Head and neck squamous cell carcinoma (HNSCC) is the sixth common cancer worldwide, with almost 650,000 new cases and 350,000 deaths each year [1]. This group of cancers represent a rather heterogeneous cohort of neoplasms originating from the oral cavity, oropharynx, hypopharynx and larynx. In the last decades, major progress has been made in the treatment of HNSCC, including the addition of concomitant chemotherapy to radiation [2], the use of altered fractionation schedules [3] and, more recently, the use of taxane-based induction chemotherapy [4]. Although outcome in terms of locoregional tumour control and overall survival has been improved significantly with these new strategies, most of these regimes have come to the expense of more severe acute and late treatment-related side-effects. Further treatment intensification will result in a further enhancement of side-effects that may hamper the feasibility of these new regimens. Therefore, in order to improve the therapeutic ratio, a more individualized approach based on specific tumour or patient characteristics that may help to differentiate better between those who will benefit from a more aggressive approach and those who will not is of increasing importance.

Alcohol and tobacco use are considered as the most important predisposing factors for HNSCC. However, there are considerable numbers of patients developing this type of cancer who have not been extensively exposed to these traditional etiologic factors.

In the United States as well in Europe, the incidence of oropharyngeal squamous cell carcinoma is rising [5, 6]. A substantial amount of data confirms that in an increasing proportion of patients with oropharyngeal squamous cell carcinoma, infection is caused by high-risk human papillomavirus (HPV) [7]. These HPV-related oropharyngeal squamous cell carcinoma have distinct epidemiological and molecular characteristics and subsequent distinct biological behaviour and clinical outcome [7] as compared with HPV-negative cases.

HPV-related oropharyngeal squamous cell carcinoma is characterized by the expression of the E6 and E7 viral oncoproteins [8]. The E6 oncoprotein causes substantial loss of p53 activity by degradation of p53 at the protein level. Consequently, normal p53 function, such as G1 cell cycle arrest or induction of apoptosis, which are important steps to allow for adequate cellular response to DNA damage, is hampered resulting in a higher susceptibility to genomic instability. The E7 oncoprotein causes degradation and inactivation of the retinoblastoma tumour suppressor gene product (pRb), preventing it from binding to the E2F transcription factor and thereby promoting cell cycle progression. As pRb normally functions as a negative regulator of p16INK4A (p16) expression, a tumour suppressor gene located on chromosome 9p21, the functional inactivation of pRb results in a reciprocal overexpression of p16 protein. As a consequence, HPV-associated oropharyngeal squamous cell carcinoma shows nuclear and cytoplasmic p16-overexpression, which is predominantly absent in HPV-negative oropharyngeal squamous cell carcinoma. On the contrary, according to the molecular progression model for tobacco-induced HNSCC, loss of 9p21 is the most frequent event and is also present in the earliest definable lesions, including dysplasia and carcinoma in situ. Hence, p16 positivity is currently regarded as a reliable surrogate marker for HPV-related oropharyngeal carcinoma.

Since 1986, the first time that HPV16 DNA was detected in an invasive HNSCC by Southern blot hybridization [9], HPV DNA sequences have been repeatedly detected in a variable proportion of HNSCC, from <10% to up to 100% [10–12] apparently biased by the anatomic location of tumours and HPV detection techniques. Weinberger et al. [13] hypothesized that p16-overexpression in oropharyngeal squamous cell carcinoma defines HPV-induced tumours with favourable prognosis. These investigators hypothesized that in HNSCC the detection of HPV DNA by itself does not prove a causal relationship as only transcriptionally active HPV DNA would be biologically and clinically relevant in the development and clinical outcome of HNSCC. P16 protein expression was used as a surrogate marker for E7 oncoprotein expression. In a cohort of 107 oropharyngeal squamous cell carcinomas treated with primary radiotherapy or surgery followed by postoperative radiotherapy, they were able to identify three distinct classes: class I consisting of HPV16-negative tumours (39%); class II consisting of HPV16-positive tumours that were p16-negative (23%); and: class III consisting of HPV16-positive tumours that were p16-positive as well (37%). Only tumours in class III showed significantly lower expression of p53 and pRb, which was not the case in class II and I. In addition, the distinction between class II and class III was also reflected in differences in clinical outcome with an overall survival of 79% in class III compared with 20% and 18% in class I and II, respectively. Comparable results were found for locoregional tumour control and disease-free survival. Taken together, these results indicate that simple p16 immunohistochemistry is sufficient for determination of the subset of patients (class III)
with biologically relevant, HPV-induced oropharyngeal squamous cell carcinoma. Based on the findings of this study, several investigators subsequently studied the association between p16 expression and HPV status combined with their prognostic relevance.

In the current issue of Annals of Oncology, Fischer et al. [14] reported on the results of a single institution retrospective study investigating the prognostic significance of p16-expression among 102 assessable patients with oropharyngeal squamous cell carcinoma treated with either radiotherapy or surgery in the early stages (stages I–II) or with combined modality treatment in the more advanced stages (stages III–IV). The authors concluded that p16 was the most relevant prognostic factor in oropharyngeal squamous cell carcinoma and that this factor should be considered for inclusion into the official staging system of HNSCC.

However, the question arises as to how these results should be interpreted in relation to the results of the prognostic and predictive value as reported by other investigators. In addition, the question arises as to whether p16-status may impact the treatment strategy to be selected and/or the design of future clinical studies.

correlation between p16 and HPV status

In many studies investigating the prognostic significance of p16-overexpression, the investigators assumed a significant correlation between HPV-positivity determined by in situ hybridisation and p16 overexpression. In situ hybridisation with an HPV16 probe has a sensitivity of one to two copies of integrated HPV DNA. When the virus is integrated, the HPV hybridisation signal is seen as punctate nuclear staining. Some investigators use HPV in situ hybridisation to determine the integration status and, presumably, the transcriptional activity of HPV in oropharyngeal tumours. They interpret punctate staining in tumours as a marker of integrated transcriptionally active virus.

Very recently, Singhi and Westra [15] reported on the results of a study in which HPV16 in situ hybridisation was tested together with p16 immunohistochemistry in 256 patients with HNSCC. A total of 182 of 256 tumours (71.2%) were HPV16-positive of which the vast majority (87%) originated in the oropharynx. In this study, all HPV16-positive cases showed p16-positive staining, while p16 positivity was present in 19 of 80 (24%) HPV16-negative tumours, out of which 6 could be explained by the presence of other HPV subtypes. The results of this study correspond to a sensitivity, specificity, positive and negative predictive value of p16 expression in relation to HPV status of 100%, 74%, 91% and 100%, respectively.

These findings are in line with those reported in another recently published series, in which p16 overexpression was found in 192 of 206 (93.2%) HPV-positive oropharyngeal squamous cell carcinoma, of which 198 (96.1%) were positive for HPV16 as assessed by in situ hybridisation. In HPV-negative patients, p16 overexpression was found in 22 of 117 patients (18.8%) [16]. In this study, paraffin-embedded tissues were first evaluated for HPV16 DNA and additional evaluations for 12 other HPV subtypes were carried out in case of HPV16 negativity. The investigators found that 198 of 206 HPV-positive patients (96.1%) were HPV16 positive. In this study, the sensitivity, specificity, positive and negative predictive values were 96%, 81%, 90% and 93%, respectively.

Smeets et al. [17] sought to determine a detection algorithm of a biologically and clinically meaningful HPV infection. The authors considered HPV E6 oncogene expression in frozen biopsies as a gold standard for a meaningful HPV infection and they evaluated the utility of the following assays on formalin-fixed paraffin-embedded tumour specimens and sera of 48 HNSCC patients: HPV DNA general primer (GP)5+/6+ PCR, viral load analysis, HPV16 DNA fluorescent in situ hybridisation detection, HPV16 E6 messenger RNA RT-PCR, p16 immunostaining, and on corresponding serum samples detection of antibodies against the HPV16 proteins L1, E6 and E7. The authors found that the most suitable algorithm with 100% sensitivity and specificity was p16 immunostaining followed by GP5+/6+ PCR on the p16-positive patients.

To summarize, p16 immunohistochemical expression appears to be a reliable surrogate marker for clinically and biologically relevant HPV infection. HPV in situ hybridisation has high specificity but will miss HPV infection caused by rare subtypes for which the corresponding probes are not included in the analysis.

p16 as prognostic and predictive factor

In the last decade, numerous investigators reported on the prognostic value of HPV/p16 positivity in HNSCC in general or oropharyngeal squamous cell carcinoma in particular and have confirmed that patients with HPV-related tumours in the head and neck region show better prognosis as compared with HPV-negative patients [16, 18, 19].

The prognostic significance of HPV/p16 positivity has been shown in particular among patients treated with radiotherapy alone or with radiotherapy combined with systemic treatment. Recently, Lassen et al. [18] reported on a retrospective analysis of 331 patients who were included in the DAHANCA-5 study in which patients were randomly assigned to receive radiotherapy alone or radiotherapy with the hypoxic modifier nimorazole. In this study, locoregional tumour control among patients with p16-positive tumours was significantly better than that observed among patients with p16-negative tumours with an 60% reduction of locoregional failures [hazard ratio (HR) = 0.40; 95% confidence interval (CI) 0.27–0.59]. In addition, Ang et al. reported on the results of a retrospective analysis of 323 patients who were included in a prospective study in which patients were randomly assigned to receive standard or accelerated fractionation. In that study, 63.8% of the patients with oropharyngeal squamous cell carcinoma were HPV positive and 61.0% were p16 positive. Overall survival was significantly better in patients with HPV-positive tumours (HR = 0.38; 95% CI: 0.26–0.55) and in patients with p16-positive tumours (HR = 0.29; 95% CI: 0.20–0.43) after adjustment for other potential confounders, such as age, tumour and nodal stage and...
treatment assignment [16]. It is important to note that p16 was a stronger survival predictor compared with in situ hybridisation. These authors also carried out a recursive partitioning analysis to identify distinct prognostic groups and were able to classify patients into three prognostic groups (low, intermediate and high risk) based on four prognostic factors including HPV status, pack-years of tobacco smoking, and T and N classification. Among patients with HPV-positive tumours, those with >10 pack-years and with N2b-N3 nodal status did significantly worse (3-year overall survival rate: 93.0%) as compared with those with ≤10 peak-years or with >10 pack-years but with N0-N2a nodal status (3-year overall survival rate: 70.8%). These results suggest that selection of different prognostic groups based on HPV status alone will not be sufficient to select those who have a very good prognosis.

Fischer et al. [20] found that p16-positive oropharyngeal squamous cell carcinoma showed better prognosis in terms of overall survival after primary radiotherapy as well as after primary surgery plus or minus adjuvant radiotherapy. Similar results, i.e. better outcome in HPV/p16-positive tumours, have been found after induction chemotherapy and/or chemoradiation [21, 22]. In other words, positive HPV/p16 status has been consistently found to be a favourable prognostic factor in terms of locoregional control and overall survival irrespective of treatment modality. Until now, it remains unclear why HPV-related oropharyngeal squamous cell carcinomas show better outcome than their HPV-negative counterparts. Although HPV/p16 status is an important prognostic factor in particular in oropharyngeal squamous cell carcinoma, most studies that reported on the prognostic significance of p16 status among patients treated within the context of randomised controlled trials did not show a difference in the benefit of new treatment strategies between the treatment arms [16, 17, 23, 24]. In other words, whether a specific patient will benefit more from one treatment strategy or another could not be predicted by p16 expression as the benefit in terms of HR was similar between p16-positive and p16-negative patients. The only exception on this was found among patients treated in randomised DAHANCA 5 trial in which the authors showed that the hypoxic cell radiosensitiser nimorazole significantly improved the outcome in HNSCC [23]. In this study, in the subset of patients with p16-negative tumours, locoregional control was worse in the placebo group than in the nimorazole group (HR 0.69; 95% CI: 0.50–0.95). However, in the subset of patients with p16-positive tumours, patients treated with nimorazole had similar locoregional control as compared with those in the placebo arm (HR 0.93; 95% CI: 0.45–1.91). The authors concluded that hypoxic modification improved outcome in p16-negative tumours but was of no significant benefit in p16-positive tumours, suggesting that hypoxic radioresistance may not be clinically relevant in these tumours.

Patients with p16-positive tumours resemble a distinct subset of HNSCC, with a different molecular profile, clinical presentation and response to treatment. In that respect, p16 expression should at least be used as stratification factor in randomised clinical trials investigating new treatment strategies in HNSCC.

However, it should be emphasized that within the p16-positive cases, subsets of patients with different prognosis can be identified. Moreover, the added value of new strategies appears to be similar among p16-positive and p16-negative patients. Therefore, based on the currently available data, selecting p16-positive patients for less aggressive treatment approaches is still not justified. Clinical trials that investigate more or less intense therapy in particular in p16-positive patients should now be undertaken. The time for molecular classification of HNSCC has come.

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