INFECTIOUS CAUSES OF CANCER

The unveiled reality of human papillomavirus as risk factor for oral cavity squamous cell carcinoma

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Abstract

The prognostic impact of human papillomavirus (HPV) in oropharyngeal cancer is generally acknowledged, and HPV-status is assessed routinely in clinical practice. Paradoxically, while the oral cavity seems the predilection site for productive HPV-infections, figures on HPV-attribution in oral cavity squamous cell carcinoma (OCSCC) differ widely, and prognostic impact is uncertain. Major obstacles are the lack of reproducible assays to detect HPV in nonoropharyngeal cancers, the relatively small cohorts studied and consequently the shortfall of convincing data. In our study, we used a validated, nucleic acid-based workflow to assess HPV-prevalence in a consecutive cohort of 1016 OCSCCs, and investigated its prognostic impact. In parallel, we analyzed p16-immunohistochemistry (p16-IHC) as surrogate marker for transforming HPV-infection and independent prognosticator. All OCSCC-patients diagnosed between 2008 and 2014 at two Dutch university medical centers were included (N = 1069). Formalin-fixed, paraffin-embedded (FFPE)-samples of 1016 OCSCCs could be retrieved. Punch biopsies were taken from the tumor area in the FFPE-blocks and tested for HPV. P16-IHC was performed on 580 OCSCCs, including all HPV-positive tumors. From 940 samples (92.5%), nucleic acids were of sufficient quality for HPV-testing. In total, 21 (2.2%) OCSCCs were HPV DNA-positive. All HPV DNA-positive tumors were E6 mRNA-positive and considered as true HPV-positive. There was no difference in survival between HPV-positive and HPV-negative OCSCCs. In total, 46 of 580 (7.9%) OCSCCs were p16-immunopositive, including all HPV-positive tumors. Survival was comparable in p16-positive and p16-negative OCSCCs. To conclude, HPV-prevalence is very low in OCSCC and neither HPV-status nor p16-status affects outcome. Based on these data, determining HPV-status in OCSCC seems irrelevant for clinical management.

1 | INTRODUCTION

Infection with high-risk human papillomavirus (HPV) is an important risk factor for head and neck squamous cell carcinoma (HNSCC). Although HPV-attribution varies between geographical regions, the oropharynx is consistently identified as the head and neck site with the highest frequency of HPV-related squamous cell carcinomas (SCCs), particularly in the Western world.[1-3] Within the oropharynx, HPV-related tumors are most abundant in the lingual tonsils and base of tongue,[4] possibly due to the reticular crypt epithelial cells that may have a high oncogenic transforming potential.[5] Compared to HPV-negative tumors, HPV-related oropharyngeal squamous cell carcinomas (OPSCCs) differ in both patient and tumor characteristics,[6,7] and have a significantly more favorable prognosis when treated with standard of care chemoradiotherapy.[8,9] HPV-related OPSCCs are therefore considered a distinct disease entity with a separate UICC TNM classification since the eighth edition.[10]

Although the prevalence and prognostic impact of HPV in OPSCC is undisputable, its role in nonoropharyngeal HNSCCs remains unclear. In the hypopharynx and larynx, the general consensus is that the proportion of HPV-positive tumors seems relatively low (1.6%-6.4% and 1.6%-7.9%, respectively).[1,11-13] The major issue lies within oral cavity squamous cell carcinomas (OCSCCs). Significant differences in HPV-attribution for OCSCCs have been reported, ranging from 2.0% to frequencies as high as 61.0%.[1,2,12,14-22] Apart from geographical variation and a relative increase in HPV-prevalence over time, several other factors might specifically contribute to this discordance. First of all, tumor site misclassification between the oral cavity and oropharynx may play a role in literature. Carcinomas arising from the oropharynx are occasionally referred to as oral cancers, and the base of tongue is sometimes considered part of the oral cavity,[23] resulting in higher HPV-attributable fractions than truly is the case. As the anatomical sites have been well-defined,[23] this should be easy to avoid. Nevertheless, larger tumors that overlap between the oropharynx and oral cavity may have found its origin in either of these sites, and thereby may confound figures.

Most importantly, however, is that there is no universal HPV-testing method available for nonoropharyngeal HNSCCs. The various HPV DNA polymerase chain reaction (PCR)-assays that are typically used are generally developed for cervical cancer screening and prevalence studies of cervical HPV-infection. These very sensitive assays may detect not only transforming HPV-infections, but also HPV DNA from productive infections. These assays are also more susceptible to contamination, leading to false-positive results.[24,25] Detection of HPV E6/E7 transcripts is therefore considered the gold standard for transforming infections in all HNSCC sites,[26] but this test is generally not feasible in daily practice as regular RNA-based assays usually demand fresh-frozen tumor material, which is not routinely collected. Therefore, alternative methods have been proposed. For OPSCC, a test

What's new

While human papillomavirus (HPV)-infection is prognostic for oropharyngeal squamous cell carcinoma (OCSCC), its role and prognostic impact in OCSCC remain uncertain. In the present study, nucleic acid-based HPV-testing of a cohort of 1016 tumor samples collected from OCSCC patients diagnosed between 2008 and 2014 reveals very low HPV-prevalence in OCSCC. In addition, no difference was found in survival between HPV-positive and HPV-negative tumors. The findings indicate that HPV is uncommon in OCSCC and, when present, has no impact on survival, providing new and important insight into the ongoing debate regarding the role of HPV in OCSCC.

algorithm for formalin-fixed, paraffin-embedded (FFPE) specimen was developed by Smeets et al, consisting of p16-immunohistochemistry (p16-IHC), followed by high-risk HPV DNA-detection with GP5+/6 +-PCR on p16-immunopositive cases.[26] The p16 protein, formally p16^{Ink4A}, is encoded by the *CDKN2A* gene, a frequently inactivated tumor suppressor gene in HPV-negative HNSCC. It typically shows increased expression in HPV-positive tumors as a functional consequence of HPV E7 oncogene signaling, making it an excellent surrogate marker for a transforming HPV-infection, at least in OPSCC. Validation of the HPV test algorithm on an external cohort resulted in an accuracy of 98% when compared to detection of HPV E6 oncogene expression in frozen tumor specimens as gold standard for a transforming HPV-infection.[27] Unfortunately, this test algorithm does not perform well on OCSCCs with a positive-predictive value of only 41.3%.[16] In 2019, Mes et al developed a rapid and contamination-free HPV-test method suited for FFPE-material from both OPSCC and non-OPSCC, called the *HPV-rTcore workflow*.[28] This method circumvents sectioning of FFPE-specimens on the microtome by taking hemotoxylin and eosin (H&E)-guided core punch biopsies from the tumor area in the FFPE-blocks. From these biopsies, total nucleic acids are isolated, which are subsequently subjected to testing for the presence of HPV DNA by targeting the E7 region of 15 high-risk HPV-types, followed by testing for the presence of type-specific HPV E6 mRNA in case of an HPV DNA-positive test result. Validation of the *HPV-rTcore workflow* on independent cohorts of OPSCCs and OCSCCs resulted in an accuracy of near 100% when compared to the earlier mentioned gold standard of HPV E6 transcript detection on RNA isolated from fresh-frozen tumor specimens.[28]

The aim of our study was to determine the HPV-attributable fraction in a large consecutive cohort of OCSCCs by using the validated

HPV-rTcore workflow, and to investigate the prognostic impact of HPV-positivity in OCSCC. Although p16-IHC is considered an inadequate surrogate marker for a transforming HPV-infection in OCSCC,[16] a survival benefit of p16-positivity has been described for non-OPSCC irrespective of HPV-status.[12,29] The secondary aim of our study was therefore to assess the performance of p16-IHC as surrogate marker for a transforming HPV-infection, as well as its role as independent prognosticator in OCSCC.

2 | MATERIALS AND METHODS

2.1 | Study design

To determine the required sample size for our study, a sample size calculation was performed first. It was assumed that the true HPV-attributable fraction in OCSCC was between 5% and 10%, and therefore we assumed an HPV-proportion of 7.5%. With an α of .05, a β of .2, an HPV-proportion of 7.5%, a hazard of 2.0, 15% baseline events per year, a censoring rate of 10% per year and an average follow-up period of 2 years, we had to include 942 patients in the analysis. Therefore, it was decided to carry out this retrospective study on a consecutive cohort of TNM-8 stage I-IVC OCSCC patients diagnosed between 2008 and 2014 in Amsterdam UMC, location VUmc and Erasmus MC Rotterdam (N = 1069). Patients were identified through the Dutch Cancer Registries, and clinical data collected from the patient files. Squamous cell carcinomas of the external lip (ICD-10 codes C00.0-C00.2 and C00.6-C00.9) were excluded, while all other OCSCC subsites were included (ICD-10 codes C00.3-C00.5, C02.0-C02.3, C02.8-C05.0, C06.0-C06.9). Of note, the oral cavity is located directly adjacent to the oropharynx, so tumors arising in the oral cavity might expand to the oropharynx and vice versa, making it challenging to establish the site of origin when tumors cross the anatomical borders. Considering that the majority of HPV-positive head and neck cancers are detected in the oropharynx, site misclassification might result in inaccurate HPV-attributable fractions for OCSCCs. We therefore excluded all overlapping lesions of the oral cavity and oropharynx, either on basis of the corresponding ICD-10 codes (C05.8 "overlapping lesion of palate" and C14.8 "overlapping lesion of lip, oral cavity and pharynx," N = 7) or on basis of reports from physical examination including panendoscopy and/or imaging that the tumor crossed the anatomical border (N = 15). FFPE-tissue from 1016 of the 1069 tumors (95%) could be retrieved from the pathology archives. HPV-attributable fraction was assessed based on all 1016 OCSCC patients, while the effect of HPV-status on OCSCC prognosis was analyzed only on OCSCC patients treated with curative intent (N = 955).

Demographic and clinical characteristics, as well as survival outcomes were obtained from the patient files. A pack year was defined as smoking 20 cigarettes a day during 1 year. A unit year was defined as consuming one alcoholic unit a day during 1 year. Histopathological features of all tumors were scored by dedicated head and neck pathologists (E. Bloemena, S. Koljenović) according to the WHO Classification of Head and Neck Tumours. In a recently published article on HPV-positive tumors of the hypopharynx, larynx and oral cavity, three clinicopathological variants (keratinizing, nonkeratinizing and warty)[30] were described, and these classifications were scored as well.

2.2 | HPV-testing

HPV-status was determined on the FFPE-samples taken for routine diagnostic workup. HPV-status was assessed by the *HPV-rTcore workflow* as described by Mes et al.[28] In short, a core biopsy of 1 mm diameter was taken from the tumor area of each FFPE-specimen by guidance of the corresponding H&E-slide using disposable biopsy punches with a plunger system (Kai Europe GmbH, Solingen, Germany). DNA and RNA were simultaneously isolated from the core biopsies using the NucliSENS easyMag (bioMérieux, Marcy-l'Étoile, France). Each isolate was first tested for the presence of HPV DNA either using a quantitative real-time PCR assay targeting the E7 region of 15 high-risk HPV-genotypes: HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67 and 68 (HPV-Risk Assay [Self-screen B.V., Amsterdam, the Netherlands] on the ViiA 7 real-time PCR system [Applied Biosystems, Foster City, CA]; or using the equivalent QIAscreen HPV PCR Test [QIAGEN, Hilden, Germany] on the Rotor-Gene Q real-time PCR cycler [QIAGEN]).[31] Four fluorescent channels were used: one for HPV16, one for HPV18, one for the other 13 (probable) high-risk HPV-types and one for the human β-globin gene. The latter served as internal control to confirm the presence of DNA with sufficient quality for reliable HPV DNA test results. For the ViiA 7, threshold cycle threshold values (Ct-values) were ≤36 for all four channels.[28] For the Rotor-Gene Q, threshold Ct-values were ≤36 for HPV16 and HPV18, ≤33.5 for the other 13 (probable) high-risk HPV-types and ≤30 for β-globin. The two assay versions were calibrated against each other and are used in diagnostics.

All HPV DNA-positive OCSCC were subsequently tested for HPV type-specific E6 mRNA with assays specifically designed for FFPE (ie, degraded RNA; Supplementary Table 1). Genotype information was derived from the real-time PCR assay for HPV16 and HPV18, and from additional testing with GP5+6+-PCR luminex for samples that were reported positive for the other 13 (probable) high-risk HPV-types.[32] In addition, 50 HPV DNA-negative OCSCCs were tested for HPV16 E6 mRNA to exclude false-negative HPV DNA test results. An OCSCC was considered truly HPV-positive if both the HPV DNA and HPV E6 mRNA were demonstrated (Figure 1).

2.3 | p16-immunohistochemistry

To determine the reliability of p16-IHC as surrogate marker for a transforming HPV-infection in OCSCC, as well as its role as independent prognosticator, p16-IHC was performed on all HPV-positive OCSCCs and 559 randomly selected HPV-negative OCSCCs. P16-status was assessed by one of two experienced head and neck pathologists independently and without knowledge of HPV-status. Equivocal staining results were evaluated by the second head and neck pathologist. Similar as in OPSCC,[9] a cutoff value of ≥70% strong and diffuse nuclear and cytoplasmic immunostaining of p16 was used. All tumors that did not meet this criterion were considered p16-negative. Of note, it is not
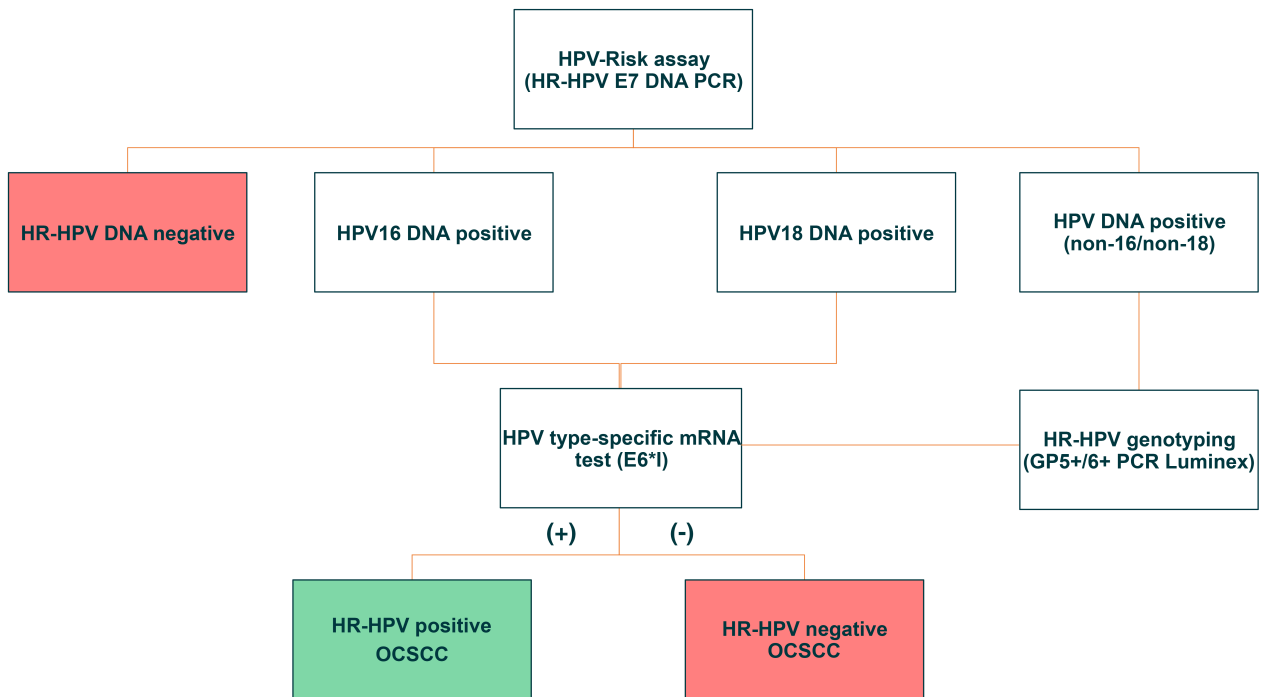
FIGURE 1 Human papillomavirus (HPV) test algorithm for oral cavity squamous cell carcinoma (OCSCC) [Color figure can be viewed at wileyonlinelibrary.com]

known whether this cutoff value, which was specifically defined for OPSCC, is valid for OCSCC as well. We therefore did not only score each tumor as being either p16-negative or p16-positive, but we paid extra attention to potential variations in staining pattern.

2.4 | Clinical endpoints

Clinical endpoints for our study were based on the "Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics" by the Food and Drug Administration (https://www.fda.gov/media/71195/download) and included 5-year overall survival (OS) and disease-free survival (DFS). OS was defined as the time from histologically confirmed OCSCC diagnosis until death from any cause. DFS was defined as the time from histologically confirmed OCSCC diagnosis until disease recurrence or death from any cause. Disease recurrence included local recurrence (within 2 cm and within 3 years of diagnosis of the index tumor), regional recurrence (lymph node metastases in previously treated levels of the neck) and distant metastases. Patients with residual disease, delayed lymph node metastases or second primary tumors were censored at the respective dates.

2.5 | Statistical analysis

Differences in demographic and clinical characteristics between de HPV-positive and HPV-negative OCSCCs were determined using the Pearson chi-square test or Fisher's exact test for categorical variables, and the Student's t test for continuous variables. OS and DFS curves were visualized using Kaplan-Meier plots, and strata compared with

the long-rank test. P-values <.05 were considered statistically significant. Analyses were performed with IBM SPSS Statistics 26.

3 | RESULTS

3.1 | Prevalence of HPV in OCSCC

From the 1016 OCSCC samples tested for HPV DNA, 940 (92.5%) samples contained DNA of sufficient quality for a reliable HPV DNA test result as determined by successful detection of the human β-globin gene. From these 940 OCSCCs, 919 tumors were HPV DNA-negative and 21 (2.2%) were HPV DNA-positive, of which 17 (81.0%) for HPV16 (Figure 2). All HPV DNA-positive OCSCCs were subsequently tested for HPV type-specific E6 mRNA. All HPV DNA-positive tumors turned out to be HPV RNA-positive as well. To verify that the HPV-Risk Assay did not miss any HPV-positive OCSCC (ie, false negative HPV DNA test results), we additionally tested 50 randomly selected HPV DNA-negative OCSCC and tested these for HPV16 mRNA, as HPV16 is the HPV type most frequently detected. All HPV DNA-negative cases were confirmed to be HPV16 mRNA-negative.

3.2 | Demographic and clinical characteristics of HPV-negative and HPV-positive OCSCCs

Demographic and clinical characteristics of the HPV-negative and HPV-positive OCSCCs are depicted in Table 1. Patients with HPV-positive OCSCCs were more frequently male (95.2% vs 57.8%, P < .001) and younger than patients with HPV-negative carcinomas (59 vs 64 years,

Attributable fraction of high-risk HPV in oral cavity squamous cell carcinoma is 2.2%
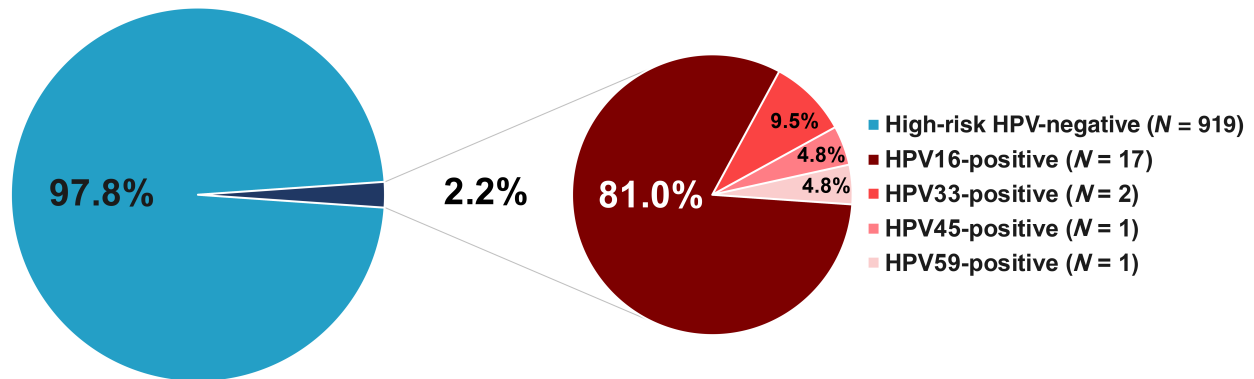

FIGURE 2 Prevalence of high-risk human papillomavirus (HPV) in oral cavity squamous cell carcinoma (OCSCC) [Color figure can be viewed at wileyonlinelibrary.com]

$P = .05$). Although the majority of HPV-negative tumors were located in the mobile tongue, more than half of the HPV-positive tumors were found in the floor of the mouth. In total, 49.7% of HPV-negative tumors were treated with surgery alone and did not receive adjuvant treatment, whereas almost half of the HPV-positive tumors received postoperative radiotherapy, and 14.3% were treated with definitive chemoradiotherapy compared to 2.0% of the HPV-negative tumors.

3.3 | Pathological characteristics of HPV-negative and HPV-positive OCSCCs

All available pathological features of the HPV-negative and HPV-positive OCSCCs are summarized in Table 2. No significant differences were observed between HPV-negative and HPV-positive tumors, except for pT stage.

3.4 | Clinicopathologic characterization of HPV-positive OCSCCs

In the light of the recently published article by Rooper et al,[30] we performed an additional morphologic analysis on the 21 HPV-positive OCSCCs. Six OCSCCs were nonkeratinizing and 14 OCSCCs were keratinizing. We identified four tumors resembling the warty variant as described by Rooper (Supplementary Figure 1). None of the HPV-positive OCSCCs had adjacent (Bowenoid) CIS.

3.5 | Prognostic impact of HPV in OCSCC

From the 940 patients with a valid HPV test result, 881 (93.7%) were treated with curative intent and were therefore included in the survival analysis. Median follow-up was 48 months. In Figure 3, the Kaplan-Meier curves of the OS (Figure 3A) and DFS (Figure 3B) are shown for HPV-negative (N = 861) and HPV-positive (N = 20) OCSCCs. Five-year OS did not significantly differ between HPV-

negative and HPV-positive OCSCCs (60.3% vs 45.3%, $P = .255$), and the same was true for 5-year DFS (52.7% vs 45.3%, $P = .685$).

3.6 | p16-status as surrogate marker for HPV in OCSCC

p16-IHC was performed on 580 OCSCCs including all 21 HPV-positive cases and 559 randomly selected HPV-negative cases. In total, 534 (92.1%) OCSCCs were p16-negative and 46 (7.6%) were p16-positive. All p16-negative OCSCCs were HPV-negative and all HPV-positive OCSCCs were p16-positive. From the 46 p16-positive OCSCCs, 25 were HPV-negative, resulting in a sensitivity, specificity, positive and negative predictive value of p16-IHC as surrogate marker for a transforming HPV-infection of 100%, 95.5%, 45.7% and 100%, respectively.

It was observed that the p16-staining pattern in OCSCC was more diverse than what is usually observed in OPSCC. OPSCCs are generally either completely p16-negative or convincingly p16-positive, with an obvious combination of nuclear and cytoplasmic immunoreactivity. In the current OCSCC cohort, however, some OCSCCs only showed nuclear immunostaining (Supplementary Figure 2A), while in other OCSCCs, the cytoplasm was exclusively immunopositive (Supplementary Figure 2B). Moreover, some tumors had weak immunostaining in the center of the tumor and strong immunostaining in the periphery (Supplementary Figure 2C). These varying patterns of immunostaining were not associated with the tumor differentiation grade or HPV DNA status (data not shown). Since these tumors did not meet the criterion for p16-positive immunostaining, they were considered p16-negative. All truly HPV-positive tumors displayed a convincing p16-immunostaining pattern with >70% of the tumor cells.

3.7 | p16-status as independent prognostic marker in OCSCC

In Figure 4, the Kaplan-Meier curves for 5-year OS (Figure 4A) and DFS (Figure 4B) are shown for p16-negative and p16-positive OCSCCs.

TABLE 1   Demographic and clinical characteristics of the cohort, as stratified by HPV-status

		HPV-negative OCSCCs (N = 919)			HPV-positive OCSCCs (N = 21)			
		Mean (SD)	N	%	Mean (SD)	N	%	P value
Age at diagnosis		64 (12)			59 (10)			.050[a]
Sex								<.001[b]
	Male		531	57.8		20	95.2	
	Female		388	42.2		1	4.8	
ACE-27								.397[b]
	None		287	31.2		10	47.6	
	Mild		303	33.0		4	19.0	
	Moderate		226	24.6		5	23.8	
	Severe		103	11.2		2	9.5	
Pack years		28 (26)			34 (20)			.146[a]
Unit years		131 (158)			167 (159)			.260[a]
Tumor location								.018[c]
	Mobile tongue		415	45.2		6	28.6	
	Floor of mouth		256	27.9		12	57.1	
	Vestibulum of mouth		122	13.3		0	0.0	
	Hard palate		12	1.3		1	4.8	
	Cheek mucosa		70	7.6		2	9.8	
	Retromolar trigone		44	4.8		0	0.0	
cT stage (TNM-8)								.096[c]
	Tis		9	1.0		1	4.8	
	T1		348	37.9		3	14.3	
	T2		290	31.6		9	42.9	
	T3		66	7.2		2	9.5	
	T4a		188	20.5		6	28.6	
	T4b		18	2.0		0	0.0	
cN stage (TNM-8)								.553[c]
	N0		723	78.7		16	76.2	
	N1		91	9.9		4	19.0	
	N2a		4	0.4		0	0.0	
	N2b		48	5.2		0	0.0	
	N2c		36	3.9		1	4.8	
	N3a		0	0.0		0	0.0	
	N3b		17	1.8		0	0.0	
Treatment received								.014[c]
	None		37	5.0		1	4.8	
	Surgery		457	49.7		5	23.8	
	Surgery + RT		309	33.6		10	47.6	
	Surgery + CRT		40	4.4		0	0.0	
	Radiotherapy		46	5.0		2	9.5	
	Chemoradiotherapy		18	2.0		3	14.3	
	Other		12	1.3		0	0.0	

Abbreviations: CRT, chemoradiotherapy; HPV, human papillomavirus; OCSCC, oral cavity squamous cell carcinoma; RT, radiotherapy.

[a]Mann-Whitney *U* test.

[b]Pearson chi-square.

[c]Fisher's exact test.

TABLE 2 Pathological characteristics of the cohort, as stratified by HPV-status

		HPV-negative OCSCCs (N = 919)			HPV-positive OCSCCs (N = 21)			
		Mean (SD)	N	%	Mean (SD)	N	%	P value
Tumor diameter (cm)		2.21 (1.46)			2.69 (1.97)			.518[a]
Tumor depth of invasion (cm)		0.82 (0.78)			1.01 (1.23)			.576[a]
Tumor pattern of invasion								.530[b]
	Cohesive		315	34.3		8	38.1	
	Noncohesive		450	49.0		8	38.1	
	Unknown		154	16.8		5	23.8	
Lymphovascular invasion								.327[c]
	No		658	71.6		13	61.9	
	Yes		116	12.6		2	9.5	
	Unknown		145	15.8		6	28.6	
Perineural invasion								.243[c]
	No		567	61.7		12	57.1	
	Yes		209	22.7		3	14.3	
	Unknown		143	15.6		6	28.6	
Differentiation grade								.238[c]
	Well differentiated		129	14.0		1	4.8	
	Moderately differentiated		481	52.3		16	76.2	
	Poorly differentiated		196	21.3		2	9.5	
	Unknown		113	12.3		2	9.5	
pT stage (TNM-8)								.048[c]
	T1		328	35.7		6	28.6	
	T2		228	24.8		1	4.8	
	T3		114	12.4		2	9.5	
	T4a		133	14.5		6	28.6	
	T4b		2	0.2		0	0.0	
	n.a. (no surgery)		110	12.0		6	28.6	
	Tx (not assessable)		4	0.4		0	0.0	
pN stage (TNM-8)								.794[c]
	N0		378	41.1		9	42.9	
	N1		89	9.7		1	4.8	
	N2a		29	3.2		0	0.0	
	N2b		43	4.7		1	4.8	
	N2c		14	1.5		1	4.8	
	N3a		0	0.0		0	0.0	
	N3b		83	9.0		2	9.5	
	n.a. (no neck dissection)		282	30.7		7	33.3	
	Nx (not assessable)		1	0.1		0	0.0	
Extranodal extension								1.000[c]
	No		524	57.0		12	57.1	
	Yes		112	12.2		2	9.5	
	n.a. (no neck dissection)		282	30.7		7	33.3	
	Unknown		1	0.1		0	0.0	
Surgical margins								.189[c]
	Clear (>5 mm)		373	40.6		5	23.8	

	HPV-negative OCSCCs (N = 919)			HPV-positive OCSCCs (N = 21)			
	Mean (SD)	N	%	Mean (SD)	N	%	P value
Close (1-5 mm)		248	27.0		6	28.6	
Involved (<1 mm)		158	17.2		4	19.0	
n.a. (no surgery)		110	12.0		6	28.6	
Unknown (not assessable)		30	3.3		0	0.0	

Abbreviations: HPV, human papillomavirus; OCSCC, oral cavity squamous cell carcinoma.
[a]Mann-Whitney U test.
[b]Pearson chi-square.
[c]Fisher's exact test.

Five-year OS did not significantly differ between p16-negative and p16-positive OCSCCs (58.2% vs 51.9%, P = .482), and the same was true for 5-year DFS (53.2% vs 41.9%, respectively; P = .262).

4 | DISCUSSION

To our knowledge, this is the first large study in which the attributable fraction of high-risk HPV in a consecutive cohort of almost a thousand OCSCCs from a European country is reported. We took several precautions to obtain the most accurate data. First, tumors crossing the anatomical borders of the oropharynx and oral cavity were excluded as this could impact both attributable fraction and prognosis. Next, we analyzed a large consecutive cohort of 940 cases to obtain convincing figures. HPV-attributions were comparable between Amsterdam UMC (2.0%) and Erasmus MC Rotterdam (2.4%) Third, HPV-testing was performed using the validated *HPV-rTcore workflow*,[28] with H&E-guided core punch biopsies taken from the identified tumor area with a disposable device. This enabled us to omit sectioning of FFPE-blocks on the microtome, which is laborious and carries the risk of sample cross-contamination. Although little literature has been published on molecular contamination of HPV during sectioning, this phenomenon is described for *Bartonella* and simian herpesviruses.[33,34]

HPV DNA-status was established with an E7-based assay, which overcomes potential false-negative results caused by viral integration with accompanying loss of HPV-regions targeted by most other HPV-assays, such as those targeting L1. The assay is validated to detect clinically relevant HPV-infection in the cervix,[31,35,36] and its sensitivity has been limited on purpose. All positive test results were verified by appropriate HPV type-specific mRNA-tests, strongly indicating that HPV DNA-assays can be used with optimally designed procedures, despite the fact that we did not wipe the surface of the blocks before punching. With ultrasensitive DNA-tests, such as the SPF-10, this picture might change. Now we observed a 100% concordance between DNA- and RNA-based testing, implying that additional mRNA-testing of HPV DNA-positive OCSCCs can be omitted when applying the DNA-assay used in our study.

The HPV-attribution in our OCSCC cohort was a mere 2.2%, which is lower than HPV-attributable fractions reported in several other studies. When exploring the existing literature, the prevalence of transcriptionally active HPV in OCSCC varies between 2.0% and 61%.[1,2,12,14-22] The HPV-attribution found in the current study likely relates to the strict criteria that were applied, but could also in part be explained by geographical variation. Castellsagué and colleagues reported data in OCSCC of 6.6% in Central-South America, 3.7% in Europe and 0% in Africa and Asia.[1] Hence, geographical variation does occur. Moreover, HPV-attribution could change in time as has been noted in OPSCC as well.[27] However, we did neither find variation between the two Dutch centers, nor any trend of increasing prevalence over time (Supplementary Figure 3).

Only few studies describe in detail the clinical characteristics of OCSCCs harboring transcriptionally active HPV. In their meta-analysis, Ndiaye et al found the highest HPV-prevalence within the oral cavity to be in carcinomas arising from the "palate not otherwise specified" (C05.0-9, HPV-prevalence 42.6%), the "gum" (C03.0-9, HPV-prevalence 39.9%) and the "tongue not otherwise specified (C01, C02.0-9, HPV-prevalence 28.0%).[2] Unfortunately, these HPV-attributions are based on HPV DNA PCR, and could therefore just as well reflect productive HPV-infections. On the other hand, Bussu et al tested 33 OCSCCs for the presence of HPV, and found one tumor to be HPV18 mRNA-positive.[14] This tumor was located in the floor of mouth. Lingen et al tested 409 OCSCC for the presence of transcriptionally active HPV, and found 24 (5.9%) OCSCCs to be HPV mRNA-positive. Nine (37.5%) of these tumors were located in the floor of mouth, six (25.0%) in the tongue, and four (16.7%) in the alveolar process.[16] Hernandez et al studied 122 OCSCC, of which 38 (31.1%) were HPV DNA-positive. Seventeen (45%) of HPV DNA-positive OCSCCs were located in the oral tongue and 10 (26%) in the floor of mouth.

In several studies, the effect of p16-status and/or HPV-status in OCSCCs was investigated. In some studies it was found that OS did not vary by HPV DNA-positivity, p16-positivity or joint positivity for both markers.[17,18,20] In contrast to the current study, two studies by Chung et al and Bryant et al found p16-status to play a comparable prognostic role in both oropharyngeal and nonoropharyngeal cancer, including OCSCC.[12,29]

Finally, we investigated the performance of p16-IHC as surrogate marker for a transforming HPV-infection, and assessed the
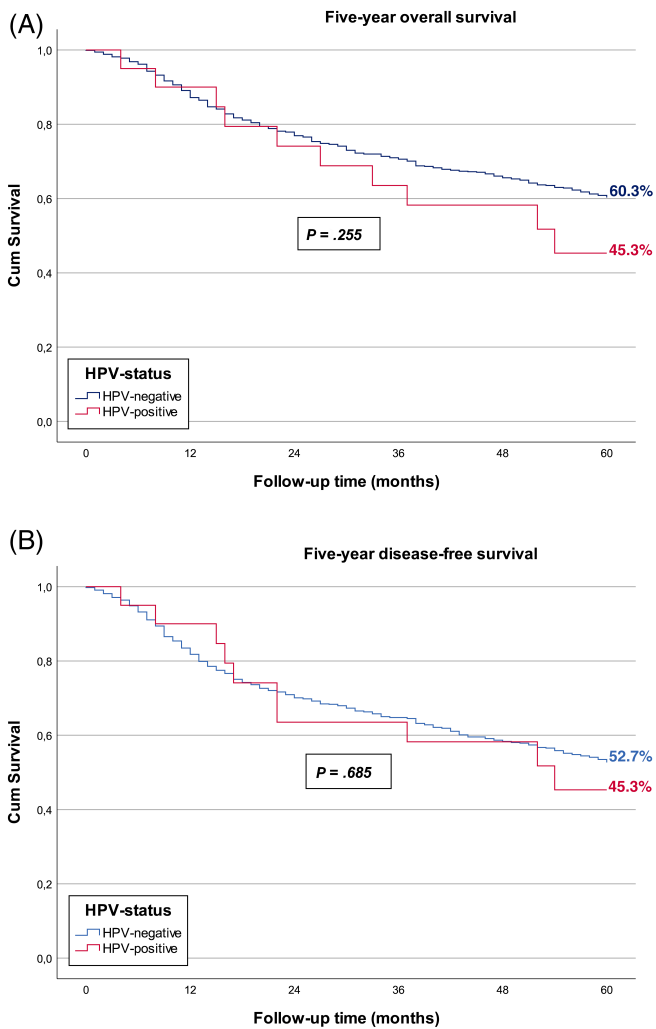
FIGURE 3 Kaplan-Meier curves of human papillomavirus (HPV)-negative and HPV-positive oral cavity squamous cell carcinomas (OCSCCs). A, Overall survival (OS) of HPV-negative (blue line, 5-year OS 60.3%) and HPV-positive (red line, 5-year OS 45.3%) OCSCC patients. Log-rank analysis showed no difference in OS between HPV-negative and HPV-positive OCSCCs (P = .255). B, Disease-free survival (DFS) of HPV-negative (blue line, 5-year DFS 52.7%) and HPV-positive (red line, 5-year DFS 45.3%) OCSCC patients. Log-rank analysis showed no difference in DFS between HPV-negative and HPV-positive OCSCCs (P = .685) [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Kaplan-Meier curves of p16-negative and p16-positive oral cavity squamous cell carcinomas (OCSCCs). A, Overall survival (OS) of p16-negative (blue line, 5-year OS 58.2%) and p16-positive (red line, 5-year OS 51.9%) OCSCC patients. Log-rank analysis showed no difference in OS between p16-negative and p16-positive OCSCCs (P = .482). B, Disease-free survival (DFS) of p16-negative (blue line, 5-year DFS 53.2%) and p16-positive (red line, 5-year DFS 41.9%) OCSCC patients. Log-rank analysis showed no difference in DFS between p16-negative and p16-positive OCSCCs (P = .262) [Color figure can be viewed at wileyonlinelibrary.com]

independent prognostic impact of p16-positivity in OCSCC. P16-IHC was carried out on 580 OCSCCs, of which 534 (92.1%) OCSCCs were p16-negative and 46 OCSCCs p16-positive. From the 46 p16-positive OCSCCs, 21 (45.7%) were HPV-positive and 25 (54.3%) were HPV-negative. To investigate whether the 25 p16-positive/HPV-negative OCSCCs had a false-positive p16-status or a false-negative HPV-status, all cases were additionally subjected to HPV16 mRNA-testing. All 25 OCSCCs remained HPV-negative when tested with for HPV16 mRNA-testing. The sensitivity, specificity, positive and negative predictive value of p16-status as surrogate marker for a transforming HPV-infection were 100%, 95.5%, 45.7% and 100%, respectively,
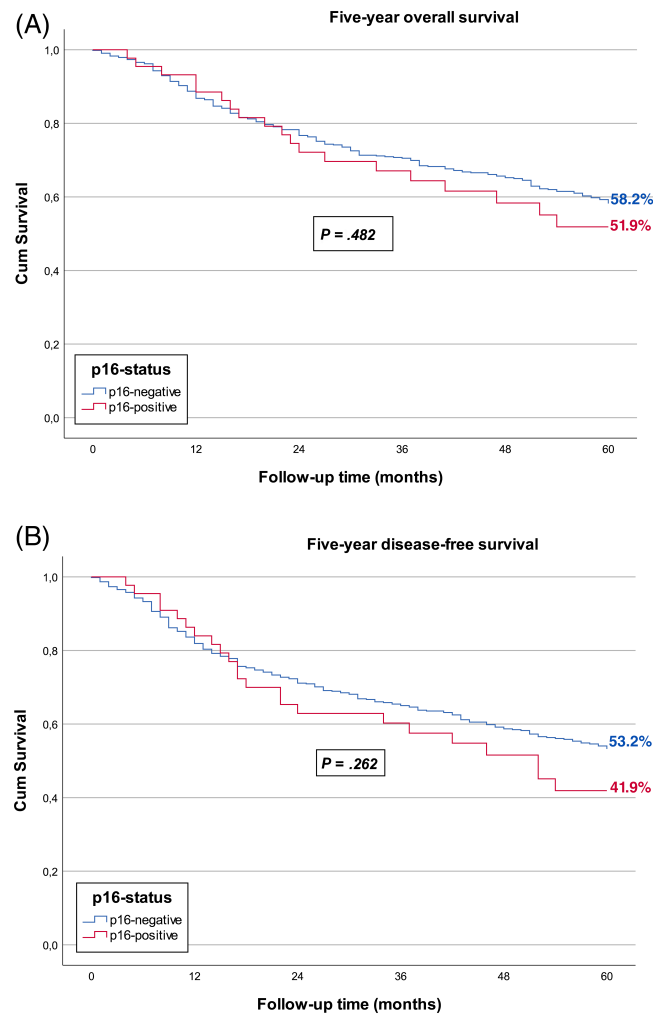
which is comparable to the values reported by Lingen et al.[16] Still, it should be acknowledged that the positive and negative predictive values of a test always relate to the disease prevalence rate in a population and may thus be different in other populations. Because of the low positive predictive value, p16-IHC alone is not sufficient to establish HPV-status in OCSCC. However, given the sensitivity and negative predictive value of 100%, it can be used as a first screening to determine which OCSCCs qualify for subsequent HPV-testing. Although some studies found a survival benefit of p16-positivity in OCSCC,[12,29] we did not find any impact of p16-status on prognosis. Our findings are in line with other studies investigating the prognostic potential of p16-status in OCSCC.[18] Of note, it should be

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acknowledged that the criteria for p16-positivity in OCSCC applied in our study—and in the literature in general—are actually set and validated for OPSCCs. The oral cavity is an independent site of the head and neck area, and the composition of the oral squamous epithelium is different from the oropharyngeal epithelium (eg, less lymphoid tissue). Moreover, in the current study it was noticed that OCSCCs display a more diverse p16-staining pattern when compared to OPSCCs. The cutoff value for p16-positivity used in OPSCC seems to be well applicable to OCSCC, albeit a subsequent HPV DNA-test is absolutely required given the suboptimal positive predictive value.

When comparing the HPV-positive OCSCCs to the HPV-negative OCSCCs, we found some remarkable differences in clinical characteristics (Table 1). For example, 49.7% of HPV-negative tumors were treated with surgery alone and did not receive adjuvant treatment, whereas almost half of the HPV-positive tumors received postoperative radiotherapy, and 14.3% were treated with definitive chemoradiotherapy compared to 2.0% of the HPV negative tumors. As depicted in Table 1, HPV-positive OCSCCs tended to have a higher T-stage than HPV-negative tumors. This may in part be explained by the fact that the majority of HPV-positive OCSCCs were located in the floor of the mouth, often with invasion of the mandible. These tumors require postoperative radiotherapy. What is more, 15% of HPV-positive tumors were too large to be treated with surgery, that is, functionally inoperable tumors. These tumors were therefore treated with definitive chemoradiotherapy.

To conclude, HPV-attribution in OCSCC is very low and neither HPV-status nor p16-status affects survival of OCSCC patients. Although when followed by a PCR test p16-IHC may fairly well serve as a first step in assessing HPV-status, determining HPV-status in OCSCC seems trivial in clinical practice and should be omitted.

CONFLICT OF INTEREST

Daniëlle A. M. Heideman has been on the speakers' bureau of QIAGEN and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb; Daniëlle A.M. Heideman is minority shareholder of Self-screen B.V., a spin-off company of VUmc; Self-screen B.V. holds patents related to the work (ie, high-risk HPV test and methylation markers). C. René Leemans has been on the advisory boards of Merck & Co. Inc., Rakuten Medical, and Nanobiotix, and reports research grants from KWF Kankerbestrijding/Dutch Cancer Society, VUmc Cancer Center Amsterdam Foundation, GenMab, BMS and the Hanarth Foundation, outside the submitted work. Ruud H. Brakenhoff reports grants from GenMab, InteRNA technologies, and Agilent and nonfinancial support from AbbVie, outside the submitted work. All the other authors declared no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (rh.brakenhoff@amsterdamumc.nl) upon reasonable request.

ETHICS STATEMENT

The study adhered to the national guideline "Human Tissue and Medical Research: Code of Conduct for Responsible Use" (https://www.federa.org/codes-conduct), and the general privacy protection regulation of the European Commission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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