Effect of HPV-Associated p16\(^{\text{INK4A}}\) Expression on Response to Radiotherapy and Survival in Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Purpose
A subset of head and neck cancers is associated with the human papillomavirus (HPV). Viral infection is closely correlated with expression of p16\(^{\text{INK4A}}\) in these tumors. We evaluated p16\(^{\text{INK4A}}\) as a prognostic marker of treatment response and survival in a well-defined and prospectively collected cohort of patients treated solely with conventional radiotherapy in the Danish Head and Neck Cancer Group (DAHANCA) 5 trial.

Patients and Methods
Immunohistochemical expression of p16\(^{\text{INK4A}}\) was analyzed in pretreatment paraffin-embedded tumor blocks from 156 patients treated with conventional primary radiotherapy alone. The influence of p16\(^{\text{INK4A}}\) status on locoregional tumor control, disease-specific survival, and overall survival after radiotherapy was evaluated.

Results
p16\(^{\text{INK4A}}\) positivity was found in 35 tumors (22%). Tumor-positivity for p16\(^{\text{INK4A}}\) was significantly correlated with improved locoregional tumor control (5-year actuarial values 58% vs 28%; \(P = .0005\)), improved disease-specific survival (72% vs 34%; \(P = .0006\)), and improved overall survival (62% vs 26%; \(P = .0003\)). In multivariate analysis, p16\(^{\text{INK4A}}\) remained a strong independent prognostic factor for locoregional failure (hazard ratio [HR], 0.35; 95% CI, 0.19 to 0.64), disease-specific death (HR, 0.36; 95% CI, 0.20 to 0.64), and overall death (HR, 0.44; 95% CI, 0.28 to 0.68).

Conclusion
Expression of p16\(^{\text{INK4A}}\) has a major impact on treatment response and survival in patients with head and neck cancer treated with conventional radiotherapy.

INTRODUCTION

Although tobacco and alcohol consumption are the primary risk factors for development of head and neck squamous cell carcinoma (HNSCC), it has recently become evident that infection with high-risk human papillomavirus (HPV) is etiologically linked to a subgroup of cancers in the head and neck region.1,2 Some 90% of HPV-positive head and neck tumors are infected with high-risk HPV16.3,4 Viral DNA is detected in some 20% of HNSCCs, the strongest association being found in oropharyngeal tumors, where carcinomas of the tonsils are particularly associated with HPV infection.4,5

Recent studies have indicated that in HNSCC, patients with HPV-infected tumors have a more favorable prognosis compared with patients whose tumors are virus-negative.8,9,10 HPV positivity seems to be associated with lower exposure to tobacco and alcohol and with younger age at time of diagnosis, factors that may by themselves positively influence prognosis regardless of tumor biology. However, evidence is accumulating that HPV-positive tumors represent a separate biologic subgroup of HNSCC distinct from those carcinomas that are induced by tobacco and alcohol. The biologic differences between these subtypes of HNSCC may per se have an impact on prognosis.1,12

The viral oncoproteins E6 and E7 play a key role in HPV-associated carcinogenesis, abrogating p53 and retinoblastoma (Rb) tumor suppressor functions, respectively.13,14 HPV-positive HNSCCs are generally associated with wild-type TP53, as opposed to tobacco-induced tumors, which are characterized by genetic alterations of the TP53 pathway.12,13 The functional inactivation of Rb by E7 leads in turn to upregulation of the p16\(^{\text{INK4A}}\)
protein. p16\textsuperscript{INK4A}, encoded by the CDKN2A tumor suppressor gene, regulates the activity of Cyclin D-CDK4/6 complexes (CDKs). When not inactivated by p16\textsuperscript{INK4A}, these CDKs phosphorylate Rb, resulting in release of the transcription factor E2F, which initiates cell cycle progression.\textsuperscript{15} In contrast, the bound Rb-E2F protein complex acts as a negative regulator, inhibiting transcription of several genes, including CDKN2A. The functional inactivation of Rb by E7 therefore results in release of the p16\textsuperscript{INK4A} gene from its transcriptional inhibition.\textsuperscript{16} Thus HPV-positive tumors are characterized by high expression of p16\textsuperscript{INK4A}.\textsuperscript{8-10,17} Moreover, because transcription of the E7 oncogene is required for p16\textsuperscript{INK4A} upregulation, it has been suggested that carcinomas overexpressing p16\textsuperscript{INK4A} represent those tumors in which HPV has been involved in the carcinogenic process.\textsuperscript{18} Thus there is good evidence that p16\textsuperscript{INK4A} positivity may be regarded as a biomarker for tumors harboring clinically and oncogenetically relevant HPV infections.

HNSCC is predominantly a locoregional disease, and achieving local tumor control is essential for survival.\textsuperscript{19} Radiotherapy can significantly improve local control and disease-specific survival in patients with HNSCC, either when used as a single treatment modality or in combination with chemotherapy\textsuperscript{20} or hypoxic modification.\textsuperscript{21,22} Over the years, radiotherapy has been optimized through modification of tumor hypoxia and by reducing overall treatment time of radiotherapy.\textsuperscript{21-24} Although this has resulted in improved treatment response for patients with HNSCC as a whole, individual patients with HNSCC still show considerable variation in clinical outcome. This indicates that other factors defining tumor response are still to be discovered.\textsuperscript{25,26} and HPV infection may be one such factor.

With the present study, we aimed to evaluate the impact of HPV-associated tumor cell p16\textsuperscript{INK4A} expression on response to radiotherapy and survival in a well-characterized and prospectively collected cohort of Danish patients with head and neck cancer treated with conventionally fractionated radiotherapy alone.

### Patients and Methods

**Patients and Tissues**

One hundred ninety-five patients with primary squamous cell carcinoma of the pharynx (stages I through IV) or supraglottic larynx (stages II through IV) were enrolled in the placebo arm of the randomized Danish Head and Neck Cancer Group (DAHANCA) 5 protocol from January 1986 to September 1990.\textsuperscript{22} Patients in this arm received primary conventionally fractionated radiotherapy (66 to 68 Gy in 33 to 34 fractions, 5 fractions/wk) as the only treatment. Routine paraffin-embedded, formalin-fixed pretreatment tumor tissues were available from the pathology archives in sufficient amounts for study in 156 of the total 195 patients (80%). The DAHANCA 5 study was designed according to the Helsinki Declaration II, and the patients gave written informed consent. Both the main study and the tumor tissue analyses were approved by the local ethics committees according to Danish law and regulations.

**Evaluation of p16\textsuperscript{INK4A} Expression**

Immunohistochemistry for p16\textsuperscript{INK4A} expression was performed on a BenchMark XT autostainer (Ventana Medical Systems, Illkirch, France) according to the manufacturer’s recommendations. Briefly, paraffin sections were cut at 5 μm on Superfrost plus charged glass slides (Menzel-Glaser, Braunschweig, Germany), heated at 60°C for 1 hour, and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 solution (CC1, Ventana Medical Systems). p16\textsuperscript{INK4A} was detected by incubating sections with antibody clone JC8 (sc-56330; Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1:25 for 32 minutes. This is a mouse monoclonal immunoglobulin G2a antibody raised against full-length human p16, particularly suitable for use on formalin-fixed, paraffin-embedded sections. Its specificity has been confirmed by Western blotting.\textsuperscript{27}

![Fig 1. (A) HPV16 DNA-positive tonsillar carcinoma. Numerous tumor cells show positive nuclear signals. (B) The same tumor showing strong specific tumor cell expression of p16\textsuperscript{INK4A}. (C) HPV16-negative tonsillar carcinoma. (D) The same tumor showing no expression of p16\textsuperscript{INK4A}. (A, C) HPV16 DNA in situ hybridization. (B, D) Immunohistochemistry with antip16\textsuperscript{INK4A} antibody clone JC8.](image-url)
Specific reactions were detected using ultraView Universal DAB Detection Kit (Ventana Medical Systems), and the slides were counterstained with hematoxylin. Sections of p16INK4A-positive cervical carcinoma were used as positive controls. p16INK4A expression was associated with distinct diffuse nuclear and cytoplasmic staining of epithelial cells. Tumors were classified dichotomously as either p16INK4A-positive (strong, diffuse nuclear and cytoplasmic staining in >10% of carcinoma cells) or negative (Fig 1).

Detection of HPV Infection

To demonstrate the correlation between HPV16 infection and tumor cell expression of p16INK4A, we performed a pilot study on a separate series of 32 tonsillar carcinomas, using in situ hybridization for detection of viral DNA. Briefly, 5-µm formalin-fixed, paraffin-embedded tumor sections were deparaffinized and treated with Dako Target Retrieval Solution (Dako, Glostrup, Denmark). Sections were digested with proteinase K (Dako, S3004) and rinsed in deionized water. Biotinylated HPV16-type specific DNA probe (Dako, code Y1407) in hybridization mixture was used according to the manufacturer’s recommendations. Denaturation and hybridization were carried out using a Hybridizer Instrument (Dako). Sections were denatured for 5 minutes at 92°C and hybridized overnight at 37°C. After stringent washing, hybridized probe was detected using the tyramide-based GenPoint Catalyzed Signal Amplification System (Dako, code K0620), with consecutive application of primary peroxidase-conjugated streptavidin, biotinyl tyramide, and secondary peroxidase-conjugated streptavidin. Signals were visualized with the chromogen 3,3-diaminobenzidine. Positive and negative control sections (SiHa, HeLa, bladder cancer J82) were included. Carcinomas were classified as HPV16 positive when a discrete signal was seen localized to the nuclei of tumor cells. Signals were categorized as either punctuate and/or diffuse, representing integrated and episomal virus, respectively.28-30 The findings of the pilot study have been previously published in abstract form.31 We found a strong correlation between HPV16 DNA detection and p16INK4A expression (Fig 1, Table 1).

Statistical Analysis

The end points used were locoregional control after radiotherapy, disease-specific survival, and overall survival. Locoregional control was defined as complete and persistent disappearance of the disease in the primary tumor (T site) and regional lymph nodes (N site) after radiotherapy. Failure was recorded in the event of recurrent tumor or if the primary tumor never completely disappeared. In the latter situation, the tumor was then assumed to have failed at the time of randomization. The end point does not, therefore, include the effect of a successful procedure with salvage surgery. Disease-specific survival was defined as death from or with the actual cancer, and overall survival was defined as death from any cause. Follow-up of the patients was completed in connection with the original publication of the study.22 All patients were observed for at least 5 years or until death. The survival status has subsequently been tracked until June 2004.

Features of p16INK4A-positive and -negative tumors were compared using the χ2 test for categoric variables. The actuarial values of the end points were evaluated by the Kaplan-Meier analysis and compared using the log-rank test. A multivariate Cox proportional hazard analysis was used to evaluate prognostic parameters and treatment with respect to the risk of locoregional failure.
significant improvement in locoregional tumor control was found in patients with p16INK4A-positive tumors as compared with patients with p16INK4A-negative tumors (58% vs 28%, respectively; P = .0005; OR, 0.26; 95% CI, 0.12 to 0.57). Looking in detail at locoregional tumor control after radiotherapy, tumor expression of p16INK4A is seen to be associated with benefit in all subgroups; however, this benefit is greater in certain subgroups compared with others (Fig 3). The more advanced the disease (N+; stage III to IV), the greater the benefit. Patients with histopathologically poorly differentiated tumors or with tumors of oropharyngeal origin profit more from having tumor cell p16INK4A positivity than do patients with moderately or well-differentiated tumors or with tumors of nonoropharyngeal origin. Moreover, women with p16INK4A-positive tumors seem to have a rather pronounced advantage over men with p16INK4A-positive tumors with regard to locoregional tumor control after radiotherapy.

The effect of the improved locoregional tumor control in the p16INK4A-positive group is reflected in the survival outcome parameters. Survival curves demonstrating the disease-specific and overall survival benefits of patients according to p16INK4A expression status are shown in Figure 4. Patients with p16INK4A-positive tumors had significantly lower disease-specific mortality (OR, 0.30; 95% CI, 0.14 to 0.66) and overall mortality (OR, 0.22; 95% CI, 0.08 to 0.56) than did patients with p16INK4A-negative tumors.

In the final Cox proportional hazards analysis with risk of locoregional failure as the end point, low tumor classification (T1/2, hazard ratio [HR], 0.56; 95% CI, 0.37 to 0.84), negative neck nodes (HR, 0.51; 95% CI, 0.33 to 0.78), and p16INK4A expression (HR, 0.35; 95% CI, 0.19 to 0.64) were independent factors associated with a good prognosis. Similarly, the same three parameters were independent prognostic factors when analyzed with death from cancer and overall death as end points: T1/2 tumors, HR, 0.61 (95% CI, 0.40 to 0.92) and HR, 0.66 (95% CI, 0.46 to 0.93), respectively; negative nodes, HR, 0.46 (95% CI, 0.30 to 0.71) and HR, 0.52 (95% CI, 0.37 to 0.75), respectively; and p16INK4A expression, HR, 0.36 (95% CI, 0.20 to 0.64) and HR, 0.44 (95% CI, 0.28 to 0.68), respectively. No other parameters were independently associated with treatment outcome.

### RESULTS

Thirty-five (22%) of the 156 tumors expressed p16INK4A. Table 2 shows patient and tumor characteristics according to p16INK4A status. The highest frequency of p16INK4A expression was seen in oropharyngeal tumors, where 24 (32%) of 74 tumors were p16INK4A-positive. Moreover, 69% of the p16INK4A-positive tumors originated in the oropharynx compared with only 41% of the p16INK4A-negative group (P = .004). Median age and sex did not differ significantly between the groups, and the vast majority of patients in both groups were diagnosed in disease stage III or IV. Similarly, no statistically significant difference between the groups regarding histopathologic tumor differentiation was found.

At the time of evaluation, 97 patients had failed to achieve persistent locoregional control within the irradiated volume. A total of 93 patients had died with or from the cancer in question, and overall, 134 patients had died. The number of locoregional failures was associated with high stage of disease (stage III to IV; P = .05) and, to a lesser extent, to nodal spread (P = .08) and high tumor classification (T3/4; P = .09). Patients with p16INK4A-positive tumors were significantly less likely to suffer locoregional recurrence than were patients with p16INK4A-negative tumors (P = .001).

Figure 2 shows the probability of locoregional tumor control 5 years after radiotherapy according to p16INK4A status. A statistically significant improvement in locoregional tumor control was found in patients with p16INK4A-positive tumors as compared with patients with p16INK4A-negative tumors (58% vs 28%, respectively; P = .0005; OR, 0.26; 95% CI, 0.12 to 0.57). Looking in detail at locoregional tumor control after radiotherapy, tumor expression of p16INK4A is seen to be associated with benefit in all subgroups; however, this benefit is greater in certain subgroups compared with others (Fig 3). The more advanced the disease (N+, stage III to IV), the greater the benefit. Patients with histopathologically poorly differentiated tumors or with tumors of oropharyngeal origin profit more from having tumor cell p16INK4A positivity than do patients with moderately or well-differentiated tumors or with tumors of nonoropharyngeal origin. Moreover, women with p16INK4A-positive tumors seem to have a rather pronounced advantage over men with p16INK4A-positive tumors with regard to locoregional tumor control after radiotherapy.

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cell expression of p16INK4A in this group of patients is associated with HNSCC treated with radiotherapy alone. This study shows that tumor therapeutic response in a prospectively collected cohort of patients with p16INK4A-positive and p16INK4A-negative carcinoma of the pharynx and supraglottic larynx treated with radiotherapy alone.

We analyzed the association of tumor p16INK4A expression with therapeutic response and survival and that p16INK4A expression status is an independent prognostic factor for both outcomes. The potent prognostic value of p16INK4A expression is emphasized by the fact that this marker proved to be an even stronger independent prognostic factor than the classical clinical parameters of tumor stage and nodal status, the only other independent variables of prognostic significance in this study.

The beneficial locoregional control rate observed in the p16INK4A-positive group indicates that these tumors respond well to conventionally fractionated radiotherapy. The biologic basis for this observation could reside in intrinsic tumor cell differences that render p16INK4A-positive cells more susceptible to radiation than p16INK4A-negative tumor cells. A possible explanation could be that tumor cells expressing p16INK4A are less hypoxic and potentially respond with less pronounced accelerated repopulation when irradiated, but the precise mechanisms are not known at the moment. Similarly, it is also not clear to what degree patients with p16INK4A-positive tumors benefit from hypoxic modification and accelerated fractionation treatment schedules, and this is an area that warrants further investigation.

In the studies by Kumar et al.11 and by Fakhry et al.,8 patients were treated with induction chemotherapy followed by concomitant chemoradiotherapy. Both groups reported treatment response rates and survival benefits for HPV-positive tumors similar to those found in our study. This indicates that p16INK4A-positive tumors in general have a better response to treatment than p16INK4A-negative tumors, regardless of treatment modality, but that the use of radiation either as monotherapy or in combination with chemotherapy seems efficacious.

The survival benefits observed for patients with p16INK4A-positive tumors in this study can partly be ascribed to the improved locoregional control rate in this group.

Analysis of known patient and tumor characteristics identified no substantial differences between the two patient groups. In particular, p16INK4A-positive tumors seemed to be more closely associated with poor histopathologic differentiation (54%) compared with p16INK4A-negative tumors (38%), but the difference was not significant at the 5% level, possibly because of lack of power. The majority of patients in both groups had nodal spread at time of diagnosis (63% and 59%, respectively), which reflects that patients with HNSCC in general are diagnosed at advanced stages regardless of p16INK4A status.

However, patients with p16INK4A-positive tumors could potentially have less comorbidity and a different risk profile in terms of exposure to tobacco and alcohol than the patients in the p16INK4A-negative group. Information on lifetime exposure to tobacco and alcohol and performance status was, however, not available. Therefore, confounding by these variables on the survival analysis cannot be completely ruled out.

Recent studies have demonstrated the correlation between HPV and p16INK4A expression status in HNSCC.9,10,17,32 Using HPV16 DNA in situ hybridization and p16INK4A immunohistochemistry on parallel sections in a series of tonsillar carcinomas, we confirmed these findings. We believe that p16INK4A expression is a reliable biomarker of infection with HPV in HNSCC. However, the importance of p16INK4A may potentially extend beyond that of merely being a surrogate marker of infection with HPV. From the molecular processes involved in viral carcinogenesis, it is possible to argue the case that p16INK4A positivity may specifically identify HPV infections in HNSCC that are biologically relevant in carcinogenesis (ie, infections in those tumors in which HPV is transcriptionally active). A similar hypothesis was proposed and tested by Weinberger et al.10 They demonstrated that double positivity in tumors for both HPV infection and
p16INK4A expression identified those tumors with a favorable prognosis and that the presence of HPV in the tumors per se did not have a substantial positive impact on prognosis.

It should be noted that expression of p16INK4A is not limited to HPV-positive tumors, and using this marker alone as an indicator of biologically relevant HPV infections inevitably entails the risk of including some (virally) false-positive results. Recently, Smets et al. proposed an algorithm for reliable detection of biologically relevant HPV infections in paraffin-embedded head and neck cancer specimens. Initial evaluation of tumors by p16INK4A immunohistochemistry, followed by polymerase chain reaction—based detection of HPV in the p16INK4A-positive cases, produced a sensitivity and specificity of 100%. The risk of both assays yielding a false-positive result in the same sample was estimated to be 2%. We believe there is sufficient circumstantial evidence to support the view that the p16INK4A-positive tumors in our cohort of patients with head and neck cancer represent those carcinomas infected with HPV.

The association between HPV and HNSCC is strongest for oropharyngeal carcinomas and, in particular, for carcinomas of the tonsil. Consistently in this cohort, tumors of oropharyngeal origin had the highest frequency of p16INK4A expression, and a detection rate of 32% in the oropharynx is in good agreement with viral detection rates observed in Swedish tonsillar carcinoma specimens from the late 1980s.

According to the Danish cancer registry, there has been a three-fold increase in the incidence of tonsillar cancer in Denmark over the past 30 years, and similar findings are reported from other parts of the Western world. This increase has occurred at the same time as there has been a pronounced decrease in the classical tobacco-induced carcinomas of the larynx and lung, and it is thought that this increase in tonsillar cancer may be attributable to an increase in HPV-associated tumors. If this hypothesis proves correct, then it suggests that we may soon face a substantial increase in the number of oropharyngeal carcinomas in the future, as the importance of HPV infection becomes apparent. In that setting, it will be essential to acknowledge the importance of the strong and so far not duly appreciated influence of p16INK4A expression on prognosis for patients with head and neck cancer treated with radiotherapy. In particular, p16INK4A status needs to be taken into account whenever a clinical trial is conducted and interpreted. Failure to recognize the importance of p16INK4A could lead to confounding, and p16INK4A status should be included as a prerandomization stratification factor in future clinical trials conducted on patients with head and neck cancer treated with radiotherapy.

The majority of patients with p16INK4A-positive tumors are diagnosed at advanced clinical stages. Despite having large tumors and nodal involvement, these patients respond extremely well to radiotherapy, which generally seems to make them complete responders and long-term survivors. Thus patients with p16INK4A-positive tumors are candidates for organ preservative therapeutic procedures, and the approach to elective neck dissection after radiotherapy in these patients should be rather cautious to minimize long-term morbidity of therapy.

In conclusion, our study shows that p16INK4A expression status should be a major determinant of future therapeutic strategies in patients with HNSCC.

REFERENCES


AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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