Human papillomavirus detection and comorbidity: critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials

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Background: The presence of human papillomavirus (HPV)-infection in oropharyngeal squamous cell carcinoma (OPSCC) is a major determinant in prognostic risk modeling. However, most risk models are based on clinical trials which only include a selected patient population. The clinical significance of HPV and other prognostic factors in patients with OPSCC remains to be evaluated in a large, unselected cohort, which also includes patients with stage I/II disease and patients with severe comorbidity.

Patients and methods: All patients diagnosed with OPSCC in 2000–2006 in two Dutch university hospitals were included. The presence of an oncogenic HPV infection was determined by p16-immunostaining, followed by a high-risk HPV general primer 5+/6+ DNA PCR on the p16-positive cases. Cox regression analysis was carried out to compare survival rates between HPV-positive and HPV-negative patients and a prognostic model was generated by recursive partitioning.

Results: In total, 163 of 841 (19.4%) tumors were HPV-positive. Patients with HPV-positive OPSCC had a more favorable overall survival [73.5% versus 40.9% after 5 years; \( P < 0.001 \); hazard ratio = 0.34, 95% confidence interval (CI) 0.25–0.48] compared with patients with HPV-negative OPSCC. Patients with p16-positive but HPV DNA-negative tumors showed a significantly less favorable survival than patients with p16-positive and HPV DNA-positive tumors \( (P < 0.001) \). A prognostic model was developed in which patients were classified into three risk groups according to HPV status, nodal stage and comorbidity. \([\text{Harrell’s concordance index of} 0.68 (95\% \text{ CI} 0.65–0.71)]\).

Conclusions: Tumor HPV status is a strong and independent prognostic factor for survival among patients with OPSCC. A prognostic risk model was proposed, based on our large, unselected cohort of patients with HPV status, comorbidity and nodal stage being the important prognostic factors. In addition, this study emphasizes the importance of performing an HPV DNA-specific test besides p16-immunostaining.

Key words: HPV, p16, survival, oropharyngeal squamous cell carcinoma, comorbidity

introduction

Over the last three decades, it has become clear that infection with high-risk human papillomavirus (HPV) is etiologically linked to the development of head and neck squamous cell carcinomas, particularly those carcinomas that arise in the oropharyngeal region.

Epidemiologic evidence has revealed a rapid increase in the prevalence rates of HPV-induced oropharyngeal squamous cell carcinomas (OPSCCs) in Europe and the United States [1–3]. HPV-associated oropharyngeal carcinomas are considered to be a different tumor entity, based on biological, epidemiological and clinical differences, when compared with the HPV-negative OPSCCs. The most important clinical difference between patients with HPV-positive and HPV-negative OPSCCs is related to the prognosis. Several retrospective and prospective studies in the United States, Australia and Western Europe have consistently demonstrated that HPV-positive OPSCC is associated with a more favorable prognosis [4–6]. However, a common limitation of most studies is the selection of the studied patient population. As most of these studies are based on protocol-driven trials with well-defined inclusion and
exclusion criteria, there is a selection bias tendency for younger and healthier patients. In addition, most clinical trials focus on patients with advanced stage disease who are treated by chemoradiation. Patients with stage I/II disease are treated differently and hence few data on this group are available. Recently, a recursive partitioning model (RPA) for patients with OPSCC has been proposed based on the Radiation Therapy Oncology Group study (RTOG 0129 study) [4]. This unique model has already been validated by others [7]. However, this model is based on a clinical trial in which only patients with stage III/IV disease and a Zubrod’s performance score of 0–1 were included. One has to wonder whether this model would also be applicable for the entire population of patients who present with OPSCC, or if additional prognostic factors need to be considered.

The first aim of this study was to determine the clinical significance of HPV and other prognostic factors in a large, unselected cohort of patients with OPSCC. The second aim was to validate the prognostic model for OPSCC patients as defined by Ang et al. [4] and to evaluate an additional prognostic model, based on this unselected cohort of patients.

**materials and methods**

**study design**

This study comprised all patients with an OPSCC diagnosed at two Dutch University hospitals between January 2000 and December 2006. The patients were identified through the Dutch Cancer Registries. Patient characteristics, information on smoking behavior (1 pack year = 20 cigarettes a day during 1 year) and alcohol consumption (1 unit year = one drink a day during 1 year) as well as clinical outcome were obtained from the patient files. Survival data were additionally linked to the Dutch Cancer Registries and were collected over a 5-year follow-up period. Comorbidity was classified according to the Adult Comorbidity Evaluation 27 (ACE-27) index calculator (http://oto2.wustl.edu/clinepi/calc.html), which divides comorbidity into three categories; mild, moderate and severe. The ACE-27 index is a comorbidity classification system based on the Kaplan–Feinstein Comorbidity index [8] and was proven to be of prognostic value [9].

The presence of HPV was detected using pretreatment formalin-fixed, paraffin-embedded (FFPE) biopsies. Eligible samples included histopathologically confirmed invasive squamous cell carcinoma of the oropharynx (International classification of diseases for Oncology, [ICD-10] codes C019, C051, C052, C090–C099 and C100–C109). In total, 841 of 906 (93%) tumor biopsies could be retrieved from the pathology archives. Approval for this study was obtained from the Institutional Review Board and secondary use of tissue specimen adheres to the guidelines for proper use of human tissue (www.federa.org).

**HPV testing**

A sample was scored as HPV-positive based on a positive p16-immunostaining and a subsequent positive GP 5+/6+ HPV DNA PCR, according to a validated algorithm [2, 10, 11].

Paraffin sections were prepared according to the sandwich method: the first and last sections were stained by haematoxylin and eosin to check for tumor presence. Sections of 3 μm were used for p16-immunostaining. Four sections of 10 μm were used for DNA isolation and HPV DNA detection. Precautions taken to avoid cross-contamination have been described before [2]. P16-immunostaining was carried out with a CINtec TM Histology Kit (Roche MTM laboratories AG, Heidelberg, Germany) which contains the mouse monoclonal antibody INK4A directed against p16, on a BONDmax (Leica) automated platform. Two independent observers carried out evaluation of the slides and consensus was achieved in all cases. Strong and diffuse nuclear and cytoplasmic immunostaining in more than 70% of the carcinoma tissue, was considered as p16-positive, whereas tissue with only faintly diffuse or no reactivity was considered to be p16-negative [12]. This definition is consistent with previously published articles and with clinical trial eligibility criteria in the United States (i.e. E1308 and RTOG 1016) [4, 13]. The GP5+/6+ PCR with enzyme-immunoassay (EIA) read-out was used for DNA detection of 14 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) [14]. Subsequent genotyping of the virus was carried out by bead-based array on the Lumixen platform [15]. Sample quality after DNA extraction was controlled by β-globin PCR (amplicon size 100 bp). When p16-immunostaining was positive, but GP 5+/6+ PCR was negative, type-specific PCR for HPV16 was carried out in addition using primers in the E7 gene [10].

**end points**

The end points were overall and progression-free survival. Overall survival (OS) was defined as the time from the date of incidence (defined as the date on which the squamous cell carcinoma was histologically confirmed) to death (any cause). Progression-free survival (PFS) was defined as the time period from date of incidence to death or the first documented relapse, which was categorized as local-regional recurrence or distant metastases.

**statistical methods**

Differences in patient characteristics between HPV-positive and HPV-negative cases were assessed using the Pearson $\chi^2$-test or Student’s $t$-test. Bonferroni correction was used to compare subgroups for specific variables. OS and PFS were estimated by means of the Kaplan–Meier method and survival curves were compared by use of the log-rank test. Gray’s test was used to compare rates of local-regional relapse, distant metastases and second primary tumors. Potential effect modifiers in the association between HPV and survival were analyzed by Cox regression analyses. Multivariable analysis was carried out using Cox regression with backward stepwise selection. The $P$-value to remove the variable from the model was set to $P < 0.1$. Statistical analysis was carried out using SPSS (version 15, SPSS, Inc., Chicago, IL). A $P$-value of $<0.05$ was considered to indicate statistical significance. To validate the recursive partitioning analysis (RPA) model based on the RTOG 0129 study (the RTOG RPA model) [4], all patients were divided into three risk categories according to this RTOG classification. We estimated the Kaplan–Meier group-stratified OS curves and comparison between the curves was carried out using the log-rank test. As a measure of model performance, the Harrell’s Concordance-index (Harrell’s C-Index) was used [16]. Exploration of risk models based on our data was carried out by RPA as well. The S-tree software (http://c2s2yale.edu/software/stree) was used to identify the factors that were most influential for overall survival and to permit classification of patients with OPSCC as having low, intermediate or high risk of death.

**results**

**patient characteristics and HPV detection**

P16-immunostaining was scored positive in 195 samples, of which 161 were GP5+/6+ -PCR-positive and 34 GP5+/6+ -PCR-negative. On this latter group, an E7 PCR was carried out, which identified two more HPV-positive cases. HPV genotyping was carried out on the HPV-positive cases; 149 (91.4%) contained HPV16, 7 (4.3%) HPV35, 3 (1.8%) HPV33, 2 (1.2%) HPV18 and 1 (0.6%) HPV45 and 1 HPV58. There was no significant
difference in HPV prevalence between the two university medical centers.

The association of patient variables and tumor characteristics were analyzed in relation to tumor HPV status (supplementary Data S1, available at Annals of Oncology online). Patients with HPV-positive OPSCC were less likely to have moderate to severe comorbidity (ACE-27 score 2–3), a history of heavy smoking (>24 pack years) and excessive alcohol consumption (>149 unit years) than HPV-negative patients (P < 0.001). HPV-positive patients had less advanced primary tumors than HPV-negative patients, but a more advanced nodal stage. A higher HPV prevalence was found in squamous cell carcinoma in the base of tongue and the tonsils compared with the other oropharyngeal subsites (P < 0.001, with Bonferroni correction). Significantly more tumors in the HPV-positive group were poorly differentiated compared with the HPV-negative group (P < 0.001). Patients with a p16-positive but HPV DNA-negative OPSCC (the ‘discordant’ group), showed more similarities to the HPV-negative group than to the HPV-positive group regarding patient variables (supplementary Data S1, available at Annals of Oncology online).

**survival analysis**

For analysis of the association of tumor HPV status with survival, we included all patients treated with curative intent (723 of 841). The baseline characteristics of patients excluded from the survival analysis are depicted in the supplementary Data S2, available at Annals of Oncology online. The median follow-up of patients who received treatment and remained alive was 4.33 years (range 0.1–12.1). There was no significant difference in survival between the different treatment groups for HPV-negative patients (P = 0.383) neither for HPV-positive patients (P = 0.149). An increase in HPV prevalence was seen in the period 2000–2006 (from 17.8% to 27.0%), but the year of inclusion did not confound OS (P = 0.481).

Patients with an HPV-positive OPSCC (n = 152) had a significantly better OS and PFS than patients with an HPV-negative OPSCC (n = 571) (P < 0.001 for both, Figure 1A and B). The 5-year OS rates were 73.5% in the HPV-positive subgroup and 40.9% in the HPV-negative subgroup. The 5-year PFS rates were 70.0% in the HPV-positive subgroup and 42.6% in the HPV-negative subgroup. OS and PFS survival rates of curatively treated patients with a p16-positive but HPV DNA-negative OPSCC (n = 26) were significantly worse compared with those of patients with an HPV-positive OPSCC (5-year OS: 46.2%, 5-year PFS: 45.8%, P < 0.001 compared with HPV-positive group) (Figure 1C and D). Analysis of treatment failure, revealed significantly less local-regional recurrences (6.6%), second primary tumors (6.6%) and distant metastases (7.2%) in HPV-positive patients compared with HPV-negative patients (19.1%, 21.5% and 13.0%, respectively).

Univariate analysis revealed that age, gender, comorbidity, pack years, unit years, tumor size, nodal stage and HPV status were all individually associated with OS and PFS outcomes. Nodal stage and comorbidity were effect modifiers on the relation between HPV status and survival. Therefore, different HPV survival curves are shown for patients with a low nodal stage (N0-N2a) versus patients with a high nodal stage (N2b-N3) and for patients with ACE-27 score 0-1 versus patients with ACE-27 score 2–3 (supplementary Data S3, available at Annals of Oncology online).

Multivariable analysis confirmed the independent association of age, gender, comorbidity, pack years, tumor size, nodal stage and HPV status with overall and progression-free survival (supplementary Data S4, available at Annals of Oncology online).

**recursive partitioning analysis**

First, the RTOG RPA model was validated with our patient data (supplementary Data S5, available at Annals of Oncology online). According to this model, 95 of 151 (62.9%) patients with HPV-positive OPSCC belonged to the low-risk group and 56 of 151 (37.1%) belonged to the intermediate risk group, because of their smoking status (i.e. >10 pack years). The Harrell’s C-Index was 0.58 [95% confidence interval (CI) 0.56–0.61].

An additional RPA model was developed, based on our consecutive patient cohort. Prognostic factors entered in the RPA were age, gender, tumor stage, nodal stage, pack years, unit years, comorbidity and HPV status. This analysis showed that HPV status of the tumor was the major determinant of OS, followed by nodal stage in the HPV-negative group and comorbidity in the HPV-positive group. According to this classification, 721 of 723 patients could be divided into three categories with respect to the risk of death: low, intermediate and high with corresponding 3-year survival rates of 88.1%, 59.1% and 33.5%, respectively (Figure 2). According to this RPA model, 127 of 151 (84.1%) HPV-positive patients belonged to the low-risk group and 24 of 151 (15.9%) to the intermediate risk group because of a higher ACE-27 score. The Harrell’s C-Index of this model was 0.68 (95% CI 0.65–0.71). Patients with early stage (stage I/II) and advanced stage (stage III/IV) disease were considered separately for the two models. Both Harrell’s C-indices remained unchanged.

**discussion**

This study evaluated the clinical significance of HPV and other prognostic factors in a large, unselected cohort of patients with OPSCC. In the period 2000–2006, 19.4% of all the OPSCCs diagnosed were HPV induced. This is a relatively low prevalence rate, compared with prevalence rates in the United States and other parts of Europe in the same period [4, 17, 18]. However, recently published data show that the proportion of HPV-induced OPSCC has been increasing rapidly at our center, from 5.1% in 1990 to 29.0% in 2010 [2].

HPV status was an independent prognostic factor for OS and PFS among patients with OPSCC. Recently, it was shown that this also counts for non-OPSCC (oral cavity, hypopharynx and larynx) [19]. Our analyses confirmed the prognostic model of the RTOG 0129 study and the 3-year survival rates were similar to those described previously [4]. However, the Harrell’s C-index was not optimal, probably because our patient cohort also included patients with stage I/II disease and patients with moderate to severe comorbidity. Therefore, we developed an additional prognostic risk model, based on our unselected
Our model revealed that the main prognostic factor in patients with an OPSCC is HPV status. In HPV-negative patients, nodal stage remains the most important prognostic factor, however in HPV-positive patients, nodal stage does not influence the prognosis. This is in concordance with several other studies [1, 3]. Comorbidity was the most important prognostic factor in HPV-positive patients and the second most important factor in HPV-negative patients. In other models, smoking appears to be one of the main prognostic factors [4]. In this study, smoking was indeed one of the prognostic determinants of survival in the univariate and multivariable analyses. However, in the RPA, comorbidity was a stronger prognostic factor than smoking. A reason for this could be that most of the patients in our cohort smoked more than 10 PY (87.1%), which is a very high percentage in comparison to other studies.

This new model is applicable for a more general group of patients and not only for patients included in clinical trials. Moreover, this model might be very suitable for a patient population with a high percentage of heavy smokers. According to the RTOG RPA model, 37.1% of the HPV-positive patients in our study belonged to the intermediate risk group, because of their smoking status (i.e. >10 pack years). However, a large part of these patients did in fact have a good prognosis. Therefore, a selection based on comorbidity seems to be more reliable in such patient populations.

One of the remarkable findings is the survival of patients with p16-positive but HPV DNA-negative OPSCC, which is significantly different compared with patients with ‘true’ HPV-positive OPSCC. The survival curve of this ‘discordant’ group almost converged the survival curve of patients with HPV-negative OPSCC. Furthermore, the patient characteristics of this ‘discordant group’ resembled those of HPV-negative patients. In 2011, similar results were reported [20]. At this moment, de-intensification trials are being conducted for which eligibility for randomization is based on a positive p16-immunostaining. However, this causes the risk to enroll patients with HPV DNA-negative tumors, who, according to our study, show a less favorable prognosis. Obviously, these findings should be interpreted with caution as they are based on a relatively small patient cohort.
Figure 2. Classification of patients into three risk groups (according to recursive partitioning analysis carried out on our patient cohort) and Kaplan–Meier overall survival curves for these three risk groups. Recursive partitioning analysis was used on our patient cohort to identify the most important prognostic factors and to classify patients into three risk-of-death categories (low, intermediate and high risk of death). The prognostic factors entered in the analysis were: age, gender, tumor stage (T1, T2, T3, T4), nodal stage (N0, N1, N2a, N2b, N2c, N3), pack years (dichotomized: 0–10 PY, 11–24 PY, >24 PY and dichotomized: \( \leq 10 \) PY, >10 PY), unit years (dichotomized: 0–100 UY, 111–149 UY, >149 UY and dichotomized: \( \leq 100 \) UY, >100 UY), comorbidity (ACE score 27: 0, 1, 2, 3) and HPV status. According to this recursive partitioning analysis, 721 of 723 patients could be divided into three categories with respect to the risk of death; low, intermediate and high. The 3-year overall survival in these three categories was 88.1% for the low-risk group (green line), 59.1% for the intermediate risk group (blue line) [HR compared with low risk: 3.50, 95% confidence interval (CI) 2.43–5.05] and 33.5% for the high-risk group (green line) (HR compared with low risk: 7.57, 95% CI 5.27–10.89) and Kaplan–Meier overall survival curves for these three risk groups [Harrell’s C-index 0.68 (95% CI 0.65–0.71)]. The associated 95% CIs are shown as well.
group. Moreover, these findings need to be validated, both clinically and biologically. Most logical explanation for p16-overexpression independent of HPV is a disruption of the pRb pathway by other as yet unidentified molecular mechanisms, such as activating CDK4 or CDK6 mutations combined with a premature senescence response of the cells. Genetic characterization might reveal whether these tumors are HPV induced or not [21].

In conclusion, tumor HPV status is a strong and independent prognostic factor for survival among patients with OPSCC. Comorbidity appeared to be another important prognostic factor for both HPV-positive and HPV-negative patients. This study highlights the importance of performing reliable HPV DNA testing besides p16-immunostaining to detect a true HPV factor for both HPV-positive and HPV-negative patients. This study identifies the importance of performing reliable HPV DNA testing besides p16-immunostaining to detect a true HPV-related OPSCC and to select patients for de-intensifying trials.

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disclosure
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references