Relationship between Epidermal Growth Factor Receptor Status, p16\textsuperscript{INK4A}, and Outcome in Head and Neck Squamous Cell Carcinoma

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Abstract

Background: Human papilloma virus (HPV) infection is a powerful prognostic biomarker in head and neck squamous cell carcinoma (HNSCC). Increased epidermal growth factor receptor (EGFR) gene copy number and protein expression have been reported to be negative predictors of outcome. This study examined the relationship between HPV status, EGFR gene copy number, EGFR protein expression, and clinical outcome in HNSCC patients treated with chemoradiation.

Methods: HPV status was determined using p16\textsuperscript{INK4A} immunohistochemistry (IHC), EGFR gene copy number was evaluated with FISH, and EGFR protein expression by IHC in 212 subjects.

Results: EGFR FISH was positive in 41 of 204 (20%) patients and was negatively correlated with failure-free survival (FFS; HR = 1.84, \(P = 0.027\)) and overall survival (OS; HR = 1.78, \(P = 0.082\)). For p16\textsuperscript{INK4A}, 85 of 200 (42.5%) patients were found to be p16 positive, including 75 of 131 (57%) with oropharyngeal cancer. Patients with p16-positive oropharyngeal cancer had significantly improved FFS (HR = 0.28, \(P < 0.001\)) and OS (HR = 0.31, \(P = 0.002\)). Only 2 of 126 (1.6%) oropharyngeal cancer patients were found to be p16\textsuperscript{+}/EGFR FISH\textsuperscript{+}. EGFR IHC was positive in 81 of 93 (87%) of patients and was associated with poorer FFS (HR = 1.98, \(P = 0.35\)) and OS (HR = 2.52, \(P = 0.22\)).

Conclusions: Increased EGFR gene copy number is largely restricted to p16\textsuperscript{INK4A}-negative oropharyngeal cancer. Although p16\textsuperscript{INK4A} and EGFR FISH are both predictive of outcome in univariate analyses, only p16\textsuperscript{INK4A} remains independently predictive.

Impact: Knowledge of HPV and EGFR status can have implications for treatment options and prognosis in HNSCC. Cancer Epidemiol Biomarkers Prev; 20(6); 1–8. \(\copyright\)2011 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC), the seventh most commonly diagnosed cancer worldwide (1), has traditionally been linked to alcohol and tobacco abuse (2) and is associated with survival rates that have remained poor over the past 30 years. To improve outcome, it is important to identify biomarkers of prognosis and response to therapy to enable better patient selection for risk-adapted treatment strategies and emerging targeted molecular therapies.

It is now evident that a significant proportion of HNSCCs, particularly oropharyngeal cancers, are caused by human papilloma virus (HPV; ref. 3). High-risk HPV subtype 16 accounts for more than 85% of all HPV-positive (HPV+) tumors in HNSCC (4). HPV-positive oropharyngeal cancer has an epidemiologic, clinical, and molecular profile that is distinct from HPV-negative (HPV−) oropharyngeal cancer (5). Patients with HPV-positive tumors are typically younger, less likely to use tobacco or alcohol, and have a higher number of lifetime sexual partners (6). Importantly, HPV-positive status is associated with improved prognosis (7–9). Increased expression of p16\textsuperscript{INK4A} (hereafter denoted as p16) results directly from functional inactivation of the retinoblastoma protein (pRb) by the HPV viral oncoprotein E7 (5), and detection of p16 by immunohistochemistry (IHC) has been shown to be a robust surrogate marker of HPV infection in HNSCC (10–12).

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor of the ERB-B family

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that is overexpressed in many tumor types including HNSCC (13). Importantly, efforts to therapeutically target EGFR in HNSCC by using the monoclonal antibody cetuximab have proven effective in combination with radiotherapy or chemotherapy (14, 15). A number of studies have investigated the relationship between high EGFR protein expression (as determined by IHC) and poor outcome in HNSCC. Results have been varied, with several groups showing correlations between EGFR protein levels and outcome (11, 16–21) whereas other groups have shown no correlation (22, 23). Increased EGFR gene copy number in HNSCC, as determined by FISH, was reported by Chung and colleagues (24), who showed that high EGFR gene copy number (as defined by high polysomy or amplification) was frequent in HNSCC and was a negative prognostic marker. In an independent study, Temam and colleagues (25) also found inferior outcome of HNSCC patients with increased EGFR copy number, albeit with a lower frequency of FISH positivity.

The relationship of HPV status with EGFR protein expression and clinical outcome has been reported in several small series (10, 11, 18, 19, 21, 26), indicating that EGFR protein levels and outcome (11, 16–21) whereas other groups showi ng correlations between gene copy status. An inverse relationship between HPV status and EGFR gene amplification has been reported (27, 28); however, to date of studies have investigated the relationship between HPV (p16) status with EGFR gene copy number and EGFR protein expression in a cohort of 212 HNSCC patients treated with chemoradiation and further examined the relationships of these markers with clinical outcome parameters.

Materials and Methods

Patients

This study comprised a cohort of 240 patients enrolled in 3 successive clinical trials (phases I, II, and III) administered from our institution, all of which have been previously described (29–31). The treatment regimens involved definitive radiotherapy concurrently with cisplatin plus tirapazamine (phase I), cisplatin plus tirapazamine or cisplatin/5-fluorouracil (phase II), or cisplatin plus tirapazamine or high-dose cisplatin alone (phase III). Major eligibility criteria for the trials included previously untreated squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx; stage III or IV disease (excluding distant metastases and T1N1 on phase II and T1–2N1 on phase III, whereas phase I patients had to have T3–4 and/or N2–3 disease); age more than 18 years, performance status [Eastern Cooperative Oncology Group (ECOG)] 0–2; adequate hematologic, renal, and liver function; no prior radiotherapy for head and neck cancer; no prior cisplatin; and no concurrent active cancer in the last 5 years. Written, informed consent was obtained from all patients, and the Institutional Ethics Committees approved the protocol. All tissue samples used for the FISH and IHC studies described in the following text were taken before the commencement of treatment.

EGFR FISH

Four-micrometer formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized in xylene and then rehydrated through graded alcohols to water. Target retrieval was performed in Heat Pretreatment Solution (Invitrogen) in a pressure cooker at 125°C for 2 minutes, followed by several washes in water. Sections were treated with Enzyme Pretreatment Reagent (Invitrogen) for 20 minutes at room temperature. Slides were dehydrated through graded ethanol and then air-dried. Three microliters of EGFR/CEP7 probe (Vysis) was added to a predefined area of the tissue section (consisting of tumor cells), which was coverslipped with a 13-mm round coverslip and sealed with rubber cement to prevent evaporation during hybridization. Slides were denatured for 5 minutes at 85°C and then hybridized for 18 hours at 37°C on a StatSpin hybridizer (Dako). After hybridization, the coverslips were removed and slides were washed for 2 minutes in a 0.5 × SSC stringent wash buffer at room temperature and then placed for 5 minutes into a 0.5 × SSC stringent wash buffer at 75°C followed by several washes in water. Finally, sections were dried and mounted in Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector), coverslipped, and sealed with nail polish. A431 (vulva) and PC3 (prostate) xenograft tumor sections were used in each FISH run as positive (amplified) and negative (nonamplified) controls, respectively. Sections were stored at 4°C in the dark before scoring.

Scoring of EGFR FISH was done on an Olympus BX51 fluorescence microscope, following the guidelines and scoring criteria as described by Varella-Garcia and colleagues (32). For each FISH slide, areas of tumor were identified at low magnification by using the DAPI filter. A total of 100 cells, typically 30 to 40 from each of 3 fields, were scored for the number of EGFR (red) and CEP7 (green) signals, using a 60× water immersion lens. Raw data was entered into a Microsoft Excel spreadsheet for calculation of EGFR gene copy status. EGFR FISH negativity was defined as disomy (>90% cells with 1–2 EGFR signals), low trisomy (>10% but <40% of cells with 3 EGFR signals), high trisomy (>40% of cells with 3 EGFR signals), or low polysomy (>10% but <40% of cells with 4 or more EGFR signals). EGFR FISH positivity was defined as either high polysomy (>40% of cells displaying >4 copies of the EGFR signal) or gene amplification (either an EGFR to CEP7 ratio >2 or >10% of tumor cells with at least 15 copies of the EGFR signal). For scoring purposes, cells with obvious large “clusters” of EGFR signal were scored as “15”, as a number was required for the calculation of the EGFR/CEP7 ratio (sum of EGFR signals divided by the sum of CEP7 signals).
p16 IHC

p16 IHC was done as previously described (9). Briefly, 4-μm FFPE tissue sections were deparaffinized in xylene and then rehydrated through graded alcohols to water. Antigen retrieval was carried out in a 10 mmol/L sodium citrate (pH 6.0) solution in a pressure cooker before loading slides onto a Dako autostainer. Incubation in mouse monoclonal anti-p16 primary antibody (Neomarkers) diluted 1:250 for 30 minutes was followed by incubation in secondary antibody/detection with EnVision+ System-HRP anti-mouse (Dako) for 30 minutes, incubation with 3,3’-diaminobenzidine (DAB) chromogen (Dako), and then counterstaining with hematoxylin. Slides were dehydrated, mounted, and coverslipped. Cervical cancer sections known to be HPV positive were used as a positive control; omission of primary antibody served as a negative control.

The pattern of p16 expression was semiquantitatively scored for both intensity and proportion of staining in the cell nucleus and cytoplasm. Intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong); proportion was scored as 0 (1%–10% of cells stained), 1 (11%–50%), 2 (51%–80%), or 3 (81%–100%). Sections scored as 0 or 1 for intensity were defined as negative, whereas those scored 2 or 3 were defined as positive.

EGFR IHC

Four-micrometer FFPE tissue sections were deparaffinized in xylene and then rehydrated through graded alcohols. Enzymatic antigen retrieval was done using Proteinase K (Dako) for 10 minutes at room temperature before loading slides onto a Dako autostainer. Incubation with mouse monoclonal anti-EGFR primary antibody (Invitrogen) diluted 1:50 for 120 minutes was followed by incubation with secondary antibody/detection with EnVision+ System-HRP anti-mouse (Dako) for 60 minutes, incubation with DAB chromogen (Dako), and then counterstaining with hematoxylin. Slides were dehydrated, mounted, and coverslipped. A431 xenograft tumor sections known to have high EGFR protein expression were used as a positive control; omission of primary antibody served as a negative control.

The pattern of EGFR expression was semiquantitatively scored for both intensity and proportion of staining in the cell membrane by 2 independent observers. Intensity was scored as 0 (none), 1 (weak), 2 (moderate), 3 (strong), or 4 (very strong); proportion was scored as 0 (1%–10% of cells stained), 1 (11%–50%), 2 (51%–80%), or 3 (81%–100%). Scores for intensity and proportion were multiplied to give a final score from 0 to 12; tumors scored as 0 to 3 were defined as negative, and those scored 4 to 12 were defined as positive.

Statistical analysis

Failure-free survival (FFS) was defined as time from start date (treatment or randomization, depending on trial) to failure (locoregional or distant) or death without preceding failure. Overall survival (OS) was defined as the time from start date until death from any cause.

Comparison of groups with respect to FFS or OS was undertaken using the log-rank test. When 2 groups were compared, an exact log-rank P value was calculated (calculated from the distribution of the sum of independent hypergeometric random variables). The effect on outcome of a dichotomous variable adjusting for the effects of another (categorical) variable was undertaken using the exact log-rank test over strata. As the data came from 3 clinical trials, analyses were done both adjusting for trial and not adjusting for trial. As conclusions were unaltered by adjusting for trial, only the unadjusted results are presented. Tests for interaction were done using Cox regression.

Results

Patient characteristics

Pretreatment tissue for molecular studies was available from 212 patients (Table 1). Ten subjects were from phase I trial, 51 from phase II trial, and 151 from phase III trial. Oropharyngeal cancer was the major cancer site (65%), but other HNSCC subsites were represented, including larynx (16%), hypopharynx (12%), and oral cavity (7%). Smoking history was not available. Given that there were

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no statistically significant differences in outcome between the 2 treatment groups (radiotherapy plus cisplatin vs. radiotherapy plus cisplatin and tirapazamine) in phase III trial (31), patients from the 3 trials were pooled for the current study.

**EGFR gene copy number and patient outcome**

EGFR gene copy number was evaluable via FISH assay for 204 of the 212 patients (Supplementary Fig. S1). Of the 163 of 204 (80%) patients scored as EGFR FISH negative (−), 101 were scored as disomy, 29 as low trisomy, 5 as high trisomy, and 28 as low polysomy. Of the 41 of 204 (20%) who were scored as EGFR FISH positive (+), 22 were scored as high polysomy and 19 as gene amplification. With regard to clinical outcome, EGFR FISH + was associated with inferior FFS (HR = 1.84, 95% CI: 1.09–3.09; P = 0.027; Fig. 1A) and OS (HR = 1.78, 95% CI: 0.90–3.50; P = 0.082; Fig. 1B). When evaluated together in oropharyngeal cancer patients, p16 expression and EGFR FISH positivity were almost mutually exclusive with only 2 of 126 (1.6%) patients found to be positive for both markers. p16 was predictive of outcome independently of EGFR FISH for both FFS (HR = 0.30, 95% CI: 0.15–0.58; P < 0.001)

**p16 expression and patient outcome**

p16 expression was observed to be generally absent or present as strong and diffuse nuclear and cytoplasmic staining (Supplementary Fig. S2). Results were obtained for 200 of the 212 patients for p16 expression; however, we restricted the analysis of p16 expression and clinical outcome to only patients with oropharyngeal cancer, of whom 75 of 131 (57%) were found to be p16 positive (p16+). Within oropharyngeal cancer, p16-positive patients had significantly improved FFS (HR = 0.28, 95% CI: 0.15–0.54; P < 0.001; Fig. 2A) and OS (HR = 0.31, 95% CI: 0.15–0.62; P = 0.002; Fig. 2B) when compared with p16-negative patients.

**Relationship of p16 status with EGFR gene copy number and patient outcome**

When evaluated together in oropharyngeal cancer patients, p16 expression and EGFR FISH positivity were almost mutually exclusive with only 2 of 126 (1.6%) patients found to be positive for both markers. p16 was predictive of outcome independently of EGFR FISH for both FFS (HR = 0.30, 95% CI: 0.15–0.58; P < 0.001).
and OS (HR = 0.29, 95% CI: 0.13–0.62; \( P = 0.001 \)), but there was no evidence for an independent effect of EGFR FISH on outcome: FFS (HR = 1.02, 95% CI: 0.39–2.64; \( P = 1.00 \)) and OS (HR = 0.63, 95% CI: 0.23–1.74; \( P = 0.60 \); Fig. 3A and B, respectively). Given previous findings from our group showing a trend for improved locoregional control in p16-negative oropharyngeal cancer patients in the tirapazamine arm of phase III trial (9), and our finding that p16 expression and EGFR FISH positivity were almost mutually exclusive, we investigated the interaction between EGFR FISH status and treatment with tirapazamine. In patients treated with tirapazamine, there was improved outcome in EGFR FISH–negative patients for both FFS (HR = 2.47, 95% CI: 1.03–5.91; \( P = 0.022 \)) and OS (HR = 2.29, 95% CI: 0.86–6.13; \( P = 0.072 \); Supplementary Fig. S3), whereas there was no difference in the cisplatin-treated patients for both FFS (HR = 1.11, 95% CI: 0.47–2.62; \( P = 0.82 \)) and OS (HR = 1.19, 95% CI: 0.46–3.12; \( P = 0.81 \); Supplementary Fig. S4). However, the \( P \) values for interaction were not significant for either FFS (\( P = 0.13 \)) or OS (\( P = 0.27 \)).

**EGFR protein expression and patient outcome**

EGFR IHC data was obtained for 93 patients of whom 81 of 93 (87%) were scored as positive: defined as moderate, strong, or very strong staining in more than 50% of tumor cells (Supplementary Fig. S5). There was a trend toward improved outcome for patients scored as negative for EGFR protein expression; however, the number of negative scores was small (\( n = 12 \)) and statistical significance was not reached: FFS (HR = 1.98, 95% CI: 0.80–4.92; \( P = 0.35 \); Fig. 4A) and OS (HR = 2.52, 95% CI: 0.94–6.80; \( P = 0.22 \); Fig. 4B). The positivity rate was 54 of 64 (84%) for patients with oropharyngeal cancer. There was no significant correlation between EGFR IHC and EGFR FISH (\( P = 0.45 \); data not shown).

**Relationship of p16 status with EGFR protein expression and patient outcome**

Analysis of the combination of p16 and EGFR protein expression in oropharyngeal cancer patients showed that p16 was significantly related to outcome, independent of EGFR protein expression: FFS (HR = 0.33, 95% CI: 0.14–0.78; \( P = 0.023 \)) and OS (HR = 0.36, 95% CI: 0.14–0.78; \( P = 0.023 \)) and OS (HR = 0.36, 95% CI: 0.14–0.78; \( P = 0.023 \)).
Discussion

The present study is the largest to date investigating the frequency of increased EGFR gene copy number in HNSCC and, to the best of our knowledge, the first to investigate the relationship between EGFR gene copy number, HPV status, and clinical outcome. We have shown that EGFR FISH positivity is negatively correlated with clinical outcome in HNSCC and confirmed that HPV (p16) positivity is strongly predictive of favorable outcome in oropharyngeal cancer. When evaluated together, EGFR FISH positivity and p16 positivity are almost mutually exclusive in oropharyngeal cancer, with EGFR FISH positivity largely restricted to p16-negative patients. We found, however, that EGFR gene copy number could not predict outcome in oropharyngeal cancer patients when stratified for p16 status.

Our finding that p16 positivity is predictive of outcome in oropharyngeal cancer is in agreement with previous work by many groups (9, 10, 12, 33) and confirms the importance of determining HPV status in HNSCC and, in particular, in oropharyngeal cancer. Several studies have discussed the issue about the optimal method for determining HPV status on FFPE tumor sections (5, 34, 35). In the current study, we have used p16 IHC as a marker of HPV status and utilized HPV PCR in a subset of patients to validate the p16 results. Close agreement between the 2 methods confirmed the use of p16 alone as a robust, clinically useful surrogate marker for HPV infection in oropharyngeal cancer (data not shown; 9). Although smoking history has been found to be a modifying factor for the positive predictive value of HPV positivity (20), we were unable to assess the modifying effects of smoking, as smoking history was not prospectively collected in this cohort.

The EGFR has attracted considerable attention as both a prognostic marker and a therapeutic target in HNSCC (13). The EGFR and its ligand TGFα are expressed at high levels in HNSCC and have been associated with inferior outcome (16). Randomized phase III studies have shown improved outcomes in HNSCC patients when cetuximab, a monoclonal antibody to the EGFR, is combined with either radiation (16) or chemotherapy (28).

Two studies have found that increased EGFR gene copy number correlated with poor outcome in patients with HNSCC. Chung and colleagues (24) reported that 58% (43 of 75) of HNSCCs had high polysomy or gene amplification (i.e., were FISH positive) and that these patients had inferior progression-free (P < 0.5) and overall (P < 0.01) survival compared with FISH-negative patients. Temam and colleagues (25) reported a rate of 17% (22 of 134) for increased EGFR gene copy in HNSCC patients and found that patients with increased gene copy number had significantly poorer clinical outcome with respect to overall, cancer-specific, and disease-free survival. Using the same scoring criteria as Chung and colleagues (24) and Temam and colleagues (25), we found a 20% FISH positivity rate (gene amplification or high polysomy) and consistent with these studies showed that patients with EGFR FISH–positive HNSCC had poorer FFS and OS compared with EGFR FISH–negative HNSCC. Our finding of EGFR gene amplification rate of 9% (19 of 204) in the largest study to date in HNSCC is consistent with previous studies in which rates of EGFR gene amplification in HNSCC range from 6% to 31% (24, 25, 27, 28, 36).

Given the heterogeneous nature of HNSCC and the known importance of HPV status, it is becoming increasingly clear that the role of novel biomarkers in HNSCC will be to further stratify p16-positive and p16-negative patients. The combination of HPV status and EGFR FISH...
status has not previously been investigated about clinical outcome parameters. We have found that \textit{EGFR} FISH positivity is largely restricted to p16-negative patients, in agreement with 2 previous studies (27, 28) that have shown, in smaller cohorts of HNSCC, that HPV positivity and increased \textit{EGFR} gene copy number are negatively correlated. In the present study, \textit{EGFR} FISH does not separate p16-negative tumors into prognostically distinct groups and only p16 remains independently predictive of outcome. A caveat of this finding is that despite this being a large study inclusive of 204 HNSCC patients with available FISH results, the subgroup of \textit{EGFR} FISH-positive patients with oropharyngeal cancer was relatively small and therefore independent validation of this finding is appropriate.

Reports of the prognostic importance of increased \textit{EGFR} protein expression in HNSCC have yielded variable results. This variation is likely due to the heterogeneity of HNSCC and the use of different antibodies and scoring criteria including manual and automated methods. Several authors found a negative correlation with outcome (11, 19, 21), whereas others found no significant outcome effects (22, 23). Our finding that 87\% of patients were \textit{EGFR} HCC+ is similar to reports from several other groups (16, 17, 19, 21, 36), though we found no significant association between \textit{EGFR} protein levels and clinical outcome parameters. This finding could be due, in part, to sample size limitations and, in particular, to the low number of patients scored as \textit{EGFR} HCC+. An effect of the \textit{EGFR} methodology used cannot also be excluded. Interestingly, however, although the number of patients was small (n=8), we observed no events for FFS and OS in the p16+/\textit{EGFR}− group, indicating that this may identify a group with excellent outcome, a finding similar to others who concluded that the combination of HPV and \textit{EGFR} protein expression had significant prognostic implications (10, 21, 23).

In conclusion, we have shown that \textit{EGFR} FISH and p16 IHC are both individually predictive of outcome in HNSCC, but in multivariate analysis, only p16 remains predictive. In oropharyngeal cancer, \textit{EGFR} FISH positivity and the expression of p16 are almost mutually exclusive, with increased \textit{EGFR} copy number largely restricted to p16-negative oropharyngeal cancer. Neither \textit{EGFR} gene copy number nor \textit{EGFR} protein levels predicted outcome in patients stratified for p16 status. Given the profound association of p16 status with clinical outcome in HNSCC, future studies investigating known and novel biomarkers in HNSCC should aim to further stratify patients whose HPV status is known so as to better select patients for risk-adapted therapy and treatment with novel targeted agents.

**Disclosure of Potential Conflicts of Interest**

No conflicts of interest were disclosed.

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