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Double positivity for HPV-DNA/p16^{ink4a} is the biomarker with strongest diagnostic accuracy and prognostic value for human papillomavirus related oropharyngeal cancer patients

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ABSTRACT

Background: The etiologic role of human papillomaviruses (HPV) in oropharyngeal cancer (OPC) is well established. Nevertheless, information on survival differences by anatomic sub-site or treatment remains scarce, and it is still unclear the HPV-relatedness definition with best diagnostic accuracy and prognostic value.
Methods: We conducted a retrospective cohort study of all patients diagnosed with a primary OPC in four Catalonian hospitals from 1990 to 2013. Formalin-fixed, paraffin-embedded cancer tissues were subjected to histopathological evaluation, DNA quality control, HPV-DNA detection, and p16^{INK4a}/pRb/p53/Cyclin-D1 immunohistochemistry. HPV-DNA positive and a random sample of HPV-DNA negative cases were subjected to HPV-*E6*I* mRNA detection. Demographic, tobacco/alcohol use, clinical and follow-up data were collected. Multivariate models were used to evaluate factors associated with HPV positivity as defined by four different HPV-relatedness definitions. Proportional-hazards models were used to compare the risk of death and recurrence among HPV-related and non-related OPC.
Results: 788 patients yielded a valid HPV-DNA result. The percentage of positive cases was 10.9%, 10.2%, 8.5%

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and 7.4% for $p16^{INK4a}$, HPV-DNA, HPV-DNA/HPV-*E6*I* mRNA, and HPV-DNA/$p16^{INK4a}$, respectively. Being non-smoker or non-drinker was consistently associated across HPV-relatedness definitions with HPV positivity. A suggestion of survival differences between anatomic sub-sites and treatments was observed. Double positivity for HPV-DNA/$p16^{INK4a}$ showed strongest diagnostic accuracy and prognostic value.

Conclusions: Double positivity for HPV-DNA/$p16^{INK4a}$, a test that can be easily implemented in the clinical practice, has optimal diagnostic accuracy and prognostic value. Our results have strong clinical implications for patients' classification and handling and also suggest that not all the HPV-related OPC behave similarly.

Introduction

About a decade ago the International Agency for Research on Cancer (IARC) established high-risk *Human papillomavirus 16* (HPV16) as a cause of oropharyngeal carcinoma (OPC) [1]. Since then, increasing amount of information on the role of HPVs in OPC has been generated. The IARC estimates that approximately 29,000 new HPV-related OPC cases occur every year, corresponding to 31% of the worldwide number of the overall incident OPC cases [2]. These estimates, as well as previous meta-analyses assessing the quantitative contribution of HPV, found high geographic heterogeneity in HPV-attributable fractions (AFs) of OPC, ranging from less than 20% in some world regions, 24% in Southern Europe to more than 60% in North America [3,4]. This low HPV-AF for OPC in Southern Europe has been recently confirmed in two recent studies conducted by our group [5,6].

HPV-related OPC differs at clinical, epidemiological and molecular level to OPC caused by classic risk factors (i.e. tobacco and alcohol) [7]. The consistent observation of improved survival and better response to treatment of HPV-related OPC has stirred up the state-of-the-art of their management. Indeed, several clinical trials of de-escalation treatments are under evaluation, aiming to achieve better results with less treatment-associated comorbidities [8]. However, the biological rationale underlying these strategies remains poorly understood, and most of schemes are extrapolated from HPV-negative OPC trials. Importantly, around 20% of HPV-related patients still fail to treatment despite its good prognosis [7].

Diagnosis algorithms for HPV-related OPC are still under development. HPV-DNA detection alone is not sufficient to classify an OPC as HPV-driven since the presence of HPV-DNA could reflect a transient or non-related infection rather than a genuine HPV-driven oncogenic process [9–11]. Additionally, the detection of high cellular $p16^{INK4a}$ expression by immunohistochemistry (IHC) is the most widely implemented technique in the clinical setting, but is not specific for HPV activity in these tumours [12,13]. Indeed, it has been demonstrated that patients with $p16^{INK4a}$ high expression but HPV-DNA-negative OPC show a significantly less favourable survival than patients with $p16^{INK4a}$ high expression and HPV-DNA-positive tumours [14,15], indicating that $p16^{INK4a}$ high expression alone may not accurately classify HPV-related OPC patients. The combination of HPV-DNA detection and $p16^{INK4a}$ IHC is starting to be recommended to diagnose HPV-related OPCs [15]. Nevertheless, there is still limited information about the accuracy and prognostic value of this combination of biomarkers.

It is imperative to identify the best HPV-relatedness definition for HPV causality and prognosis in OPC. This is a prerequisite to provide a sound approach to study differences in survival of HPV-related OPC by factors such as anatomical sub-site [16,17] and by treatment [18].

In an attempt to elucidate these gaps, we conducted a study in OPC to assess the association of different HPV-relatedness definitions with patients' overall survival (OS) and progression-free survival (PFS), stratified by anatomical sub-site or treatment.

Methods

Study design and population

We designed a retrospective cohort study of all patients diagnosed with a primary OPC in four hospitals of Catalonia from 1990 to 2013 (Catalan Institute of Oncology-ICO-Hospital Universitari de Bellvitge, Hospital de Sant Pau, Hospital del Mar and Hospital Parc Taulí). Protocols were approved by the ethics committee of each participating hospitals.

Cancer cases were identified from medical records/pathology reports of the centers of origin. We included cases that fulfilled the following criteria: to be diagnosed with primary invasive cancer of the oropharynx (any histology; codes from the International Classification of Diseases for Oncology version 3: C01.9, C02.4, C05.1, C05.2, C09, C10, C14.2), and to have access to medical records on demographic and clinical information.

From all eligible cases, we reviewed medical records of the patients and accessed information on demographics, smoking and alcohol consumption, clinical and follow-up data; and formalin-fixed paraffin embedded (FFPE) tumour samples from the diagnosis previous to treatment when available.

In order to assess potential carryover HPV contamination at the local level, we additionally included a set of control samples selected by local investigators (5% of the number of cases evaluated, corresponding to tissue samples of patients with diagnoses non- related with HPV processed in the same laboratory).

FFPE blocks processing and histopathological evaluation

All specimens processing was centralized at ICO. FFPE blocks were re-embedded whenever necessary. First and last sections were used for histopathological evaluation after hematoxylin and eosin (H&E) staining. Two in-between sections were used for HPV-DNA testing, genotyping and *E6*I* mRNA detection; four additional slides were obtained to assess expression of cellular proteins by IHC. A block was classified as "adequate" for HPV testing if invasive cancer was observed in the two H&E stained sections of the specimen. Pathology review was performed blind with respect to the original local diagnosis and followed a pre-established algorithm for diagnostic consensus involving three pathologists, as reported elsewhere [5]. Pathological classification was based on the World Health Organization pathological criteria for head and neck cancer [19].

FFPE blocks were processed under strict conditions of pre/post polymerase chain reaction (physical separation), and blank paraffin blocks were systematically tested in parallel to serve as sentinels for contamination as previously published [20].

HPV-DNA detection and genotyping

The detailed methods used for HPV-DNA detection and genotyping have been reported elsewhere [21]. Briefly, we used a PCR with the consensus primers SPF_{10} PCR and a DNA enzyme immunoassay (DEIA) to test for the presence of HPV-DNA. Virus genotyping was performed using reverse hybridization line probe assay (LiPA25_v1) on all samples testing positive for viral DNA, targeting 25 HPV types with different oncogenic risk (Laboratory Biomedical Products Rijswijk, The Netherlands). DNA quality was evaluated in all HPV-DNA negative samples by testing for the *tubulin*-β gene (21). All DEIA and LiPA25_v1 assays were performed at ICO.

*HPV-E6*I mRNA detection*

All HPV-DNA positive samples underwent RNA extraction and HPV-*E6*I* mRNA detection at DKFZ, Heidelberg, Germany [22]. Briefly, the assays target a total of 20 HPVs types. For each sample, type-specific *E6*I* mRNA reverse transcription quantitative PCR (RT-qPCR) was performed for all available HPVs types detected at the DNA level and additionally for HPV16. A random selection (10%) of HPV-DNA negative cancers was tested for HPV16-*E6*I* mRNA, and all of them were mRNA negative. Detection of housekeeping gene *ubiquitin C* mRNA was used for RNA quality control in all tested samples.

Immunohistochemistry

Protein expression patterns were evaluated for $p16^{INK4a}$, pRb, p53, and Cyclin-D1 in all samples, independently of HPV results. All IHC assays were performed at Hospital General de L'Hospitalet, L'Hospitalet de Llobregat, Spain, under the manufacturer's standards: Roche mtm Laboratories AG (Heidelberg, Germany) for $p16^{INK4a}$, Vision Biosystems Novocastra (Newcastle, USA) for pRb, and Dako (Denmark) for p53 and Cyclin-D1. We used the predefined algorithm developed by Halec and colleagues [21] to determine the cutoff values for high vs low expression of pRb, p53, and Cyclin-D1. For $p16^{INK4a}$, the intensity of nuclear and cytoplasmic staining within the tumours was scored and those with a strong staining of > 70% were considered $p16^{INK4a}$ high [23]. The expected pattern for HPV-related cancers was high expression of $p16^{INK4a}$ and low expression of the other three cellular markers.

Statistical analyses

Cancer samples having tested negative for both viral and human DNA were excluded from the analyses. In line with work from several authors [22], we established that in order to explore algorithms to classify an OPC as HPV-related we needed to consider biomarkers of HPV infection (HPV-DNA detection), biomarkers of transcriptional activity of HPV oncogenes (HPV-*E6*I* mRNA), and surrogate biomarkers of HPV-related cellular transformation ($p16^{INK4a}$, pRb, p53, and Cyclin-D1). We used HPV-mRNA positivity as the gold standard for viral activity. We assumed that 90% of HPV-DNA negative cases not tested for *E6*I* mRNA were also mRNA negative. We assessed the accuracy of the four IHC, alone and combined, and of double positivity for HPV-DNA/$p16^{INK4a}$ by estimating the sensitivity, specificity, odds ratios, and area under the receiver operating characteristic (ROC) curves (AUC), and compared the AUC. Descriptive, bivariate and unconditional logistic regression analyses were performed to identify independent factors (i.e. age, sex, tobacco-alcohol use, clinical data) associated with HPV etiological involvement in OPC according to six different HPV-relatedness definitions: (1) HPV-DNA positivity; (2) $p16^{INK4a}$ high expression; (3) Double positivity for HPV-DNA/$p16^{INK4a}$; (4) Double positivity for HPV-DNA/HPV-*E6*I* mRNA; (5) Double positivity for HPV-DNA and ($p16^{INK4a}$ or HPV-*E6*I* mRNA) and (6) Triple positivity for HPV-DNA/HPV-*E6*I* mRNA/$p16^{INK4a}$. Crude and adjusted odds ratios and their

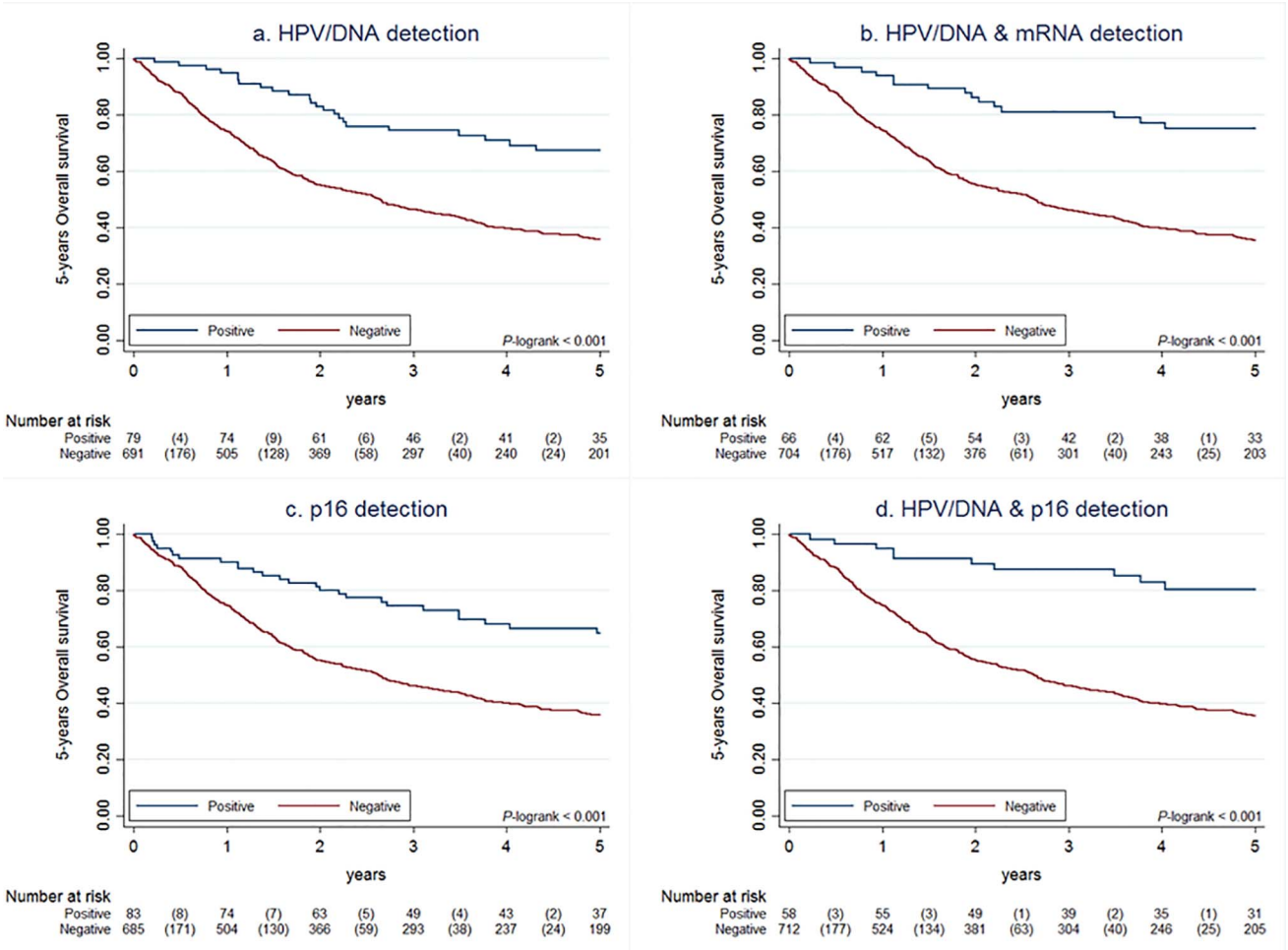

Fig. 1. 5 years Overall Survival by HPV status according to four different HPV-relatedness definitions. Legend: Data on 5 years Overall Survival by HPV status according to four different HPV-relatedness definitions. Panel "a" showed Kaplan-Meier curve for HPV/DNA detection. Panel "b" showed Kaplan-Meier curve for HPV/DNA and HPV mRNA detection. Panel "c" showed Kaplan-Meier curve for $p16^{INK4a}$ detection. Panel "d" showed Kaplan-Meier curve for double positivity for HPV-DNA/$p16^{INK4a}$. Panel "d", double positivity for HPV-DNA/$p16^{INK4a}$ showed the best prognostic value, since it classified better HPV-related cases and showed improved 5 years OS.

95% confidence intervals were estimated. Histological variables were not considered in multivariate analyses as previously described [21]. Survival time was calculated from the date of histological diagnosis to time of death for any cause (OS) or cancer recurrence (PFS). OS and PFS estimates were assessed up to 5 years. The cumulative probability of survival was estimated by Kaplan–Meier analysis. Survival curves were compared with the log-rank test, which was adjusted for multiple testing when making comparisons among the different HPV-relatedness definitions or when comparing treatments. Pairwise comparisons of survival curves between group levels when considering combinations of HPV-DNA detection and $p16^{INK4a}$ expression results or when examining the combined variable of HPV-status and tobacco use were also performed. All corrections were performed using the Benjamini-Hochberg procedure. Multivariate Cox's proportional hazards models to explore the effect of the HPV status as a prognostic factor were performed, in all sites and stratified by anatomical sub-sites. Metastasic patients (stage IVc, 7th edition TNM) were excluded from survival analyses.

Results

Fig. S1 describes the workflow of the OPC targeted cases, samples collected, processed, tested and finally included in the statistical analysis. A total of 1381 OPC cases were identified and included in the study, of which 555 (40.2%) had unavailable FFPE blocks at diagnosis. Cases provided by Sant Pau's Hospital, diagnosed in older periods (1991–1994), located on the base of tongue (BOT) or patients who underwent a palliative treatment had lowest proportion of FFPE blocks available compared to other variable categories (data not shown).

After pathology evaluation, samples from 802 OPC (58.1%) were tested for HPV-DNA. A total of 788 OPC samples yielded a valid DNA result and were finally included in the analysis. HPV-DNA positivity was found in 80 (10.2%) samples. The percentage of HPV-related cases when considering only $p16^{INK4a}$ high expression was 10.9%, and it dropped to 8.5% and 7.4% respectively for double positive HPV-DNA/HPV-*E6*I* mRNA, and HPV-DNA/$p16^{INK4a}$. Results of double positivity for HPV-DNA and ($p16^{INK4a}$ or HPV-*E6*I* mRNA) were equivalent to those of double positivity for HPV-DNA/HPV-*E6*I* mRNA, and the same was observed between double positivity for HPV-DNA/$p16^{INK4a}$ and triple positivity for HPV-DNA/HPV-*E6*I* mRNA/$p16^{INK4a}$. Thus, only four different HPV-relatedness definitions were further considered. The most common HPV type among HPV-DNA positive cases was HPV16 (67/80 cases, 83.8%), followed by HPV33 (6.3%), HPV18 (2.5%) and HPV31, 51 and 58 (1.3% each). All HPVs were detected as single infections. In three cases (3.8%) the HPV present in the sample could not be genotyped. Positivity of HPV16 for cases double positive for HPV-DNA/HPV-*E6*I* mRNA, and HPV-DNA/$p16^{INK4a}$ was 89.6% and 93.1%, respectively.

Table S1 shows the demographic and clinical characteristics of the

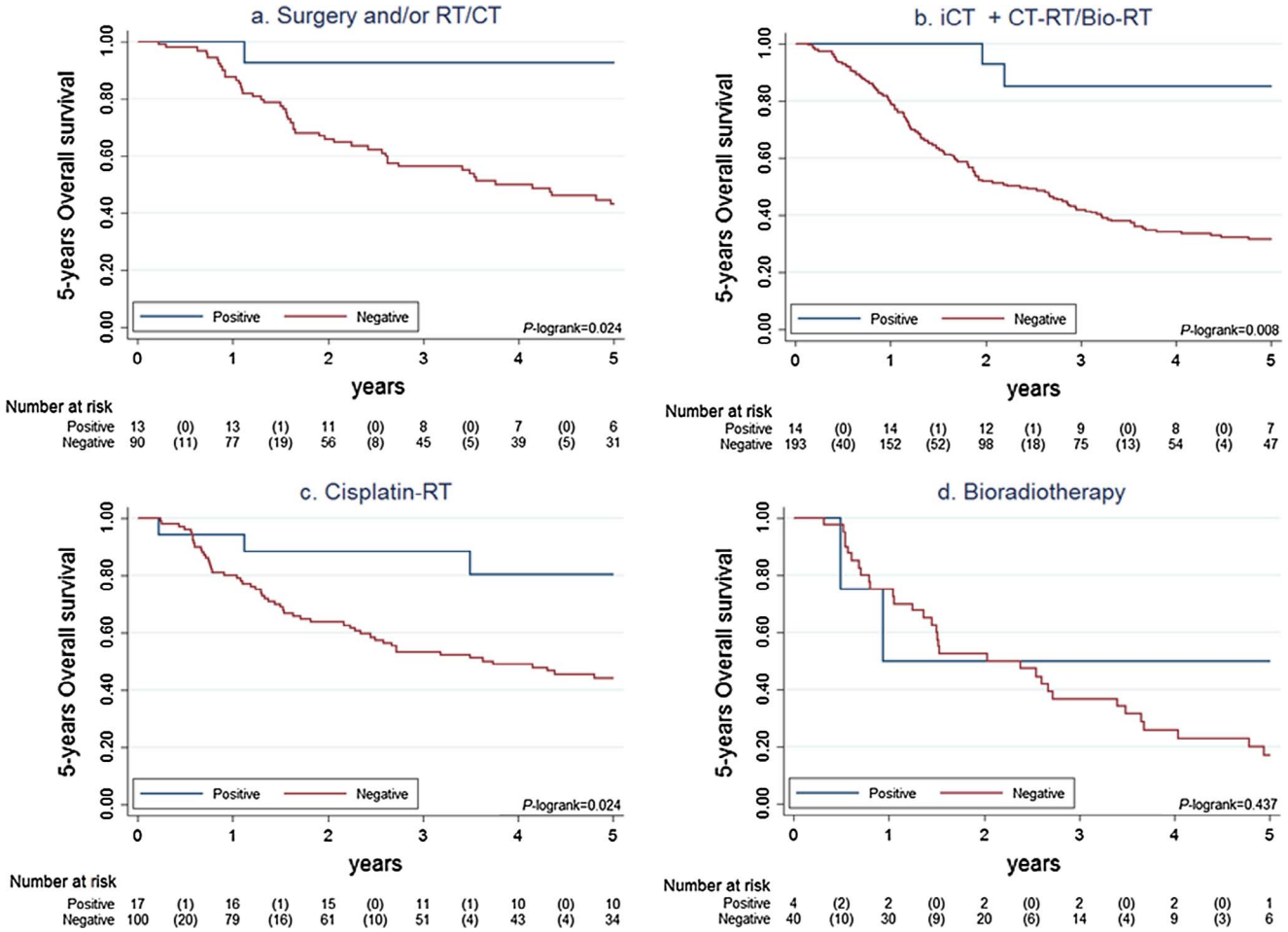

Fig. 2. 5 years Overall Survival by standard treatment for locally advanced OPC patients (stages III, IVa and IVb) and HPV status according to double positivity for HPV-DNA/$p16^{INK4a}$. Legend: Data on 5 years Overall Survival by standard treatment for locally advanced OPC patients (stages III, IVa and IVb) and HPV status double positivity for HPV-DNA/$p16^{INK4a}$. Panel "a" showed Kaplan-Meier curve for patients who underwent surgery with/without adjuvant chemo-radiotherapy. Panel "b" showed Kaplan-Meier curve for patients who underwent induction chemotherapy followed by chemo-radiotherapy or bioradiotherapy. Panel "c" showed Kaplan-Meier curve for patients who underwent cisplatin-radiotherapy. Panel "d" showed Kaplan-Meier curve for patients who underwent cetuximab-radiotherapy. Improved OS was not observed on panel "d". RT: radiotherapy; CT: chemotherapy; iCT: induction chemotherapy; bio-RT: bioradiotherapy (radiotherapy-cetuximab).

788 OPC patients included in the analysis, as well as the crude and adjusted measures of associations between those and double positivity for HPV DNA/p16^{INK4a}. The equivalent results for HPV-DNA detection alone, p16^{INK4a} high expression alone and double positivity for HPV-DNA/HPV-*E6*I* mRNA are presented in Table S2. Patients were mostly male (89.2%), heavy smokers (75.6%) and heavy drinkers (51.8%), with a locally advanced keratinizing grade 3 squamous cell carcinoma (SCC). Of note, 10 samples were defined as sarcomatoid SCC (n = 3), undifferentiated carcinoma (n = 4) and neuroendocrine carcinoma (n = 3), and all of them were primary tumours. The tonsil was the most common anatomical sub-site (40.0%). After adjusting for significant covariates, HPV-related patients were significantly more likely to be non-smokers and non-drinkers and to have a SCC of the tonsil, consistently across the four HPV-relatedness definitions analyzed. Association of HPV-positivity and female gender was observed in all univariate but none multivariate analyses.

As described in Table S3a, double positivity for HPV-DNA/p16^{INK4a} was the biomarker combination that showed the highest AUC. Among surrogate biomarkers of HPV-related cellular transformation alone, p16^{INK4a} high expression was the one that showed best accuracy for diagnosis. Best accuracy parameters were observed in tonsillar cancers (Table S3b).

We examined the crude OS and PFS of OPC patients based on Kaplan–Meier curves stratified by HPV positivity according to the four different HPV-relatedness definitions (Figs. 1 and S2, respectively). Double positivity for HPV-DNA/p16^{INK4a} showed the best prognostic value. Moreover, it classified better HPV-related cases and showed improved five years OS and PFS irrespective of having an early or locally advanced OPC stage (Figs. S3 and S4). However, when examining crude OS of locally advanced OPC patients based on Kaplan–Meier curves stratified by standard treatments, better OS were not observed for patients' double positive for HPV-DNA/p16^{INK4a} treated with bioradiotherapy (anti-EGFR concomitant with radiotherapy), as it was observed for other treatments (Fig. 2). Improved PFS were observed in patients' double positive for HPV-DNA/p16^{INK4a} for all treatment schemes herein evaluated (Fig. S5), although those were not statistically significant. We also analyzed crude OS of OPC patients according to the four possible combinations of HPV-DNA detection and p16^{INK4a} expression results. Pairwise analyses showed that only patients double positive for HPV-DNA/p16^{INK4a} had a statistically better OS compared to any other combination of those biomarkers (Fig. 3). Importantly, HPV-DNA-negative/p16^{INK4a} positive patients displayed OS similar to HPV-DNA-negative/p16^{INK4a}-negative or HPV-DNA-positive/p16^{INK4a}-negative ones.

Hazard ratios (HR) for death and for recurrence by HPV status according to the four HPV-relatedness definitions, after adjustment for age (only for death), tobacco use, stage and treatment, are presented in Table 1. Statistically significant improved OS and PFS among patients with HPV-related OPC were only observed in tonsillar cancer. Double positivity for HPV-DNA/p16^{INK4a} was the biomarker with strongest prognostic value (OS adjusted HR 0.21, 95%CI 0.11–0.40). A statistically significant interaction between HPV status and tobacco use was observed in the multivariate Cox's proportional hazards model for death for all anatomical sites. This interaction was not consistent across the four HPV-relatedness definitions and did not substantially improve the model. Thus, it was not further considered in the model. However, we explored the interaction further by creating a combined variable of HPV-status (as defined by double positivity for HPV-DNA/p16^{INK4a}) and tobacco use and examining the OS of each combination (Fig. S6), as well as stratifying the analyses by HPV status (Tables S4a and S4b). Age was a prognostic factor for death in both HPV-positive and HPV-negative patients, consistently for all HPV-relatedness definitions. However, tobacco use was only a prognostic factor for death in HPV-positive (for all HPV-relatedness definitions with the exception of double positivity for HPV-DNA/p16^{INK4a}), but not in HPV-negative cases. On the other hand, stage and treatment scheme were prognostic factors in HPV-negative but not HPV-positive cases (with the exception of high expression of p16^{INK4a} for treatment). Adjusted HRs for death were also examined for all cellular protein biomarkers and their combinations (Table S5). A better OS was observed for positivity to all markers, either individually or combined, except for low pRb and/or p53 expression. Again HPV-DNA/p16^{INK4a} showed the strongest association with survival.

Discussion

Mounting evidence supports the etiologic role of oncogenic HPVs in certain OPCs and the potential implications in the management of HPV-related patients. Our knowledge remains however incomplete regarding differences in prognosis by anatomic sub-site or treatment, or about the differential performance in terms of diagnostic accuracy and prognostic values between HPV-related biomarkers that can be easily implemented in the clinical setting.

To the best of our knowledge, this study represents the first attempt to address jointly all these issues in a large retrospective series of unselected patients. In an era of de-escalation clinical trials, this information is crucial in order to unequivocally identify patients who can really benefit from de-escalate protocols and to avoid worsening their outcomes.

The epidemiology of HPV-related OPC in our cohort differed in some aspects from what is observed in other high-income countries. HPV-AFs were slightly higher in women than in men, as has already been observed in other series [5], in contrast with what is observed in the United States in cohorts from the same time periods [24]. This discrepancy may reflect distinct temporal, geographical, and socio-demographic trends in population exposure to both tobacco use and/or oral HPV infection, leading to a rapid shift in the epidemiology of HPV-related OPC.

We examined the HPV-diagnostic accuracy of several biomarkers with a previously validated robust and comprehensive methodology [5]. In line with our previous results [5] and a recent meta-analysis [15], double positivity for HPV-DNA/p16^{INK4a} showed higher AUCs than any other combinations of biomarkers. Importantly, the double testing for HPV-DNA/p16^{INK4a} can be easily implemented in the clinical setting.

We examined the prognostic value of HPV-related biomarkers in OPC as defined by four different HPV-relatedness definitions. We found that HPV-positivity had stronger prognostic value than stage (7th edition TNM), consistently for all tests, since HPV-related locally advanced OPC patients had better OS and PFS than stage I-II HPV-non-related

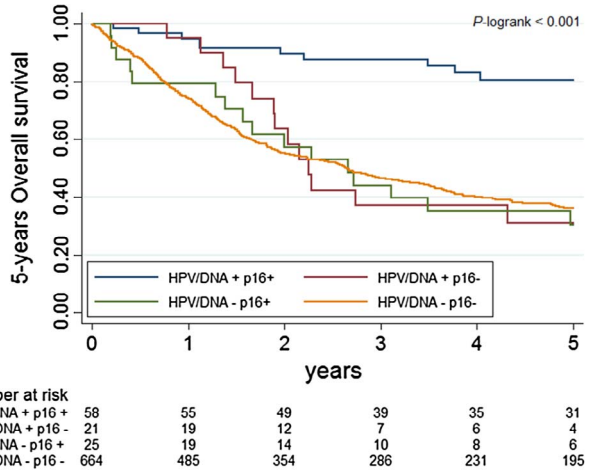

Fig. 3. 5 years Overall Survival by HPV-DNA detection and p16^{INK4a} high expression. Legend: Pairwise analyses showed that only patients double positive for HPV-DNA/p16^{INK4a} had a statistically better OS compared to any other combination of those biomarkers.

Table 1
Hazard ratios for death and recurrence for OPC patients, all sites and stratified by anatomical sub-site (stage VIc patients are excluded).

Five-years overall survival												
HPV biomarker	All sites			Tonsil			Base of tongue			Others		
	Cases/deaths	HR crude (95%CI)	HR adjusted[a] (95%CI)	Cases/deaths	HR crude (95%CI)	HR adjusted[a](95%CI)	Cases/deaths	HR crude (95%CI)	HR adjusted (95%CI)	Cases/deaths	HR crude (95%CI)	HR adjusted (95%CI)
DNA									–			–
–	691/426	Ref.	Ref.	259/165	Ref.	Ref.	151/98	Ref.		262/149	Ref.	
+	79/23	**0.37 (0.24–0.56)**	**0.37(0.24–0.58)**	49/11	**0.27 (0.15–0.50)**	**0.24 (0.12–0.48)**	14/6	0.53 (0.23–1.20)		16/6	0.51 (0.23–1.16)	
DNA/mRNA									–			–
Other	704/434	Ref.	Ref.	263/168	Ref.	Ref.	152/99	Ref.		270/153	Ref.	
+/+	66/15	**0.27 (0.16–0.46)**	**0.26 (0.15–0.45)**	45/8	**0.20 (0.10–0.41)**	**0.18 (0.08–0.39)**	13/5	0.47 (0.19–1.17)		8/2	0.30 (0.07–1.21)	
p16									–			–
Low	685/422	Ref.	Ref.	252/159	Ref.	Ref.	152/99	Ref.		263/150	Ref.	
High	83/26	**0.41 (0.27–0.61)**	**0.32 (0.21–0.50)**	55/16	**0.36 (0.22–0.61)**	**0.26 (0.14–0.46)**	12/5	0.59 (0.24–1.45)		15/5	0.45 (0.19–1.10)	
DNA/p16									–			–
Other	712/439	Ref.	Ref.	267/171	Ref.	Ref.	155/101	Ref.		271/153	Ref.	
+/high	58/10	**0.20 (0.11–0.38)**	**0.21 (0.11–0.40)**	41/5	**0.13 (0.05–0.33)**	**0.11 (0.04–0.29)**	10/3	0.38 (0.12–1.20)		7/2	0.35 (0.09–1.41)	
Five-years progression-free survival												
	Cases/ recurrences	HR crude (95%CI)	HR adjusted[b](95%CI)	Cases/ recurrences	HR crude (95%CI)	HR adjusted[b](95%CI)	Cases/ recurrences	HR crude (95%CI)	HR adjusted (95%CI)	Cases/ recurrences	HR crude (95%CI)	HR adjusted (95%CI)
DNA									–			–
–	691/194	Ref.	Ref.	259/87	Ref.	Ref.	151/37	Ref.		262/63	Ref.	
+	79/10	**0.33 (0.18–0.63)**	**0.32 (0.16–0.62)**	49/6	**0.26 (0.12–0.60)**	**0.18 (0.07–0.45)**	14/2	0.50 (0.12–2.06)		16/2	0.37 (0.09–1.50)	
DNA/mRNA									–			–
Other	704/197	Ref.	Ref.	263/89	Ref.	Ref.	152/37	Ref.		270/64	Ref.	
+/+	66/7	**0.27 (0.13–0.58)**	**0.26 (0.12–0.57)**	45/4	**0.18 (0.07–0.50)**	**0.12 (0.04–0.36)**	13/2	0.55 (0.13–2.29)		8/1	0.36 (0.05–2.58)	
p16									–			–
Low	685/193	Ref.	Ref.	252/87	Ref.	Ref.	152/38	Ref.		263/62	Ref.	
High	83/11	**0.36 (0.20–0.67)**	**0.35 (0.19–0.67)**	55/6	**0.24 (0.10–0.54)**	**0.16 (0.06–0.40)**	12/1	0.29 (0.04–2.14)		15/3	0.69 (0.22–2.19)	
DNA/p16									–			–
Other	712/200	Ref.	Ref.	267/91	Ref.	Ref.	155/38	Ref.		271/64	Ref.	
+/high	58/4	**0.17 (0.06–0.46)**	**0.16 (0.06–0.44)**	41/2	**0.10 (0.02–0.39)**	**0.06 (0.01–0.26)**	10/1	0.32 (0.04–2.36)		7/1	0.41 (0.06–2.97)	

[a] Adjusted by age, tobacco consumption, stage and treatment.
[b] Adjusted by tobacco consumption, stage and treatment.

ones. However, double positivity for HPV-DNA/p16^{INK4a} was the only biomarker showing the best prognostic value for HPV-related patients as also reported in a recent meta-analysis [25].

When examining the prognostic value of double positivity for HPV-DNA/p16^{INK4a} in locally advanced OPC patients by their standard treatments, we found that HPV-related OPCs showed improved OS for all treatment schemes with the exception of those who underwent bioradiotherapy. A recent study also suggested better outcomes in locally advanced HNSCC patients receiving concurrent cisplatin over cetuximab (anti-EGFR therapy) regardless of HPV/p16^{INK4a}status [26]. These findings have strong clinical implications because cetuximab is being explored as an alternative to cisplatin when given concurrently with radiotherapy as one main de-escalation strategies for HPV-related OPC patients aiming to reduce toxicities [8]. However, our results should be interpreted with caution since the number of HPV-positive patients treated with bioradiotherapy was very small and thus underpowered to draw firm conclusions. Noteworthy, anti-EGFR therapies are not currently recommended for treatment of anogenital HPV-related cancer [27,28]. To date, the available evidence supporting the use of anti-EGFR therapies in HPV-related OPC is therefore not conclusive; and we must wait for results of ongoing de-escalation clinical trials.

We also wanted to elucidate the differences in OS and PFS according to HPV-status by anatomical sub-sites within the oropharynx. For all four HPV-relatedness definitions herein evaluated, HPV had significant prognostic value only in tonsillar carcinoma, and double positivity for HPV-DNA/p16^{INK4a} was the biomarker with best prognostic value. This has also been reported for OS in a recent study of a large cohort of Danish patients [16]. However, this Danish study found equivalent results for BOT carcinoma, while in our case, although HPV-related BOT carcinoma displayed higher OS with lowest mortality observed for double positivity for HPV-DNA/p16^{INK4a}, the results were not significantly different. This could be partially explained by the lower HPV prevalence in BOT carcinoma in our Spanish cohort (5.8%) as compared to the Danish one (46%). On the other hand, our results on other locations than tonsil or BOT were in line with previous results from Sweden [17], where HPV-DNA and p16^{INK4a} status had no impact on clinical outcome in OPCs other than tonsil or BOT. However, the HRs of around 0.5 in these locations were in the same direction as those for tonsillar cancers, as it was observed for BOT cancers, despite their wide confidence intervals. Again, these results should be interpreted with caution due to small number of cases.

When we examined adjusted HRs for death stratified by HPV status, we found differences between HPV-positive and negative OPC patients. The lack of prognostic advantage of non-smokers among HPV-negative patients could be partially explained by the limitation of self-reported data and warrant further research with biomarkers of tobacco use. On the other hand, the fact that stage was not a prognostic factor in HPV-positive patients evidences the limitation of the 7th edition of TNM to accurately classify HPV-positive OPCs.

Finally, when we evaluated the prognostic value of cellular biomarkers of protein expression alone or combined, none of them showed better HR than double positivity for HPV-DNA/p16^{INK4a}, but we found better OS for p16^{INK4a} overexpression alone than previous publications [29]. The discrepancy may be due to the differences in the difficulties for comparing cut-off points for p16^{INK4a} expression between studies.

Our study has several limitations. The retrospective nature of our cohort may have hampered the thorough characterization of the patients according to risk factors such as tobacco-alcohol use, since this kind of information could only be partially obtained from medical records. Also, paraffin blocks were not available at diagnosis for an important number of cases, notably BOT carcinoma, a location particularly more difficult to biopsy, as well as for cases from older periods. For HPV-diagnostic accuracy analyses, we assumed that the 90% of HPV-DNA negative cases not tested for HPV-*E6*I* mRNA were mRNA negative. Our classification of other sub-sites than tonsil or BOT comprised many different locations, including oropharynx specified or overlapping lesions that could include also tonsil and BOT. In addition, we have a low rate of HPV-related OPC patients included in the analysis (i.e. Kaplan-Meir analysis by treatment), because HPV-related OPC AFs in our country is still low in comparison with other geographic regions like United States or Northern Europe.

Conclusion

Our findings from a large cohort of unselected OPC Spanish patients provide robust evidence that double positivity for HPV-DNA/p16^{INK4a} has optimal diagnostic accuracy and prognostic value as compared with a broad battery of HPV-related biomarkers. Noteworthy, this is a test that can be easily implemented and used in the clinical practice. Moreover, our results suggest that one of the main de-escalation treatment strategies for HPV-related OPC being currently evaluated in clinical trials (anti-EGFR/radiotherapy) may not be appropriate for HPV-related patients. Our results also suggest that there may be differences between OPC sub-sites regarding diagnostic accuracy and prognostic value of HPV-related biomarkers and thus, the need to address the management of the patients accordingly. Finally, our results have strong clinical implications as they contribute to a better classification of the patients to provide them with the best personalized treatment.

Conflict of interest statement

RM has received personal fees and non-financial support from Merck, and personal fees from AstraZeneca and MSD. MT has received non-financial support from Merck and Archimedes, and personal fees from Sanofi Pasteur and Merck. Cancer Epidemiology Research Program (LA MM SdS FXB SM ST BQ OC MT MTo MP) has received sponsorship for grants from Merck and co. The rest of authors have declared no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2018.01.010.

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