Oral Oncology 162 (2025) 107209

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Histological and genetic criteria define a clinically relevant subgroup of HPV-positive oropharyngeal carcinoma☆

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ARTICLE INFO

Keywords:
Human papillomavirus (HPV)
Oropharyngeal squamous cell carcinoma (OPSCC)
Survival
Prognosis
Genetic alterations
Histomorphology

ABSTRACT

Introduction: Subgroups with a poorer prognosis exist among patients with human papillomavirus positive oropharyngeal squamous cell carcinoma (HPV-positive OPSCC). This study aims to identify histological and genetic differences within HPV-positive OPSCC and correlate these findings with patient outcomes.
Methods: The study included 102 OPSCC patients, all tested positive for high-risk HPV DNA and p16INK4a expression. Based on histomorphological classification (HPV Prediction Classification, HPV PC), all cases were categorized as either classic HPV-positive OPSCC (cHPV) or non-classic HPV-positive OPSCC (non-cHPV). Next-generation sequencing (NGS) of selected genes was performed on 55 tumor samples, correlating results with morphological status and survival.
Results: Of all cases, 49 % (n = 50/102) were categorized as non-cHPV, histomorphologically resembling HPV-negative OPSCC, and showed significantly poorer overall survival (p = 0.004) and five-year survival rate (5YS: 83.9 % vs. 58.4 %). Multivariate analyses identified HPV PC as an independent prognostic marker ($p = 0.027$). NGS revealed loss-of-Function (LOF) mutations in TP53 in three non-cHPV samples. Additionally, PIK3CA/PTEN mutations were found in 35.7 % (10/28) of non-cHPV cases. The cumulative burden of gene mutations was higher in the non-cHPV subgroup compared to the cHPV subgroup ($n = 53$, $p = 0.1$).
Conclusion: HPV PC distinguished two histomorphological subgroups within HPV-positive OPSCCs: cHPV with excellent prognosis and non-cHPV with poorer overall survival. Non-cHPV tumors also exhibited higher overall mutation rates, notably LOF-TP53 and PIK3CA/PTEN mutations. These morphological subtypes, along with their corresponding mutational profiles, warrant further investigation as potential biomarkers for de-escalation intervention trials.

☆ This article is part of a special issue entitled: 'HPV and Head and Neck Cancers' published in Oral Oncology.

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https://doi.org/10.1016/j.oraloncology.2025.107209
Received 3 August 2024; Received in revised form 19 January 2025; Accepted 25 January 2025
Available online 1 February 2025

Introduction

Head and neck squamous cell carcinomas (HNSCC) are among the ten most common cancers worldwide, with an incidence of 900,000 cases per year [1]. Within this class, especially the incidence of human papilloma virus (HPV) related oropharyngeal squamous cell carcinoma (OPSCC) is steadily increasing and has already surpassed the incidence of HPV-associated cervical carcinomas in the USA and the UK [2–4]. Persistent infection with high-risk HPV is considered the oncogenic driver in these tumors, with HPV 16 being the most frequent subtype (approx. 90 %) [5–7]. Numerous studies have already demonstrated the superior survival of patients with HPV-positive in contrast to those with HPV-negative OPSCC [6–8]. However, a subgroup within HPV-positive OPSCC patients demonstrates a distinctively unfavorable survival [6,7]. Concordantly, up to 25 % of all HPV-positive OPSCC patients develop locoregional recurrences or metastatic disease [9]. Despite this prognostic difference, treatment is applied independent of HPV-status and consists of either surgery +/- risk adapted (chemo)radiotherapy or primary chemoradiotherapy in a curative setting. Those treatment modalities are often accompanied by severe long-term side effects as dysphagia, dryness of the mouth and stiffness of the neck. Attempts to de-intensify therapy for patients with low-risk HPV-positive OPSCC, such as replacing cisplatin with cetuximab, have failed until now [10–13]. Furthermore, there is still a lack of biomarkers to reliably predict prognosis, potentially providing an indication for therapy de-intensification.

In previous studies, OPSCC could be classified into keratinizing and non-keratinizing subgroups. A non-keratinizing cell pattern is more common in HPV positive OPSCC and is associated with better survival [14–16]. Other studies have also linked survival to the viral integration status [17]. However, an absence of desmoplastic stromal reaction, a basaloid nuclear morphology and an abundance of tumor-infiltrating lymphocytes (TIL) are similarly typical characteristics of HPV-association [18]. For example, higher infiltration by TIL has been associated with HPV and improved survival [19–22]. Previously, we demonstrated that a deep learning-based algorithm can successfully recognize parts of these histological characteristics by identifying not only HPV-positive patients among OPSCC, but also HPV-positive OPSCC with a distinctively favorable prognosis [18].

Heavy tobacco and/or alcohol consumption causes DNA damage in oncogenic and tumor suppressor genes, fueling the development of HPV-negative OPSCC [23]. However, in HPV-positive OPSCC patients, however, consumption of these toxic substances is less frequent and mutational burden is reduced compared to HPV-negative OPSCC. Independent of HPV, the most frequently mutated genes in OPSCC are *TP53*, *SOX2*, *CDKN2A/2B*, *PIK3CA*, *TP63* and *NOTCH1* [24]. In particular, mutations in the tumor suppressor gene *TP53*, as a regulator of the cell cycle and apoptosis, play a central role in the carcinogenesis of HPV-negative OPSCC and is significantly less frequent in HPV-positive cases [24,25], but typically harbor activating mutations in *PIK3CA* [25–27]. Additionally, HPV-negative tumors are often associated with *CDKN2A* mutations [25,28]. In contrast to HPV-negative cancers, the carcinogenesis of HPV-positive OPSCC is dependent on the activity of the viral oncoproteins E6/E7. E6 enhances the proteolytic degradation of p53, whereas E7 increases the degradation of retinoblastoma protein (Rb). This decreases cell cycle control, leading to unrestricted cell replication and reduced apoptosis. The development of HPV-positive tumors therefore does not rely on *TP53* driver mutations, which are rarely detected (approx. 8–16 %) in contrast to HPV-negative cases (*TP53* mutation in about 75 %) [24,25,27,28]. Mutations in *TP53* have already been demonstrated to be predictors of poor survival in head and neck squamous cell carcinoma (HNSCC) and OPSCC [29], in a HPV-independent fashion [30].

With a frequency of 20–30 %, *PIK3CA* mutations are among the most common mutations in HPV positive OPSCC [26,31]. *PIK3CA* wild-type status appears to be associated with an increased chance of recurrence [32]. Controversially, in a de-intensification trial with chemo-radiotherapy, HPV-positive tumors with *PIK3CA* mutations recurred more frequently [31]. In contrast, an activating mutation in *PI3K* was reported to be associated with better treatment response in metastatic HPV-positive OPSCC [33].

Other mutations, such as in *NOTCH1* are associated with poor survival in HPV-positive OPSCC, whereas in HPV-negative carcinomas, mutations in *SOX2* lead to poorer survival [24]. Among HPV-negative OPSCC with and without distant metastasis, the mutation profile is largely the same, whereas metastasized HPV-positive OPSCC display a different mutation profile compared to HPV-positive OPSCC without distant metastasis, involving mutations in *TP63*, *PIK3R1*, *HRAS* and *STK11* [34]. HPV-positive OPSCC with distant metastasis present a mutation pattern similar to HPV-negative with the exception of *TP53*. *TP53* mutations appear to have a lower incidence in HPV-positive tumors [34].

Despite the scientific progress, there are no reliable biomarkers for therapy de-escalation for HPV-positive OPSCC. Due to the morphological and genetic differences between HPV-positive and HPV-negative OPSCC, and furthermore due to histomorphological variation within the HPV-positive OPSCC group, in the present study, we combined respective analyses. We assumed that HPV-positive OPSCC histomorphologically resembling HPV-negative OPSCC are associated with poorer survival and show a higher mutation frequency compared to HPV-positive OPSCC with classic morphology. To test this hypothesis, we examined a large cohort of HPV-positive OPSCC according to morphological criteria and molecular characteristics using NGS.

Materials and methods

Patient cohort

In this study, patients were included from two different hospital sites. A total of 105 patients diagnosed with HPV DNA-positive and p16-positive OPSCC (C09, C10, International Classification of Diseases for Oncology (ICD-O)) between 1999 and 2020 were included. The recruiting hospitals were the University Hospital of Cologne (63 patients) and the University Hospital of Giessen (39 patients). Therapy consisted of either surgery with risk-adapted (chemo)radiotherapy or definitive chemoradiotherapy. Formalin-fixed paraffin-embedded (FFPE) cancer tissue was mandatory for the implementation of the gene analysis and was available for 53 patients. Data were collected retrospectively from the cancer registry of the Centre for Integrated Oncology Cologne (CIO) and the Giessen Tumor Documentation System (GTDS). The clinicopathological characteristics of the cohorts are shown in Tables 1 and 2.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the respective ethics committee (IRB number: 19-1288). Informed consent was obtained from all patients.

The assessment was made according to the current guidelines for each case (7th and 8th edition of the International Union Against Cancer (UICC), TNM classification and WHO criteria for squamous cell carcinomas of the oral mucosa).

DNA isolation

After tumor cell content assessment by a pathologist tumor areas were macrodissected from 10 μm thick FFPE tissue sections. DNA extraction was performed using the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega, Mannheim, Germany) on a Maxwell 16 instrument (Promega) following the manufacturer's instructions as described before [35].

HPV analysis

After amplification of the viral DNA via PCR, reverse hybridization of

Table 1
Patients characteristics of the cohort.

Characteristics	n	%
Total	102	100 %
Age		
<60 years	46	45.1 %
≥60 years	56	54.9 %
Sex		
Female	28	27.5 %
Male	74	72.5 %
Smoking		
Yes (>10 py)	62	60.8 %
No (≤10 py)	40	39.2 %
Location		
Tonsils	57	55.9 %
Other than tonsils	44	43.1 %
Unknown	1	1.0 %
HPV prediction score		
cHPV	52	51.0 %
non-cHPV	50	49.0 %
HPV type		
HPV16	93	91.2 %
HPV18	3	2.9 %
HPV33	2	2.0 %
HPV35	3	2.9 %
HPV52	1	1.0 %
p16		
Positive	102	100 %
Negative	0	0 %
Therapy		
Surgery	74	72.5 %
Radiotherapy	28	27.5 %
T		
T1-2	80	78.4 %
T3-4	22	21.6 %
N		
N0	16	15.7 %
N+	86	84.3 %
M		
M0	102	100 %
M1	0	0 %

the amplicons was performed using the LCD-Array 3.5 HPV 3.5 LCD Array Kit on the CHIP-Scanner PF7250u (CHIPRON GmbH, Berlin, Germany) according to the manufacturer's recommendations.

Next generation sequencing (NGS)

DNA concentrations were measured with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) using the Qubit dsDNA HS Assay Kit. Quality control and fragment length estimation was performed using the 4200 TapeStation system (Agilent, Santa Clara, CA, USA) with the High Sensitivity D1000 DNA Kit (Agilent, Santa Clara, CA, USA). After enzymatic fragmentation, library preparation was performed via a hybrid capture method using a customized panel (Tab. S2) (Twist Bioscience HQ, South San Francisco, USA). Libraries were sequenced on the NextSeq system (Illumina, San Diego, CA, USA) following the manufacturer's recommendations.

Variant calling and classification

Variant calling was performed using an in-house pipeline. For protein effect classification of each variant, the following databases were used: https://ckb.jax.org; https://www.oncokb.org/; https://www.ncbi.nlm.nih.gov/; https://tp53.isb-cgc.org/; https://cancer.sanger.ac.uk/. The NGS Gene content is presented in Table S2.

Histomorphology of HPV Prediction Classification

Tissue microarrays (TMAs) of a total of 102 carcinomas of the oropharynx were available from previous analyses, all of which were HPV DNA-positive and p16 IHC-positive [18].

Two board-certified pathologists analyzed the H&E-stained standard morphology of the OPSCCs in a blinded fashion according to previously defined histomorphological criteria. These characteristics were taken from previous publications describing the features of OPSCCs [15,18–21] and included: 1. tumor cell pleomorphism (monotonous versus pleomorphic appearance of the tumor), 2. nuclear morphology (basaloid, hyperchromatic nuclei versus vesicular nuclear chromatin), 3. stromal reaction (desmoplasia versus absence of desmoplastic stromal reaction and 4. keratinization by the tumor cells (detectable versus undetectable). Examples of these features compared with the HPV-negative OPSCC sample are illustrated in Fig. 1. In addition, we compared the results with the results of the same cohort determined using artificial intelligence (deep learning-based Prediction Score) [18].

We applied these criteria to all cases to define two histomorphological groups, labeled as the HPV Prediction Classification (HPV PC): a) tumor clearly resembles HPV-positive OPSCC (cHPV) b) tumor certainly appears like not HPV-positive OPSCC (non-cHPV).

Statistical analysis

Statistical analyses were performed using SPSS Statistics (IBM SPSS Version 28.0, Armonk, New York, USA) and GraphPad PRISM (GraphPad Software, Inc., Boston, Massachusetts, USA). In order to evaluate the differences in relation to the clinicopathological characteristics and the results of the gene analysis, Fisher's exact test or Pearson's chi-quadrat test were performed. Survival results were calculated using the log-rank test and the Kaplan-Meier method. Univariate and multivariate analyses by applying the Cox proportional-hazards model were performed to estimate hazard ratio (HR) and a 95 % confidence interval (CI). For all tests p-values ≤0.05 were considered significant.

Table 2
Clinicopathological characteristics according to HPV Prediction Classification in the cohort (bold values represent significant values ≤0.05).

		All		cHPV		non-cHPV		
Risk factors		(n = 102)	100 %	(n = 52)	%	(n = 50)	%	p
Age	<60 y	46	45.1 %	25	48.1 %	21	45.7 %	0.538
	≥60 y	56	54.9 %	27	51.9 %	29	58 %	
Sex	female	28	27.5 %	17	32.1 %	11	22 %	0.226
	male	74	72.5 %	35	67.3 %	39	78 %	
Smoking history	yes (>10 py)	62	60.8 %	30	57.7 %	32	64.0 %	0.514
	no (≤10 py)	40	39.2 %	22	42.3 %	18	36.0 %	
Tumor characteristics								
T	1–2	80	78.4 %	45	86.5 %	35	70 %	**0.42**
	3–4	22	21.6 %	7	13.5 %	15	30 %	
N	N0	16	15.7 %	9	17.3 %	7	14 %	0.646
	N+	86	84.3 %	43	82.7 %	43	86 %	
M	0	102	100 %	52	100 %	50	100 %	–
	1	0	0 %	0	0 %	0	0 %	

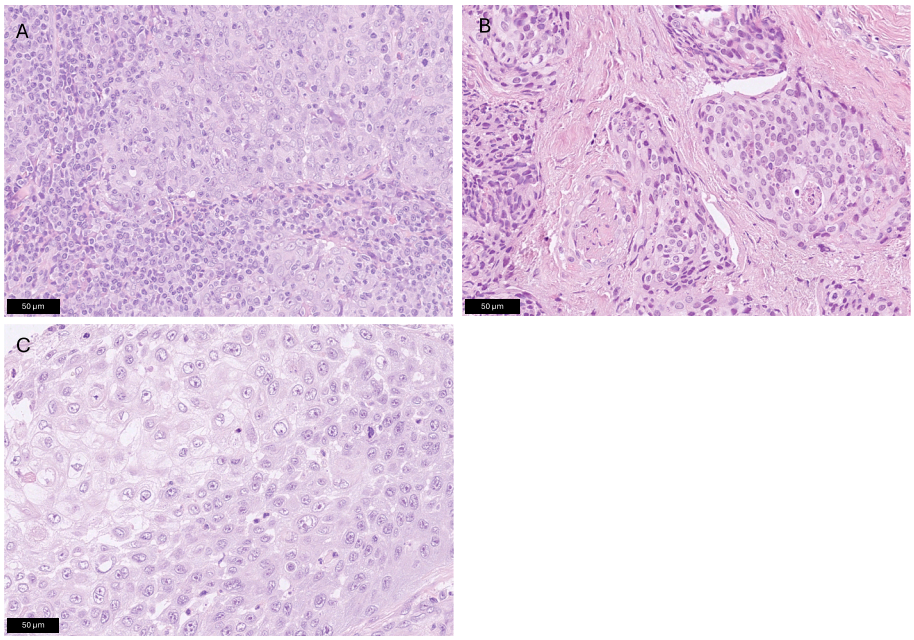

Fig. 1. Different histomorphology in TMAs (H&E staining) of (A) cHPV, (B) non-cHPV and (C) HPV negative OPSCC.

Results

Patients and clinicopathological characteristics

A total of 102 HPV/p16-positive OPSCC were included in this bicentric study (Cologne, $n = 63$; Gießen, $n = 39$). Clinicopathological characteristics are shown in Tables 1 and 2. High-risk HPV16 was detected in 91.2 % of all tumors. Other detected high-risk types were HPV18 (2.9 %), HPV33 (2.0 %), HPV35 (2.9 %) and HPV52 (1.0 %). The study population comprised 28 female and 74 male patients. 46 patients were younger than 60 years and 56 patients were at least 60 years old. Smoking history was positive (>10 pack-years (py)) in 60.8 % of the cases. The tonsils were the most frequent localization (55.9 %). The primary therapy was surgery in 72.5 % of the cases, whereas 27.5 % of all patients received primary (chemo)radiotherapy. Of all OPSCC, 78.4 % were of low T-stage (T1-2) and 84.3 % presented with lymph node metastasis (N+). No patients presented with metastatic disease.

We described two histomorphological groups: classical HPV (cHPV) and non-classical HPV (non-cHPV). cHPV OPSCC resembled histologically like HPV-positive OPSCC, while non-cHPV OPSCC resembled like HPV-negative OPSCC (Fig. 1). We termed this the HPV Prediction Classification (HPV PC). Through the HPV PC 51.0 % were classified as cHPV and 49.0 % as non-cHPV. Patients with cHPV OPSCC had a significantly lower T-stage at time of diagnosis ($p = 0.042$). In the group of tumors with HPV16, 53.8 % belonged to the non-cHPV tumor group, whereas in the group with an HPV type other than HPV16, 77.8 % were classified as non-cHPV ($p = 0.089$). There was no difference in the number of smokers between the cHPV and non-cHPV groups. No other

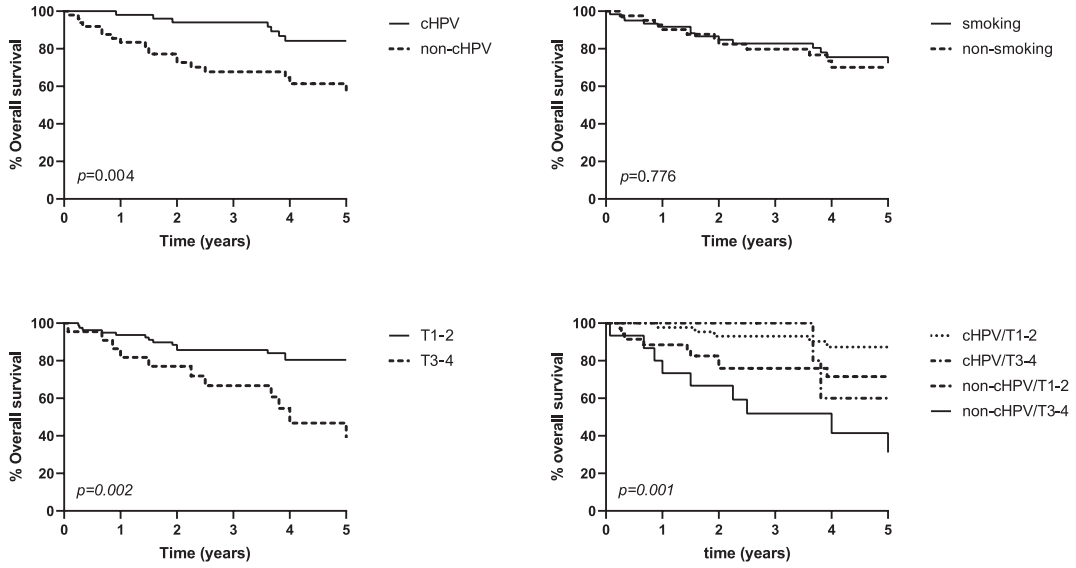

Fig. 2. Overall survival of a group of HPV-positive OPSCC (n = 102) (A) according to HPV Prediction Classification separated in cHPV (n = 52) and non-cHPV (n = 50), (B) stratified by smoking history separated in smoking (n = 62) and non-smoking (n = 40), (C) dichotomized by T stage in T1/2 (n = 80) and T3/4 (n = 22) and (D) according to HPV Prediction Classification and T Stage separated in cHPV/T1-2 (n = 45), cHPV/T3-4 (n = 7), non-cHPV/T1-2 (n = 35) and non-cHPV/T3-4 (n = 15) (value was derived by log-rank/Mantel-Cox test).

differences in clinicopathological characteristics were found between our histomorphological subgroups (Table 2). When a Bonferroni correction was applied to mitigate the risk of an alpha error accumulation, no statistically significant difference was observed.

Survival analysis and prognostic significance

cHPV OPSCC displayed better overall survival (OS) and five-year survival (5YS) than non-cHPV OPSCC, as illustrated by the Kaplan-Meier curve in Fig. 2 ($n = 102$, log-rank test: $p = 0.004$, 5YS: 83.9 % vs. 58.4 %) and estimated using a univariate Cox proportional hazard model (Table 3, HR 0.3, CI 0.124–0.719, $p = 0.007$). During the observation period, 9 patients in the cHPV group died, compared to 23 in the non-cHPV group. In addition, the univariate Cox analysis revealed that a low T stage was a factor for more favorable OS (HR 3.331, CI 1.509–7.353, $p = 0.003$). Both, cHPV and lower T stage, were associated with better OS in the multivariate analysis (HR 0.364, CI 0.149–0.89, $p = 0.027$; HR 2.649, CI 1.179–5.962, $p = 0.018$; Table 3, Fig. 2). A lower T stage (T1-2) was also associated with better OS compared to higher T stage (T3-4) in the Kaplan-Meier analysis (Fig. 2, $n = 102$, log-rank-test: $p = 0.002$, 5YS: 80.4 % vs. 39.0 %). Among low T stage tumors (T1-2), patients with cHPV (cHPV/T1-2) displayed a trend towards better OS and 5YS than those with non-cHPV (non-cHPV/T1-2) (Fig. 2, $n = 80$, log-rank test: $p = 0.059$, 5YS: 87.3 % vs. 71.5 %). Overall, only a small subset of patients presented with a high T stage (T3-4, $n = 22$) and no significant difference was observed between cHPV and non-cHPV groups (cHPV/T3-4 vs. non-cHPV/T3-4, Fig. 2, $n = 22$, log-rank test: 0.203, 5YS: 60.0 % vs. 31.1 %). The best OS and 5YS was documented for cHPV/T1-2, whereas the poorest OS was observed for non-cHPV/T3-4 (Fig. 2, $n = 60$, log-rank test: $p < 0.001$, 5YS 87.3 % vs. 31.1 %). Smoking history, age, sex, N stage and M stage were not associated with patient outcome in the overall cohort (Table 3, Fig. 2).

Inter-rater reliability and deep learning-based Prediction Score

The initial analyses were performed by a single pathologist. To determine the inter-rater reliability, a further examination was performed by a second pathologist, where the strength of agreement was almost perfect between the two observers ($n = 47$, $\kappa = 0.869$, $p < 0.001$). In 3 (6.4 %) cases, the observer reached different results. A survival analysis based on the results of the second observer also demonstrated a significantly better overall survival in the group of cHPV OPSCC, despite the smaller sample size ($n = 47$, log-rank test: $p = 0.043$, 5YS 78.7 % vs. 47.7 %).

We also used a deep learning-based Prediction Score as an additional observer and compared the results. The deep learning-based Prediction Score was previously developed by our research group and has already been published [18]. As the deep learning-based Prediction Score was a metric value, we divided it by the median and categorized it as 'high' or 'low' to match the HPV PC. We found a significant concordance with a fair strength of agreement between the deep learning-based prediction score and HPV PC ($n = 95$, $\kappa = 0.296$, $p = 0.004$, Fig. S1). In 34 cases, the deep learning-based Prediction Score came to different results. A survival analysis based on the results of the deep learning-based Prediction Score showed no significant differences in overall survival ($n = 95$, log-rank test: $p = 0.468$, 5YS 77.0 % vs. 68.0 %).

Frequency of gene mutations according to HPV PC

cHPV OPSCC patients tended to have fewer mutations than non-cHPV OPSCC patients ($n = 53$, $p = 0.10$; Table 4). Overall, 7 of 25 (28 %) cHPV OPSCC had mutations in at least one of the analyzed genes, compared to 14 of 28 (50 %) of the non-cHPV group, which had at least one detectable mutation Table 5.

In non-cHPV OPSCC three (3/28, 10.7 %) *TP53* Loss-of-Function (LOF) mutations were detected, whereas none (0/25, 0 %) were detected in cHPV ($p = 0.238$; Table 4). Furthermore 12.0 % (3/25) of cHPV and 28.6 % (8/28) of the non-cHPV OPSCC ($p = 0.183$, Table 4) had activating *PIK3CA* mutations. No (0/25) *PTEN* mutations were detected in cHPV OPSCC, whereas 3 (3/28, 10.7 %) were found in non-cHPV OPSCC (Table 4). In summary, 13 (13/53, 24.5 %) mutations were found in the *PIK3CA/PTEN* pathway and were summarized in the group *PIK3CA/PTEN*. In cHPV OPSCC, 12 % (3/25) of cases had mutations in *PIK3CA/PTEN* while in the group of non-cHPV, mutations in *PIK3CA/PTEN* were detected in 35.7 % (10/28) of the cases. This revealed a statistical trend toward an increased number of PIK3CA/PTEN alterations in non-cHPV OPSCC ($n = 53$, $p = 0.59$, Table 4). The other analyzed genes were mutated less frequently with similar distribution across cHPV and non-cHPV.

There were no significant genetic differences according to smoking history (Tab. 5). At least one mutation was detected in 39.6 % (21/53) of the patients, but there was no difference in smoking status ($n = 53$, $p = 0.533$). All *TP53* mutations (100 %, 3/3) and seven *PIK3CA/PTEN* mutations (53,8%, 7/13) were identified in the smoking group.

Considering all observed criteria, cHPV defines a histomorphological subgroup with different genetic and clinical characteristics (Table S1) compared to non-cHPV.

Discussion

Patients diagnosed with HPV-positive OPSCC generally exhibit a more favorable OS compared to those with HPV-negative OPSCC. However, a recent study highlighted that this survival advantage is predominantly observed in patients with double-positive OPSCC, characterized by both HPV DNA positivity and p16 overexpression. Conversely, patients with single-positive HPV status (either HPV DNA or

Table 3
Univariate and multivariate analysis according to HPV Prediction Classification (HPV PC) and clinicopathological characteristics (bold values represent significant values ≤0.05).

		Univariate					Multivariate			
		n	HR	CI		p	HR	CI		p
		102		lower	upper			lower	upper	
HPV PC	cHPV	52	0.3	0.124	0.719	**0.007**	0.364	0.149	0.89	**0.027**
	non-cHPV	50								
Smoking history	yes (>10 py)	62	0.844	0.383	1.860	0.674				n.s
	no (≤10 py)	40								
Age	<60	46	1.337	0.6	2.982	0.478				n.s
	≥60	56								
Sex	Female	28	0.81	0.323	2.028	0.653				n.s
	Male	74								
T	1–2	80								
	3–4	22	3.331	1.509	7.353	**0.003**	2.649	1.179	5.952	**0.018**
N	N0	16								n.s
	N1	86	1.277	0.691	4.271	0.691				

Table 4
Results of genes analysis (NGS) according to the HPV PC (cHPV/non-cHPV) in a subgroup of HPV positive OPSCC (n = 55).

		All		cHPV		non-cHPV		
Gene (NGS)		**(n = 53)**	**%**	**(n = 25)**	**%**	**(n = 28)**	**%**	**p**
Mutation rate	≥1 Mut.	21	39.6 %	7	28 %	14	50 %	*0.10*
	no Mut.	32	60.4 %	18	72 %	14	50 %	
TP53	mut[1]	3	5.7 %	0	0 %	3	10.7 %	0.238
	WT	50	94.3 %	25	100 %	25	89.3 %	
PIK3CA	mut[2]	11	20.8 %	3	12 %	8	28.6 %	0.183
	WT	42	79.2 %	22	88 %	20	71.4 %	
PTEN	mut	3	5.7 %	0	0 %	3	10.7 %	0.238
	WT	50	94.3 %	25	100 %	25	89.3 %	
PIK3CA/PTEN	mut[2]	13	24.5 %	3	12 %	10	35.7 %	*0.059*
	WT	40	75.5 %	22	88 %	18	64.3 %	
HRAS	mut	2	3.8 %	1	4 %	1	3.6 %	1.0
	WT	51	96.2 %	24	96 %	27	96.4 %	
KRAS	mut	1	1.9 %	1	4 %	0	0 %	0.472
	WT	52	98.1 %	24	96 %	28	100 %	
ERBB2	mut	1	1.9 %	1	4 %	0	0 %	0.472
	WT	52	98.1 %	24	96 %	28	100 %	
KEAP1	mut	2	3.8 %	1	4 %	1	3.6 %	1.0
	WT	52	96.2 %	24	96 %	27	96.4 %	
FGFR4	mut	1	1.9 %	1	4 %	0	0 %	0.472
	WT	52	98.1 %	24	96 %	28	100 %	
FGFR3	mut	1	1.9 %	1	4 %	0	0 %	0.472
	WT	52	98.1 %	24	96 %	28	100 %	
FGFR2	mut	1	1.9 %	0	0 %	1	3.6 %	1.0
	WT	52	98.1 %	25	100 %	27	96.4 %	
NRAS	mut	1	1.9 %	1	4 %	0	0 %	0.472
	WT	52	98.1 %	24	96 %	28	100 %	

[1]Loss-of-Funtion (LOF) Mutation. [2]Activating Mutation of PIK3CA.

Table 5
Results of genes analysis (NGS) according to the smoking history in a subgroup of HPV positive OPSCC (n = 55).

		All		Smoking		Non smoking		
Gene (NGS)		**(n = 53)**	**100 %**	**(n = 33)**	**%**	**(n = 20)**	**%**	**p**
Mutation rate	≥1 Mut.	21	39.6 %	12	36.4 %	9	45 %	0.533
	no Mut.	32	60.4 %	21	63.6 %	11	55 %	
TP53	mut[1]	3	5.7 %	3	9.1 %	0	0 %	0.282
	WT	50	94.3 %	30	90.9 %	20	100 %	
PIK3CA	mut[2]	11	20.8 %	7	21.2 %	4	20 %	1.0
	WT	42	79.2 %	26	78.8 %	16	80 %	
PTEN	mut	3	5.7 %	1	3 %	2	10 %	0.549
	WT	50	94.3 %	32	97 %	18	90 %	
PIK3CA/PTEN	mut[2]	13	24.5 %	7	21.2 %	6	30 %	0.471
	WT	40	75.5 %	26	78.8 %	14	70 %	

[1]Loss-of-Funtion (LOF) Mutation. [2]Activating Mutation of PIK3CA.

p16) demonstrate inferior survival outcomes compared to their double positive counterparts [6,7].

In this study, we stratified 102 cases of HPV double-positive (HPV DNA-positive and p16 positive) OPSCC into two distinct subgroups based on comprehensive histomorphological criteria. The prognostically favorable group, designated classic HPV-positive OPSCC (cHPV), was characterized by a uniform tumor cell morphology with basal, hyperchromatic nuclei and the absence of keratinization defects and stromal desmoplasia. In contrast, the prognostically unfavorable group, labeled non-classic HPV-positive (non-cHPV), exhibited morphological features similar to ordinary oropharyngeal squamous cell carcinomas, typified by marked tumor cell pleomorphism, stromal desmoplasia, and cornification defects.

Furthermore, by stratifying these groups according to T stage, we found that cHPV OPSCC with a low T stage exhibited the most favorable survival outcomes, outperforming non-cHPV OPSCC with low T stage. Conversely, non-cHPV OPSCC with high T-stage demonstrated the poorest OS rates. Genetically, our analysis revealed a discernible trend towards an increased mutation burden and a higher number of mutations *PIK3CA/PTEN* in the non-cHPV group, with all *LOF-TP53* mutations being localized within this subgroup. Notably, pathogenic *LOF-TP53* mutations were conspicuously absent in all cHPV OPSCC samples analyzed ($n = 25$). No significant molecular differences were observed according to smoking history, whereas all three *TP53* mutations were found in the smoking group. However, the overall frequency of mutations in the genes we analyzed was low, and no significant results were found regarding genetic results.

The histomorphological criteria for HPV-positive OPSCC and for HPV-negative OPSCC have been previously described [15,19–21]. A high number of TIL and a non-keratinizing morphology seem to be associated with a better survival and were typically detected in HPV-positive OPSCC [15,19–21]. However, previous studies have only examined small cohorts with partially discordant HPV status or only tested for HPV DNA or p16 alone. Other studies found that the frequency of *TP53* mutations and other gene mutations in HPV-positive OPSCC did not differ significantly between smokers and non-smokers [27,36] and in a small cohort of smokers and non-smokers with HPV positive OPSCC, patients had equal OS [37].

For the first time, this work combines two aspects that have so far only been considered separately: a) histomorphological features and b) genomic background. We applied morphological criteria to standard hematoxylin and eosin (H&E) staining and included 102 HPV double positive OPSCC. The primary aim was to analyze whether these criteria could prognostically stratify HPV-driven OPSCC according to their

outcome. We recently demonstrated that Artificial Intelligence (the so called 'deep learning-based Prediction Score') correctly predicts HPV association in OPSCC [18]. This work, which analyzed 594 OPSCC cases, highlighted that within the group of HPV-positive OPSCC (histologically defined as HPV DNA and p16 positive) different prognosis groups appeared. Another study showed that the deep learning algorithm even outperforms traditional HPV DNA/p16 testing in predicting prognosis of HPV-positive OPSCC [38]. We also compared the results of the HPV PC with the results of the deep learning-based Prediction Score of the same cohort and found significant overlaps. However, it was also evident that the misclassified tumors (non-cHPV OPSCC) had a significantly worse prognosis than the correctly classified tumors (cHPV OPSCC) (Fig. 1). To date, no study has shown that within the group of HPV DNA-positive, p16 immunohistochemistry (IHC)-positive OPSCC, histomorphological criteria have a discriminating prognostic effect. In addition, the impact of *TP53* mutations in HPV-positive OPSCC has not been clarified because of the low incidence of *TP53* mutations compared to HPV-negative OPSCC [24,27]. For this purpose, NGS was used in this study to analyze 53 OPSCC. Previous authors have already identified mutations in PIK3CA and PTEN in HPV-positive OPSCC [39]. These mutations influence tumor progression as part of the PI3K/AKT/mTOR signaling pathway and contribute to tumor growth and showed a negative impact on survival [40–42]. In this study, mutations in PIK3CA/PTEN were significantly less frequent in the cHPV OPSCC group compared to non-cHPV OPSCC.

One limitation of the study is the small cohort size, with only approximately half of the patients having undergone NGS analysis, which may restrict the generalizability of the findings to detect associations with less frequent mutations. Furthermore, as a retrospective study, potential biases related to patient selection, treatment protocols, and incomplete data cannot be excluded.

We chose to evaluate the histomorphological differences in H&E-stained TMAs because the tumor cell number present in a TMA spot corresponds to the minimal tumor cell number in a biopsy. Usually, the tumor cell count is even exceeded in routine. This ensured that histological characteristics can also be evaluated in pre-therapeutic biopsy material. This allows the application of our criteria in the context of therapy studies. An important task in future oncology will be to define patient subgroups with an excellent prognosis who do not benefit from aggressive therapy and can be preserved of its toxic side-effects. Since these patients have a high probability of surviving their tumor disease (treated with standard, non-escalated therapy) for decades, the probability of suffering from long-term side effects of overly aggressive therapy must be minimized. We provide a prognostically favorable subgroup of HPV-positive OPSCC that could qualify for tailored therapy with the goal of minimal toxicity. Given the excellent prognosis of cHPV OPSCC, associated with a lower incidence of *TP53* and *PIK3CA/PTEN* mutations and a low mutational burden, a therapy with reduced radiation dose compared to the current standard or even surgery alone should be discussed and considered in the context of prospective studies.

Conclusion

This study represents a novel integration of histomorphological and genetic characteristics to stratify two distinct prognostic subgroups of HPV-positive (OPSCCs) – namely, classic HPV-associated (cHPV) and non-classic HPV-associated (non-cHPV) tumors. Our findings reveal that a clinically significant subgroup (cHPV) with markedly improved survival can be histologically identified using standard H&E staining. cHPV tumors exhibit superior survival outcomes primarily attributable to histological features. The described histological features may indicate an underlying genetic causality, potentially linked to mutations in *PIK3CA/PTEN* and *TP53*, along with the associated reduced survival.

Based on our findings, we recommend an allocation for the future classification of HPV-positive OPSCC into two distinct subgroups with therapeutic implications: 1. classic OPSCC (cHPV) with low T-stage showing an excellent prognosis, suggesting the potential for therapy de-escalation, and 2. non-classic, mutation-driven OPSCC (non-cHPV) with high T stage showing a poor prognosis, necessitating intensified therapy. Patients within the prognostically favorable subgroup may benefit from less aggressive treatment approaches, as their outcomes are unlikely to be significantly enhanced by standard (chemo)radiotherapy regimens.

We propose that our subgrouping strategy could inform and optimize the design of future de-escalation therapy trials for OPSCC, ultimately contributing to more tailored and effective treatment strategies.

CRediT authorship contribution statement

Malte Suchan: Writing – original draft, Visualization, Validation, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Nora Wuerdemann:** Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Steffen Wagner:** Writing – review & editing. **Christine Langer:** Writing – review & editing. **Christoph Arens:** Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Conceptualization. **Jannik Johannsen:** Writing – review & editing. **Johanna Prinz:** Writing – review & editing. **Shachi Jenny Sharma:** Writing – review & editing. **Arthur Charpentier:** Writing – review & editing. **Marcel Mayer:** Writing – review & editing. **Charlotte Klasen:** Writing – review & editing. **Philipp Zimmermann:** Writing – review & editing. **Hans Eckel:** Writing – review & editing. **Christopher Kopp:** Writing – review & editing. **Christian U. Huebbers:** Writing – review & editing. **Sebastian Klein:** Writing – review & editing. **Janna Siemanowski:** Writing – review & editing. **Jörn Meinel:** Writing – review & editing. **Jens Peter Klussmann:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **Alexander Quaas:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Christoph Arolt:** Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Conceptualization.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions: All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

None

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2025.107209.

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