. From the subnetwork, four genes were differentially expressed and are listed as follows, EGFR, SNF (SWI/SNF

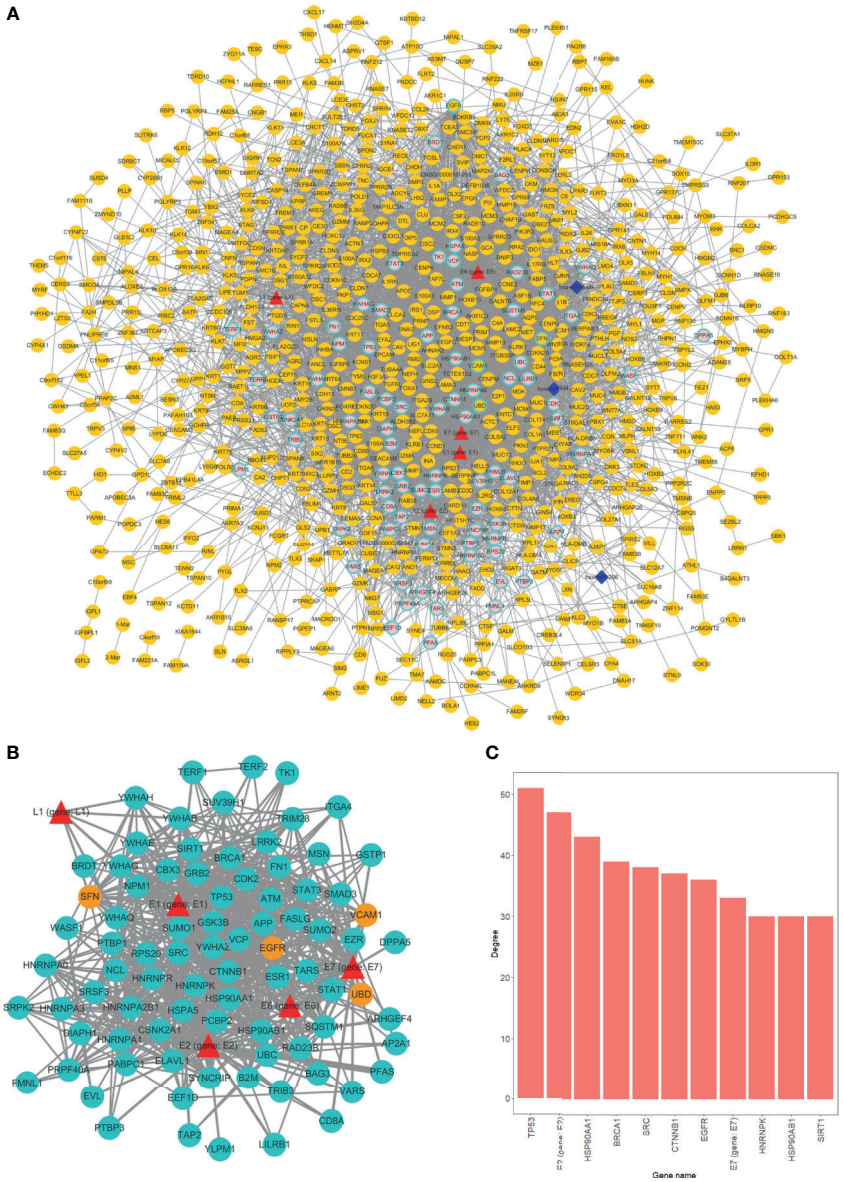

FIGURE 3 | Overview of interaction network. **(A)** Global network of miRNA-mRNA-protein interactions. HPV proteins are marked as red triangles; miRNAs are represented as deep blue diamonds; differentially expressed genes (DEGs) are orange circle; nondifferentially expressed genes are in pink. Circles with light blue border are HPV directly interacted human genes. **(B)** HPV proteins directly regulated subnetwork. Turquoise circles are HPV directly manipulated nondifferentially expressed genes; red triangles are HPV proteins; orange circles are HPV directly manipulated DEGs. **(C)** Top 11 genes degree distributions of HPV proteins directly regulated subnetwork.

related member), ubiquitin D (UBD), and vascular cell adhesion molecule 1 (VCAM1) (**Figure 3B**). Further findings indicated that the variation between HPV+ cancer and HPV− cancer can possibly be attributed to the effect of HPV regulation of those four genes. Through degree analysis of subnetwork, we showed that degrees of tumor suppressor gene TP53 (regulated by HPV E7 protein) are the highest of all genes (**Figure 3C**). This result indicates that the regulation of TP53 by HPV is a crucial mechanism for HPV+ tumor to occur.

2.4 EGFR Is the Crucial Gene That Regulate HPV+ Tumor Differentially Expressed Genes

We used degree = 55 to screen hub nodes of miRNA-mRNA-protein network, and 22 nodes were selected. Out of the 22 genes, eight genes are DEGs. Whereas 15 genes are direct HPV-regulated genes and nodes like TP53, BRCA1, EGFR, and CTNNB1 are classic tumor-related genes (**Figure 4A**). Remarkably, EGFR is the only hub node that belongs to both DEGs and directly interacts with

HPV (**Figure 4B**). We further extracted a subnetwork constructed with EGFR and its first neighbor. The result showed that EGFR interacts with a considerable amount of DEGs, and it is also regulated by hsa-miR-944 and HPV protein E7 (**Figure 4C**). This indicates that EGFR is an essential gene that regulates the differentially expressed genes.

Furthermore, we predicted that EGFR is regulated by hsa-miR-944 and that the upregulation of has-miR-944 caused the downregulation of EGFR (**Figures 1J**, **4B**). In order to confirm whether hsa-miR-944 combine stably with EGFR, RIsearch2 software was used for RNA combined analysis. The result shows that EGFR and hsa-miR-944 have low-energy binding site at 3′ end of hsa-miR-944 (**Figure 4D**). Our findings revealed that EGFR is regulated by both E7 protein and hsa-miR-944. This shows that E7 protein does not only induces carcinogenesis in HPV+ tissues but also causes the difference in appearance in HPV+ and HPV− tumor.

A module with EGFR was identified using module analysis of global network. Since gene in the same module interacts closely, there is a possibility that they can participate in the same biological process. HPV protein E2, a key protein that plays a pivotal role in HPV infection from the early stage to the late stage, was also identified (**Figure 4E**). This finding suggests that EGFR participates in all HPV infection stages and could probably influence tumor development and prognosis. Likewise, GO and KEGG enrichment analyses revealed that the module genes are enriched with EGFR downstream pathways and participates in several functions, including development regulation, epithelial regulation, and mitogen-activated protein kinase (MAPK) pathway (**Figure 4F**). This implies that EGFR downstream pathways are regulated by E2, and it can influence oncogenesis at late stage of infection.

2.5 Activation of EGFR-Related Pathway Is an Important Factor That Decreases Survival

In order to figure out how EGFR influence prognosis, we merged reverse-phase protein array (RPPA) data of CESC and HNSC and a total of 133 proteins were obtained. Student's *t*-test was used to test for the differences between HPV+ and HPV− groups.

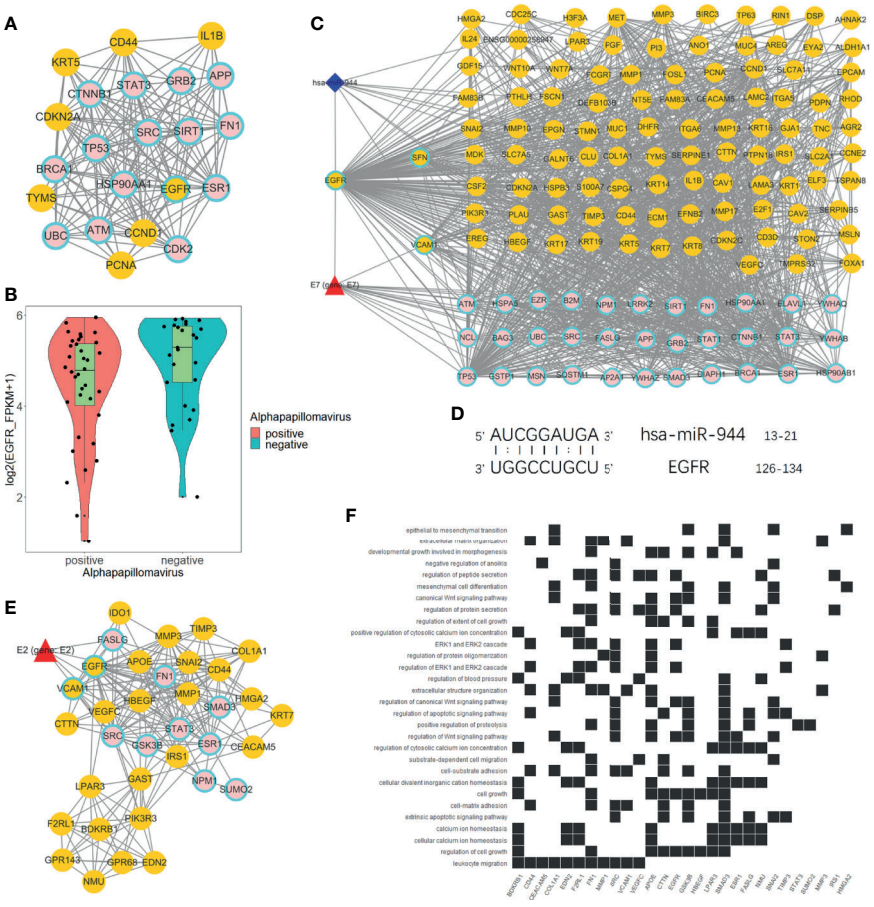

FIGURE 4 | Analysis of EGFR-related network. **(A)** Hub genes of global network, including 15 HPV manipulated genes; seven DEGs are not regulated by HPV and one HPV regulated DEG. **(B)** EGFR express status in HPV+ and HPV− samples respectively according to RNA-Seq FPKM from ICGC PCAWG project CESC and HNSC data. **(C)** Subnetwork of EGFR first neighbors. **(D)** EGFR RNA binding prediction site with hsa-miR-944 using RIsearch2 software. **(E)** MCODE-calculated EGFR containing network cluster. **(F)** EGFR containing cluster GO and KEGG enrichment analysis.

Interestingly, 123 proteins were differentially expressed and only 10 proteins were not significant. Yet, TP53 was not differentially expressed at the protein level. Subsequently, COX proportional hazard regression model was used to evaluate the correlation between survival time and deferentially expressed proteins in HPV+ tumor patients. The results suggest that the expressed level of EGFR did not have any significant relationship with prognosis. Notably, there was a negative correlation between all tyrosine residue phosphorylated forms of EGFR and patients' survival. Phosphorylation on site pY1068 and pY1173 representing EGFR was activated to form a dimer that binds with its ligand thus, further activating downstream pathways like PI3K/Akt, MAPK, and WNT pathway. We also highlighted that amphiregulin (AR), an EGFR ligand, and some significant proteins belong to PI3K/Akt or MAPK pathway. Those downstream proteins also showed similar properties of phosphorylated form of EGFR that are significantly negatively correlated with survival (**Figure 5**).

2.6 EGFR Modulates Cancer Prognosis Through the Regulation of Immune and DNA Repair Pathway

In order to further find out the prognosis-related pathways, we carried out KEGG enrichment analysis of HPV+ *versus* HPV− DEGs and mRNA targets of differentially expressed miRNAs (DEmiRs). The top enriched term list showed "human papillomavirus infection," demonstrating that HPV activates a unique pathway different from HPV− cancer. For DEG enrichment analysis, immune-related pathway (such as IL-17 signaling pathway and TNF signaling pathway), cancer-related pathway, and DNA-related pathway were shown, and it could be correlated with prognosis. Remarkably, EGFR-related pathways, "PI3K/Akt pathway" and "ECM-receptor interaction," were also included in the top list (**Figure 6A**). For DEmiR target enrichments, numerous cancer-related pathways were shown, and EGFR-related pathway, "PI3K/Akt pathway", was also enriched (**Figure 6B**). These results suggest that HPV+ cancer

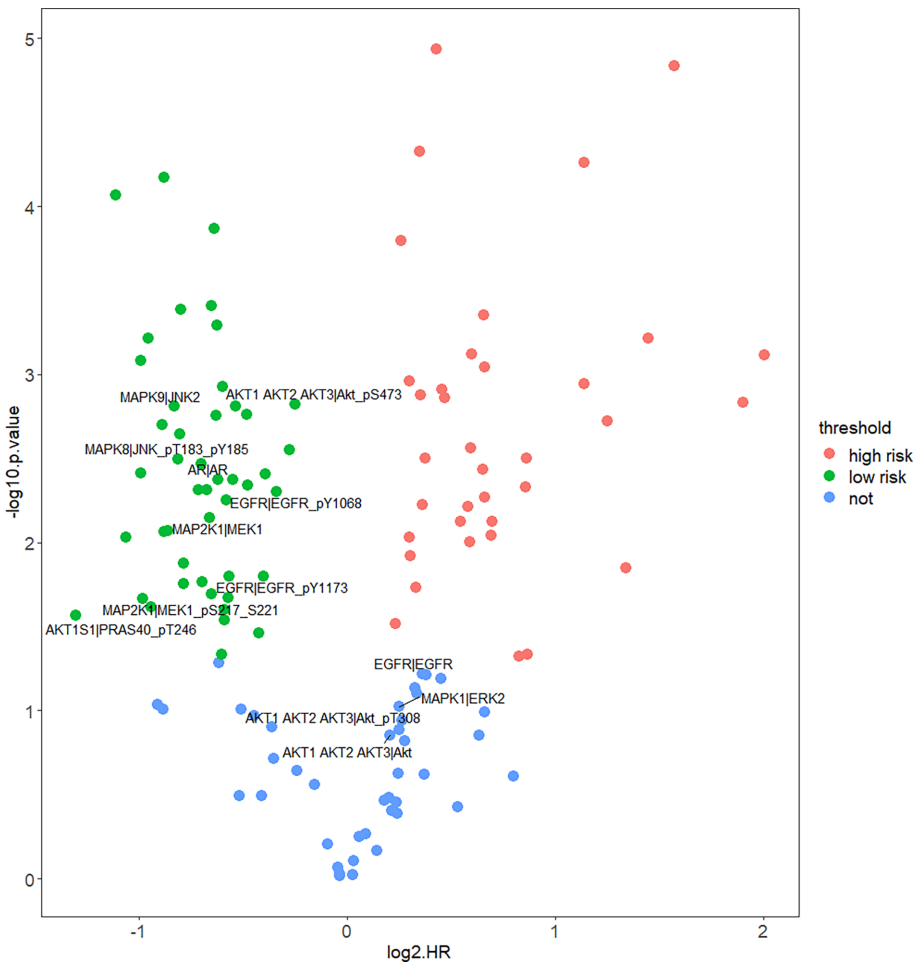

FIGURE 5 | Correlation of protein expression and HPV+ patients' survival. Figure shows COX proportional hazard regression. It measured correlation significance of protein expression and HPV+ patients' survival. Only differentially expressed proteins (DEPs) of HPV+ *vs.* HPV− are displayed. Proteins which $p < 0.05$ are significantly correlated with HPV+ patients' survival, log2HR >0 is positively correlated with survival, log2HR <0 is negatively correlated with survival.

shows different prognosis-related pathway activation compared with HPV− cancer, even in cancer-related pathway. Gene Set Enrichment Analysis (GSEA) further showed that immune-related pathways and tumor-related pathways were inactivated and DNA repair pathways were activated in HPV+ cancer groups compared with HPV− groups. All these activated and inactivated pathways can possibly enhance better prognosis of HPV+ cancer (**Figures 6C–K**). Since EGFR is the hub gene of DEG network, EGFR can possibly affect the prognosis of tumors through the regulation of immune, tumor, and DNA repair pathway.

3 DISCUSSION

HPV is a tissue-specific oncogenic virus that specifically infects epithelium tissues. The mechanisms of HPV-induced squamous cell carcinoma are probably not similar to the mechanisms of HPV nonassociated squamous cell carcinoma. Since both mechanisms cause tumorigenesis, it suggests that their gene expression patterns are somewhat common. Based on this hypothesis, our study compared HPV+ and HPV− tumors at multiomics level in order to identify similar and different underlining molecular mechanisms. Although HPV can infect

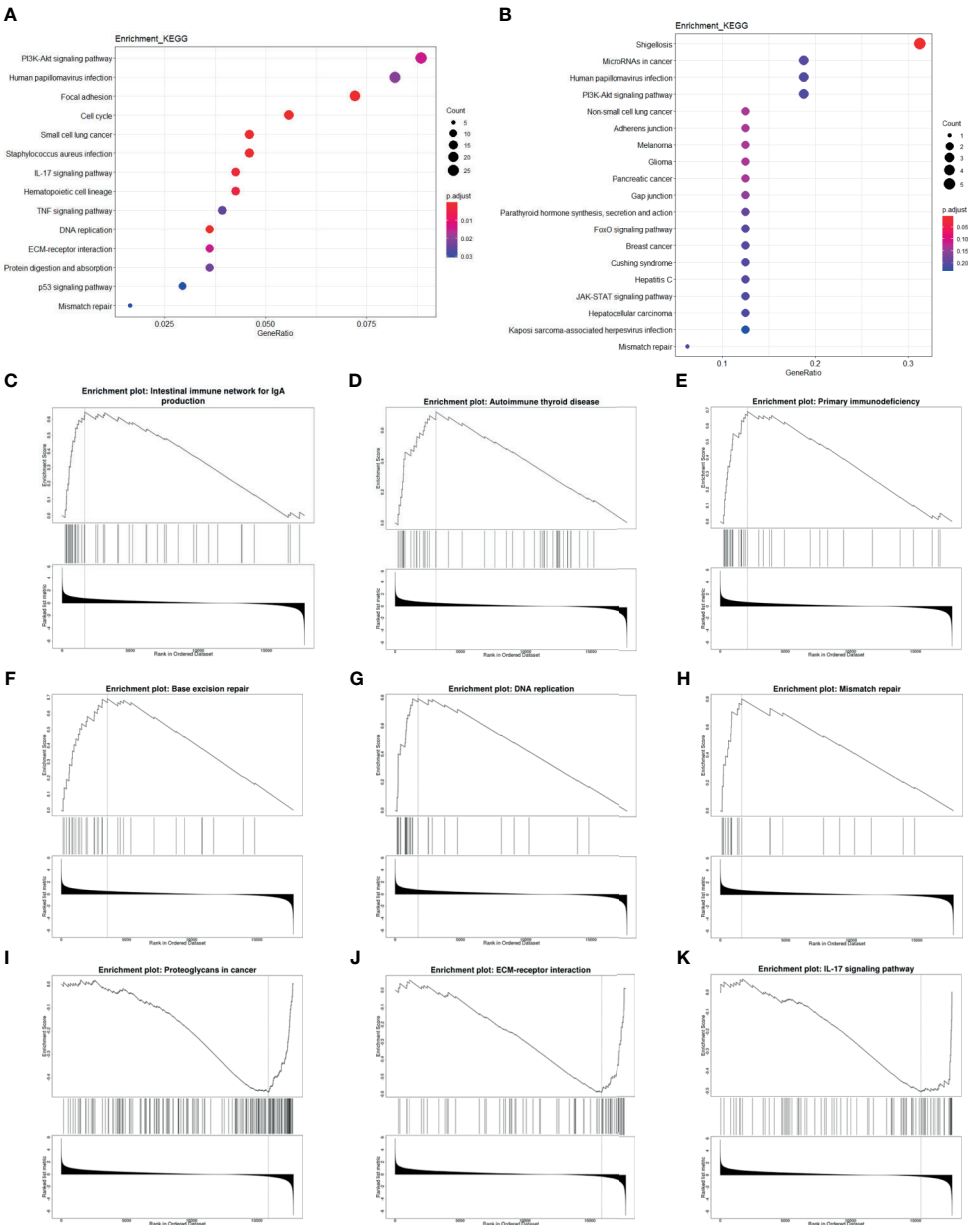

FIGURE 6 | Enrichment analysis of mRNA and miRNA targets. **(A, B)** Top KEGG-enriched terms of differentially expressed genes and differentially expressed miRNA target genes, respectively. **(C–K)** GSEA-enriched terms, in which genes were sorted by log2FC.

various types of epidermal tissues, CESC and HNSC are the most common types of HPV-induced squamous cell carcinoma. Considering the representative data and limited data volume, we combined both CESC and HNSC data into our study. Also, since our study focuses on HPV-induced tumorigenesis rather than ontogenesis, we merged the two cancer data for analysis instead of separate analysis. All HNSC samples included in this study were oropharynx carcinomas, which avoids the influence of non-HPV-associated HNSCs on the final results.

Recent reports revealed that HPV+ patients show better prognosis than HPV− patients in certain types of tumor (23). In addition, our study is consistent with this result despite merging data from two different types of cancer. It implies that the better prognosis of HPV+ tumor is consistent across different tumor types. This effect is probably related to the difference in the expression pattern of prognosis-associated genes. Although, different in mechanisms, both HPV− and HPV+ tumors express similar pattern in tumor-associated genes. Virus infection influence host gene expression pattern mainly through direct regulation by virus-derived proteins, and this process rarely induce gene mutations (24). The tumors that are not driven by virus usually show mutations at oncogenes and/or tumor suppressor genes. In the total of 84 HPV-regulated genes, only four genes (EGFR, SNF, UBD, and VCAM1) were differentially expressed when compared with HPV− tumor. Among the four genes, SNF and VCAM1 directly interact with EGFR through string estimation; this suggests that HPV-regulated DEG tends to interact, and that they participate in similar biological processes that affect patient's survival.

Our RNA-Seq PCA showed that HPV+ tumors are distributed in different areas against HPV− tumor at the first principal component. Although the dispersion within group is not obvious, we believe that HPV infection status probably influences gene expression more than the primary tumor site. The 834 DEGs obtained from differentially expressed analysis further confirm that gene expression patterns of HPV+ tumors are different from HPV− tumors. Although the clustering analysis showed that the clustering of samples was basically the same as that of HPV infection, a small number of HPV+ and HPV− clustered together. There are two possible reasons for this phenomenon. First, the HPV expression level of the samples may be very low, which may induce their gene expression patterns closer to that of HPV− tumors. Second, these samples were probably infected by low-risk HPV that rarely induce cancer, which implies that their oncogenesis ability is relatively lower. In spite of diversity in mRNA expression, miRNA expression between HPV+ and HPV− shows little differences. However, based on the enrichment analysis of DEmiRs target genes, we showed that there are several differences in tumor-related pathways of HPV+ and HPV− cancers. One possible reason for this is that only a small percentage of miRNAs are differentially expressed in HPV+ and HPV− tumors. In addition, these differentially expressed miRNAs are highly correlated with tumor-related biological processes.

Since certain mutations occur across different cancer types (25), we therefore focus on differences in mutation types between HPV+ and HPV− tumors. Our study showed that TP53 mutation rate in HPV+ tumor is dramatically lower than in HPV− tumor. TP53 is an important tumor suppressor gene. The mutation of tumor suppressor gene is considered more serious than its dysfunction. HPV+ cancer patients showed better outcome than HPV− cancer patients and that can probably be attributed to low TP53 mutation rate (7, 26). We also showed that genes that belong to the same family or participate in the same pathway have higher mutation rate. For instance, mucin glycoprotein-encoded genes MUC16, MUC17, and MUC4 ranked among the top 30 mutated genes. This suggests that mucin glycoprotein mutation is a signature of HNSC and CESC (27). Moreover, MUC4 mutation rate in HPV+ samples are obviously higher compared with HPV− samples. We believe that mucin glycoprotein subtype and their mutation rates could be a latent biomarker for tumor classification.

Virus protein expression and regulation of biological function are usually diverse in HPV infection stage. For HPV, functional proteins like E1, E2, E5, E6, and E7 are highly expressed at early infection stage. Using these early stage proteins, HPV can hijack DNA and protein synthesis machinery of the cell for self-proliferation. At the final stage, HPV capsid proteins, L1 and L2, are expressed for virus assembly and escape from the cell (28). Reports suggest that, E2, an early-stage protein, possibly participates in the late stage of HPV replication by activating DNA damage response (29). Our result shows that, EGFR is regulated by HPV oncoprotein E7 and that it takes part in E2-related regulation unit. This suggests that EGFR is involved in E2-regulated DNA damage response (30). Also, DNA-related functions of HPV+ cancers are significantly activated compared with that of HPV− cancers. Beside HPV proteins, differentially expressed miRNAs also participate in EGFR regulation. Although differentially expressed miRNAs and their targets are rare, it is not accidental that differentially expressed miRNA targets EGFR (hypergeometric test $p < 0.001$).

Since EGFR is a potent oncogene, EGFR dysregulation will cause several forms of cancer. High proportion of nonsmall-cell lung carcinomas expresses EGFR and the EGFR mutant as its signature (31). Likewise, EGFR has become a biomarker of HNSC (15). In our study, EGFR shows different express pattern for HPV+ and HPV− cancers. EGFR is a critical receptor that transduces epithelium growth and developmental signal into the cells. It plays an important role in epithelial stem cell division and differentiation. HPV does not only infect the basal cells of epithelium tissues but it also requires epithelial development for its replication. Hence, regulation of basal cell differentiation is a crucial control strategy for HPV replication. On the other hand, HPV− cancers do not require epithelium differentiation for its replication. Therefore, EGFR downregulation is a possible potential strategy that targets HPV-specific lifecycle. Tyrosine-phosphorylated EGFR is the activated form of EGFR. Only the activated form of EGFR can serve as a receptor and receive extracellular signals. Downregulation of the overall expressed EGFR can possibly decrease the expression of phosphorylated EGFR, thus inhibiting the downstream signaling pathway of EGFR (32). It has been reported that continuous

hyperfractionated accelerated radiotherapy is more effective for HNSCs with high EGFR expression than HNSCs with low EGFR expression (33). Considering that HPV+ cancer induces higher EGFR express level upon study provides us a potential specific HPV+ cancer therapeutic method.

Although, there are limitations to this study. We only considered the effect of HPV infection on tumors but ignored the potential effect of different subtypes of HPV (HPV-16, HPV-33, HPV-18) on gene expression. Whether EGFR expression level is higher in some HPV subtypes or lower in other subtypes during infection still need to be explored.

In summary, HPV+ cancer is significantly different from HPV- cancer in many aspects like DNA mutation, mRNA and protein expression. The initiation of cancer in HPV+ cells results from the regulation of biological processes related to host development by viral proteins. In contrasts, HPV− cancer is activated by several categories of risk factors and is highly related to TP53 mutation. Although distinct in mechanisms, both HPV+ and HPV− cancers are triggered by onco-related gene dysregulation. This study showed that EGFR is possibly the core molecule that affects immune and cancer-related biological processes and it can eventually cause prognosis differences between HPV+ and HPV− cancers.

4 MATERIALS AND METHODS

4.1 Data Source

CESC and HNSC RNA-Seq read counts data of the PCAWG project were obtained from the ICGC database (https://icgc.org/). In the PCAWG project, information on HPV infection in each of the samples was from a study recently published by Zapatka et al. (34). CaPSID, P-DIP, and SEPATH pipelines were used for detection of HPV reads from PCAWG samples in the study by Zapatka, and samples with HPV reads that were detected in at least two pipelines were considered infected. After screening, 20 CESC samples (19 HPV+, one HPV−) and 41 HNSC samples (17 HPV+, 24 HPV−) were included in this study. Simple nucleotide variation (SNV) data, including 305 CESC samples and 510 HNSC samples, are from Genomic Data Commons Data portal (GDC, http://portal.gdc.cancer.gov/). The source of reverse-phase protein array (RPPA) level 3 data and clinical data of 173 CESC samples and 212 HNSC samples were obtained from the FireBrowse database (http://firebrowse.org/).

4.2 Principal Component Analysis

PCA was applied for data dimension reduction. Sample distribution confidence intervals of each sample groups were displayed. The samples located far away from confidence interval ellipse were considered outliers and were deleted in the subsequent exploration.

4.3 Differentially Expressed Analysis

Limma package of R was used for RNA-Seq data and miRNA-Seq data differentially express analysis. DEGs and DEmiRs were identified by *p. adjust.* <0.05 and |log2FC| >1.2. BH method was applied for *p*-value adjustment. Student's *t*-test was used to analyze differentially expressed RPPA data. Proteins with *p-value* <0.05 were regarded as differentially expressed proteins (DEPs).

4.4 Survival Analysis

Clinical data from FireBrowse database were used for the prediction in survival differences of the two groups. To compare the data from the HPV+ tumor patients and HPV− tumor patients, Kaplan-Meier method was used for survival rate prediction and Kaplan-Meier plot (KM plot) was used to determine the survival curve. COX proportional hazard regression model was used to predict the correlation between differentially expressed protein (from RPRA data) and HPV+ patients' survival time. $p < 0.05$ was considered to significantly correlate with survival time, HR >1 was considered positive correlation with survival time, and HR <1 was considered to be negative correlation with survival time.

4.5 miRNA Target Prediction

TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/) database were used for differentially expressed miRNA target prediction. Targets that appeared in both databases were considered well predicted. RIsearch2 software was used for further verification of specific interesting miRNA-mRNA interaction.

4.6 SNV Analysis

Single nucleotide variation (SNV) data of CESC and HNSC project were downloaded from the TCGA GDC data portal. The processed MAF data used in this study was downloaded from MUSE software processed. Maftools R package was used for MAF file data mining. Maftools was likewise used for statistical analysis of gene mutation and data visualization.

4.7 HPV-miRNA-mRNA Network Construction and Analysis

HPV-human protein interaction prediction data were downloaded from P-HIPSTer (http://phipster.org/), a database that predicts virus-human protein interactions based on structural information. Differentially expressed mRNA interactions were predicted by String database (https://string-db.org/). MCODE plugin of Cytoscape (version 3.7.0) was used for network module identification. The parameters for MCODE were set as default. The degree of calculation was determined by NetworkAnalyzer, and genes with degree higher than the threshold were defined as hub genes.

4.8 Enrichment Analysis

KEGG pathway enrichment analysis was determined using R package clusterProfiler. Hypergeometric test was used for terms

significance testing. The *p. adjust.* <0.2 was set as the threshold of significantly enriched terms. Webtools WEBGESTALT (http://www.webgestalt.org/) was used for GSEA enrichment analysis and result visualization (35).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JQ, FH, and YW conceived the study. JQ, FH, and YW designed the experiment. JQ performed the experiment and computational analysis. YG, ZD, HN, and YC collected the data. JQ wrote the manuscript. OO, WZ, YQ, and LL provided valuable suggestions to improve the manuscript. TS and ZZ provided the professional consulting support. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Desrichard A, Kuo F, Chowell D, Lee KW, Riaz N, Wong RJ, et al. Tobacco Smoking-Associated Alterations in the Immune Microenvironment of Squamous Cell Carcinomas. *J Natl Cancer Inst* (2018) 110(12):1386–92. doi: 10.1093/jnci/djy060
2. Mirzaei H, Goudarzi H, Eslami G, Faghihloo E. Role of Viruses in Gastrointestinal Cancer. *J Cell Physiol* (2018) 233(5):4000–14. doi: 10.1002/jcp.26194
3. Nakagawa M, Nawa N, Takeichi E, Shimizu T, Tsuchiya J, Sato A, et al. Mac-2 Binding Protein Glycosylation Isomer as a Novel Predictive Biomarker for Patient Survival After Hepatitis C Virus Eradication by DAAs. *J Gastroenterol* (2020) 55(10):990–9. doi: 10.1007/s00262-020-02681-x
4. Chiramel AI, Best SM. Role of Autophagy in Zika Virus Infection and Pathogenesis. *Virus Res* (2018) 254:34–40. doi: 10.3390/ijms19061775. Human Papilloma Virus and Autophagy. Int J Mol Sci 19(6).
5. Rawle DJ, Li D, Wu Z, Wang L, Choong M, Lor M, et al. Oxazole-Benzenesulfonamide Derivatives Inhibit HIV-1 Reverse Transcriptase Interaction With Cellular Eef1a and Reduce Viral Replication. *J Virol* (2019) 93(12):e239–e219. doi: 10.1128/jvi.00239-19
6. Shaikh MH, Bortnik V, McMillan NA, Idris A. cGAS-STING Responses are Dampened in High-Risk HPV Type 16 Positive Head and Neck Squamous Cell Carcinoma Cells. *Microb Pathog* (2019) 132:162–5. doi: 10.1016/j.micpath.2019.05.004
7. Li S, Hong X, Wei Z, Xie M, Li W, Liu G, et al. Ubiquitination of the HPV Oncoprotein E6 Is Critical for E6/E6AP-Mediated P53 Degradation. *Front Microbiol* (2019) 10:2483–93. doi: 10.3389/fmicb.2019.02483
8. Budhwani M, Lukowski SW, Porceddu SV, Frazer IH, Chandra J. Dysregulation of Stemness Pathways in HPV Mediated Cervical Malignant Transformation Identifies Potential Oncotherapy Targets. *Front Cell Infect Microbiol* (2020) 10:307. doi: 10.3389/fcimb.2020.00307
9. Hemmat N, Bannazadeh Baghi H. Association of Human Papillomavirus Infection and Inflammation in Cervical Cancer. *Pathog Dis* (2019) 77(5):ftz048. doi: 10.1093/femspd/ftz048
10. Hoppe-Seyler K, Bossler F, Braun JA, Herrmann AL, Hoppe-Seyler F. The HPV E6/E7 Oncogenes: Key Factors for Viral Carcinogenesis and Therapeutic Targets. *Trends Microbiol* (2018) 26(2):158–68. doi: 10.1016/j.tim.2017.07.007
11. Sher YP, Lee C, Liu SY, Chen IH, Lee MH, Chiu FF, et al. A Therapeutic Vaccine Targeting HPV E6/E7 With Intrinsic Toll-Like Receptor 2 Agonist Activity Induces Antitumor Immunity. *Am J Cancer Res* (2018) 8(12):2528–37.
12. Becker M, Greune L, Schmidt MA, Schelhaas M. Extracellular Conformational Changes in the Capsid of Human Papillomaviruses Contribute to Asynchronous Uptake Into Host Cells. *J Virol* (2018) 92(11):e02106–17. doi: 10.1128/jvi.02106-17
13. Henderson S, Chakravarthy A, Su X, Boshoff C, Fenton TR. APOBEC-Mediated Cytosine Deamination Links PIK3CA Helical Domain Mutations to Human Papillomavirus-Driven Tumor Development. *Cell Rep* (2014) 7(6):1833–41. doi: 10.1016/j.celrep.2014.05.012
14. Kobayashi K, Hisamatsu K, Suzui N, Hara A, Tomita H, Miyazaki T. A Review of HPV-Related Head and Neck Cancer. *J Clin Med* (2018) 7(9):241. doi: 10.3390/jcm7090241
15. Pectasides E, Rampias T, Sasaki C, Perisanidis C, Kouloulias V, Burtness B, et al. Markers of Epithelial to Mesenchymal Transition in Association With Survival in Head and Neck Squamous Cell Carcinoma (HNSCC). *PloS One* (2014) 9(4):e94273. doi: 10.1371/journal.pone.0094273
16. Devaraja K, Aggarwal S, Verma SS, Gupta SC. Clinico-Pathological Peculiarities of Human Papilloma Virus Driven Head and Neck Squamous Cell Carcinoma: A Comprehensive Update. *Life Sci* (2020) 245:117383. doi: 10.1016/j.lfs.2020.117383
17. Ilahi NE, Bhatti A. Impact of HPV E5 on Viral Life Cycle via EGFR Signaling. *Microb Pathog* (2020) 139:103923. doi: 10.1016/j.micpath.2019.103923
18. Keck MK, Zuo Z, Khattri A, Stricker TP, Brown CD, Imanguli M, et al. Integrative Analysis of Head and Neck Cancer Identifies Two Biologically Distinct HPV and Three non-HPV Subtypes. *Clin Cancer Res* (2015) 21(4):870–81. doi: 10.1158/1078-0432.Ccr-14-2481
19. Taberna M, Torres M, Alejo M, Mena M, Tous S, Marquez S, et al. The Use of HPV16-E5, EGFR, and pEGFR as Prognostic Biomarkers for Oropharyngeal Cancer Patients. *Front Oncol* (2018) 8:589. doi: 10.3389/fonc.2018.00589
20. He C, Mao D, Hua G, Lv X, Chen X, Angeletti PC, et al. The Hippo/YAP Pathway Interacts With EGFR Signaling and HPV Oncoproteins to Regulate Cervical Cancer Progression. *EMBO Mol Med* (2015) 7(11):1426–49. doi: 10.15252/emmm.201404976
21. Basto DL, Chaves CBP, Felix SP, Amaro-Filho SM, Vieira VC, Martins LFL, et al. The Papillomavirus E5 Gene Does Not Affect EGFR Transcription and Overall Survival in Cervical Cancer. *J Med Virol* (2020) 92(8):1283–9. doi: 10.1002/jmv.25624
22. House R, Majumder M, Janakiraman H, Ogretmen B, Kato M, Erkul E, et al. Smoking-Induced Control of miR-133a-3p Alters the Expression of EGFR and HuR in HPV-Infected Oropharyngeal Cancer. *PloS One* (2018) 13(10):e0205077. doi: 10.1371/journal.pone.0205077
23. Wookey VB, Appiah AK, Kallam A, Ernani V, Smith LM, Ganti AK. HPV Status and Survival in Non-Oropharyngeal Squamous Cell Carcinoma of the Head and Neck. *Anticancer Res* (2019) 39(4):1907–14. doi: 10.21873/anticanres.13299
24. Shanmugasundaram S, You J. Targeting Persistent Human Papillomavirus Infection. *Viruses* (2017) 9(8):229. doi: 10.3390/v9080229
25. Wang B, Li X, Liu L, Wang M. β-Catenin: Oncogenic Role and Therapeutic Target in Cervical Cancer. *Biol Res* (2020) 53(1):33. doi: 10.1159/000495680

26. Scheel A, Bellile E, McHugh JB, Walline HM, Prince ME, Urba S, et al. Classification of TP53 Mutations and HPV Predict Survival in Advanced Larynx Cancer. *Laryngoscope* (2016) 126(9):E292–299. doi: 10.1002/lary.25915
27. Kannan A, Hertweck KL, Philley JV, Wells RB, Dasgupta S. Genetic Mutation and Exosome Signature of Human Papilloma Virus Associated Oropharyngeal Cancer. *Sci Rep* (2017) 7:46102. doi: 10.1097/pas.0000000000000543
28. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The Biology and Life-Cycle of Human Papillomaviruses. *Vaccine* (2012) 30(Suppl 5):F55–70. doi: 10.1016/j.vaccine.2012.06.083
29. Graham SV. Human Papillomavirus E2 Protein: Linking Replication, Transcription, and RNA Processing. *J Virol* (2016) 90(19):8384–8. doi: 10.1016/j.semcancer.2013.12.003. Banerjee, N.S., Moore, D., Parker, C.J., Broker, T.R., and Chow, L.T. (2019). Targeting DNA Damage Response as a Strategy to Treat HPV Infections. Int J Mol Sci 20(21). doi: 10.3390/ijms20215455.
30. Khan K, Valeri N, Dearman C, Rao S, Watkins D, Starling N, et al. Targeting EGFR Pathway in Metastatic Colorectal Cancer- Tumour Heterogeniety and Convergent Evolution. *Crit Rev Oncol Hematol* (2019) 143:153–63. doi: 10.1016/j.critrevonc.2019.09.001
31. da Cunha Santos G, Shepherd FA, Tsao MS. EGFR Mutations and Lung Cancer. *Annu Rev Pathol* (2011) 6:49–69. doi: 10.1038/s41416-019-0573-8
32. Bossler F, Hoppe-Seyler K, Hoppe-Seyler F. PI3K/AKT/mTOR Signaling Regulates the Virus/Host Cell Crosstalk in HPV-Positive Cervical Cancer Cells. *Int J Mol Sci* (2019) 20(9):2188. doi: 10.3390/ijms20092188
33. Bentzen SM, Atasoy BM, Daley FM, Dische S, Richman PI, Saunders MI, et al. Epidermal Growth Factor Receptor Expression in Pretreatment Biopsies From Head and Neck Squamous Cell Carcinoma as a Predictive Factor for a Benefit From Accelerated Radiation Therapy in a Randomized Controlled Trial. *J Clin Oncol* (2005) 23(24):5560–7. doi: 10.1200/jco.2005.06.411
34. Zapatka M, Borozan I, Brewer DS, Iskar M, Grundhoff A, Alawi M, et al. The Landscape of Viral Associations in Human Cancers. *Nat Genet* (2020) 52 (3):320–30. doi: 10.1038/s41588-019-0558-9
35. Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: Gene Set Analysis Toolkit With Revamped UIs and APIs. *Nucleic Acids Res* (2019) 47 (W1):W199–w205. doi: 10.1093/nar/gkz401

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