Published in final edited form as:
J Clin Oncol. 2008 July 1; 26(19): 3128–3137. doi:10.1200/JCO.2007.12.7662.

EGFR, p16, HPV Titer, Bcl-xL and p53, Sex, and Smoking As Indicators of Response to Therapy and Survival in Oropharyngeal Cancer

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Abstract

Purpose—To prospectively identify markers of response to therapy and outcome in an organ-sparing trial for advanced oropharyngeal cancer.

Patients and Methods—Pretreatment biopsies were examined for expression of epidermal growth factor receptor (EGFR), p16, Bcl-xL, and p53 as well as for *p53* mutation. These markers

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Presented in part at the 4th International Meeting on Cancer Molecular Markers, September 8-10, 2006, Stone Mountain, GA and at the Multidisciplinary Head and Neck Cancer Symposium, January 18-20, 2007, Rancho Mirage, CA.

Authors' Disclosures of Potential Conflicts of Interest: Although all authors completed the disclosure declaration, the following author (s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: David M. Kurnit, SensiGen (U), SensiGen (C) **Consultant or Advisory Role**: David M. Kurnit, SensiGen (U) **Stock Ownership**: David M. Kurnit, SensiGen **Honoraria**: None **Research Funding**: None **Expert Testimony**: None **Other Remuneration**: None

Author Contributions: Conception and design: Bhavna Kumar, Julia S. Lee, Gregory T. Wolf, Susan G. Urba, Douglas B. Chepeha, Theodoros N. Teknos, Avraham Eishbruch, Kun Yang, David M. Kurnit, Joshua A. Bauer, Carol R. Bradford, Thomas E. Carey
Financial support: Carol R. Bradford, Thomas E. Carey
Administrative support: Gregory T. Wolf
Provision of study materials or patients: Kitrina G. Cordell, Mark E. Prince, Gregory T. Wolf, Susan G. Urba, Douglas B. Chepeha, Theodoros N. Teknos, Avraham Eishbruch, Christina I. Tsien, Nisha J. D'Silva, Carol R. Bradford
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Final approval of manuscript: Bhavna Kumar, Kitrina G. Cordell, Julia S. Lee, Francis P. Worden, Mark E. Prince, Huong H. Tran, Gregory T. Wolf, Susan G. Urba, Douglas B. Chepeha, Theodoros N. Teknos, Avraham Eishbruch, Christina I. Tsien, Jeremy M.G. Taylor, Nisha J. D'Silva, Kun Yang, David M. Kurnit, Joshua A. Bauer, Carol R. Bradford, Thomas E. Carey

were assessed for association with high-risk human papillomavirus (HPV), response to therapy, and survival. Patient variables included smoking history, sex, age, primary site, tumor stage, and nodal status.

Results—EGFR expression was inversely associated with response to induction chemotherapy (IC) ($P = .01$), chemotherapy/radiotherapy (CRT; $P = .055$), overall survival (OS; $P = .001$), and disease-specific survival (DSS; $P = .002$) and was directly associated with current smoking ($P = .04$), female sex ($P = .053$), and lower HPV titer ($P = .03$). HPV titer was significantly associated with p16 expression ($P < .0001$); p16 was significantly associated with response to IC ($P = .008$), CRT ($P = .009$), OS ($P = .001$), and DSS ($P = .003$). As combined markers, lower HPV titer and high EGFR expression were associated with worse OS ($\rho_{EGFR} = 0.008$; $\rho_{HPV} = 0.03$) and DSS ($\rho_{EGFR} = 0.01$; $\rho_{HPV} = 0.016$). In 36 of 42 biopsies, p53 was wild-type, and only one HPV-positive tumor had mutant *p53*. The combination of low p53 and high Bcl-xL expression was associated with poor OS ($P = .005$) and DSS ($P = .002$).

Conclusion—Low EGFR and high p16 (or higher HPV titer) expression are markers of good response to organ-sparing therapy and outcome, whereas high EGFR expression, combined low p53/high Bcl-xL expression, female sex, and smoking are associated with a poor outcome. Smoking cessation and strategies to target EGFR and Bcl-xL are important adjuncts to the treatment of oropharyngeal cancer.

Introduction

In the preceding article, we report the results of an organ-sparing regimen for advanced squamous cell carcinoma of the oropharynx (SCCOP) that used induction chemotherapy (IC) to select patients for subsequent chemotherapy/radiotherapy (CRT).[1] In this trial, 70.4% OS and 75.8% DSS rates were achieved at 4 years. However, in patients who failed to respond to IC or CRT, survival was poor. To improve outcome, it is necessary to identify patients who will respond to a particular therapy. It is important to understand the biology of resistance in those who will not respond and to develop effective alternative treatments. Thus, it is of significant interest to identify molecular biomarkers of individual disease risk and prognosis and to identify tumors for target-specific therapies. This is accomplished best in the context of clinical studies, in which response to therapy and outcome can be evaluated without the conflicting variables of different treatments or sites with differing prognoses.

High-risk human papillomavirus (HPV) is an important etiologic factor in oropharyngeal cancer[2,3] and is thought to be the cause of the increasing incidence of this cancer type,[4-6] particularly among younger patients.[7,8] It is also noted that patients with HPV-positive oropharynx tumors have a survival advantage.[2,9-11] By using a sensitive quantitative assay that detects 13 high-risk HPV types,[12,13] Worden et al[1] found HPV16 in 27 (64%) of 42 pretreatment biopsies of oropharynx tumors from this trial. HPV copy number per cell was significantly associated with a better response to IC and CRT as well as with improved DSS and OS.[1] In this article, we prospectively examine other biomarkers for their association with HPV and outcome.

$p16^{INK4a}$ (CDKN2A) is a cyclin-dependent kinase inhibitor that inhibits pRb phosphorylation and blocks cell cycle progression at the G1 to S check point.[14] Loss of p16 expression by deletion, mutation, or hypermethylation is common in head and neck squamous cell carcinomas (HNSCC).[15,16] However, functional inactivation of pRb by HPV E7 protein results in overexpression of $p16^{INK4a}$, which makes it a surrogate marker for HPV.[9,17-19] Similarly, HPV E6 protein can inactivate p53; thus, there is a lower incidence of *p53* mutations in HPV-positive tumors.[2,9]

Aberrant epidermal growth factor receptor (EGFR) function[20] affects cell cycle progression, apoptosis, angiogenesis, tumor-cell mobility, and metastasis[21] and correlates with poor prognosis in HNSCC.[22-24] In SCCOP, some reports found high tumor EGFR expression to be associated with inferior disease-free survival,[25] whereas others[26,27] found no correlation between EGFR expression and survival.

p53 is mutated frequently,[28-30] is overexpressed[31,32] in HNSCC, and is associated with poor prognosis in some studies [33,34] but not in others.[35-38] Thus controversy remains regarding the prognostic significance of p53. Overexpression of the antiapoptotic proteins Bcl-2 and Bcl-xL is associated with chemotherapy and radiation resistance,[39,40] and the combination of p53 status with Bcl-xL is associated with cisplatin resistance in HNSCC cells in vitro.[41]

We examined pretreatment biopsies from patients with SCCOP who were enrolled on a University of Michigan Cancer Center organ-sparing trial (UMCC 9921)[1] for the expression of EGFR, p16, p53, Bcl-xL, *and p53* mutations. Biomarkers, sex, smoking status, and HPV presence and titer were analyzed individually and together for effects on response to therapy and on survival.

Patients and Methods

Study Population

The full study included 66 patients with stage III to IV SCCOP.[1] We retrieved blocks that contained pretreatment biopsies from 50 patients (38 men and 12 women). The treatment schema and outcome for these 50 patients are shown in Appendix Figure A1. Patients received IC as one cycle of cisplatin (100 mg/m^2/d for 1 day) or carboplatin (AUC 6) and fluorouracil (1,000 mg/m^2/d for 5 days). Responders (ie, those with > 50% response at the primary site) received CRT; nonresponders received surgery and radiation.[42,43] Adjuvant paclitaxel was offered after CRT. Seven patients experienced treatment failure with IC; all have died—six as a result of their tumors and one as a result of an unknown cause. Forty-two of 50 patients achieved a greater than 50% response at the primary tumor site; one died as a result of distant metastases before CRT; and 41 received CRT. Three patients experienced treatment failure with CRT (two had salvage surgery; one refused): all died as a result of their tumors. Thirty of 38 responders to CRT have remained alive; eight have died—six as a result of their tumors. The institutional review board, in accordance with Department of Health and Human Services assurance, approved the study. All patients gave written informed consent to allow examination of their tissues and medical records.

Tissue Microarray and DNA Isolation

A tissue microarray (TMA) was constructed from 50 pretreatment biopsies.[44] When sufficient tumor was available in the biopsy (42 of 50 patient cases), a core was taken for DNA extraction. [1] Tissue was not available from 16 of 66 patients with outside diagnostic biopsies.

Immunostaining and Scoring

TMA slides were deparaffinized, were rehydrated, and were peroxidase-quenched (Dako Cytomation, Glostrup, Denmark). For antigen retrieval, slides were incubated with pepsin (EGFR;10 minutes at 37°C) or with citrate buffer (p16, p53, Bcl-xL; 30 minutes at 92°C) and were blocked with horse serum (30 minutes at 25°C). Primary antibody, EGFR/31G7 (Zymed Laboratories, South San Francisco, CA), p16/16P04, p53/DO1, and Bcl-xL/7D9 (Lab-Vision, Fremont CA) were added for 1 hour and were probed with avidin/biotin peroxidase (ABC Kit; Vector Laboratories, Burlingame, CA). Antibody binding was scored by a pathologist (K.G.C., who was blinded to the clinical outcome) using a continuous scale (ie, 10%, 30%, 90%, etc) for the proportion of EGFR-positive tumor cells in each core. For p16, p53, and Bcl-xL, a scale

of 1 to 4 was used: 1 was less than 5%; 2, 5% to 20%; 3, 21% to 50%; and 4, 51% to 100% tumor staining. Intensity was scored as 1 equal to no staining; 2, low intensity; 3, moderate; and 4, high intensity. Scores for multiple cores from each patient were averaged.

HPV and p53 Mutation Analysis

HPV type and copy number were reported in Worden et al.[1] *p53* exons 4 to 9 were amplified by using the primers given in Appendix Table A1. Products from two polymerase chain reactions were sequenced in both directions and were analyzed by using Mutation Surveyor version 2.61 (Soft Genetics Inc, State College, PA,) and by manual review.

Statistical Analysis

Patient-level averages for EGFR, p16, p53, and Bcl-xL expression and the titer of HPV16 (log-transformed) were used in the analysis. The *p53* status was categorized as wild-type or mutant. Patients were categorized as current, past (quit > 6 months ago), or never smokers. For the assessment of univariate association between markers and covariates of interest, rank-based statistical methods were employed. Spearman rank correlation was used to assess the correlation between marker scores and continuous variables. The univariate association between two-level variables and response to IC was assessed by the Cochran-Armitage trend test. The Wilcoxon rank sum test and Kruskal-Wallis statistics were used to compare the marker distribution between categoric covariates and response to IC when appropriate. The Kaplan-Meier method and the log-rank test were used to compare the homogeneity of survival rates between categories of discrete clinical variables. DSS was defined as the time to death from oropharynx cancer. Patients who were alive at last follow-up or who died as a result of reasons other than SCCOP were censored. Cox proportional hazards models were used to assess time to event outcomes. Clinical variables, T class, and smoking status were analyzed as ordinal variables; N class was dichotomized at N0 class; and age was a continuous variable. Thus, there was one degree of freedom spent for each clinical variable. For each time to event outcome, three models were constructed: a model with biomarkers alone; a model with clinical variables alone; and a model with clinical variables and biomarkers. Models 2 and 3 were used to assess marker effects beyond the effects of clinical variables. Models 1 and 2 were used to assess clinical variable effects beyond the effects of biomarkers. Likelihood ratio statistics were used to compare models. The power of the tests may be limited by the sample size and the number of events in the model. Although a nonsignificant result may be a false negative, a significant result would indicate that our data are sufficient to achieve a stable positive finding. All statistical analyses were done with SAS version 9.0 (SAS Institute, Carey, NC). A two-tailed *P* value of .05 or less was considered statistically significant.

Results

The influence of patient characteristics and biomarkers on response and outcome for the 50 patients represented on the TMA are given in Table 1. There was no significant difference in the rate of response to IC among those included on the TMA and those who were not ($P = .47$).

EGFR, p16, HPV16, Bcl-xL, and p53 As Single Markers

HPV16 viral load was significantly associated with response to therapy and outcome in this patient set.[1] Similarly, p16 expression (Fig 1) was significantly associated with HPV copy number ($P < .0001$), younger age ($P = .002$), response to IC ($P = .008$; Fig 2), CRT ($P = .009$), OS ($P = .001$), and DSS ($P = .003$; Fig 3). EGFR intensity (Fig 4) was significantly associated with poorer response to IC ($P = .01$; Fig 5), marginally poorer response to CRT ($P = .055$), and poorer OS ($P = .001$) and DSS ($P = .002$; Fig 6). A *p53* mutation (all missense, all overexpressed) was found in 6 (14.3%) of 42 tumors but was not associated with treatment

response or survival. Only 1 of 6 was HPV positive and had less than 1 copy/cell. Of the tumors with wild-type *p53*, HPV, and p53 expression data, 15 (seven HPV negative and eight HPV positive) had low p53 expression, and 19 (three HPV negative and 16 HPV positive) had high p53 expression. Stain proportion and intensity were significantly correlated for p53 ($P < .0001$) and for Bcl-xL ($P < .0001$; Figs 7A and 7B). As single markers, neither p53 nor Bcl-xL expression was significantly associated with treatment response or survival outcomes.

Multimarker Analysis

HPV titer and p16 expression were inversely associated with EGFR expression (HPV $P = .03$; p16 $P = .007$). Higher HPV titer and lower EGFR intensity as combined markers were associated with better OS (ρ_{HPV}, 0.03; ρ_{EGFR}, 0.008) and DSS (ρ_{HPV}, 0.016; ρ_{EGFR}, 0.01; Fig 8A), as were high p16 combined with low EGFR (OS ρ_{p16}, 0.004; OS ρ_{EGFR}, 0.03; DSS ρ_{p16}, 0.003; DSS ρ_{EGFR}, 0.04; Fig 8B). The effect of EGFR remained after adjusting for p16 (OS $P = .03$; DSS $P = .04$). The prognostic value for p53 was dependent on Bcl-xL expression status. Patients with low p53 and low Bcl-xL expression had better OS and DSS than those with low p53 and high Bcl-xL or those with high p53 and high or low Bcl-xL (OS $P = .005$; DSS $P = .002$; Fig 8C).

Sex, Biomarkers, and Smoking Influence Outcome

Current smoking status and female sex were significantly associated with worse OS and DSS in the UMCC 9921 trial.[1] This was true for all 66 patients in the trial and for the subset of 50 patients used to build the TMA (Fig 9A and 9B). Never and former smoking history were significantly correlated with higher p16 expression ($P = .01$; Fig 9C), lower EGFR ($P = .04$; Fig 9D), and lower Bcl-xL expression ($P = .015$; Fig 9E). All six patients with mutant *p53* were former (n = 3) or current (n = 3) smokers. After analysis was adjusted for smoking status, p53 and Bcl-xL combined and EGFR alone each held prognostic significance for OS and DSS. Similarly, after analysis was adjusted for smoking status, p16 still held prognostic value for OS (Table 2, column A). After analysis accounted for single marker effect or combined p53 and Bcl-xL, smoking status held prognostic value for OS and DSS, depending on the biomarker (Table 2, column B).

The expression of p16, p53, and Bcl-xL and *p53* mutation status did not differ significantly between men and women. EGFR intensity was marginally higher in tumors from women than men ($P = .053$; Fig 9F). After accounting for sex, p53 and Bcl-xL combined, p16, and EGFR held prognostic value (Table 2, column C). After analysis was adjusted for p53 combined with Bcl-xL or for each of the single markers, female sex held a significant negative prognostic value for OS and DSS (Table 2, column D).

Sex is tied to smoking status in this cohort: all women were either former (5 of 12) or current (7 of 12) smokers. After analysis accounted for smoking status and each biomarker, sex still held prognostic value for OS and DSS (Table 2, column E). Conversely, after analysis accounted for both gender and individual biomarkers, the prognostic value of smoking for OS and DSS depended on the biomarker in the model (Table 2, column F). Further, after analysis accounted for smoking and sex, EGFR, p16, and p53 combined with Bcl-xL still affected OS and DSS (Table 2, column G).

The poorer prognosis of patients with lower p16 expression and higher EGFR expression was maintained after adjustments for age, sex, smoking status, T class, N class, and primary site (p16: OS $P = .004$, DSS $P = .02$; EGFR: OS $P = .02$, DSS $P = .04$). The poorer prognostic profile of lower HPV titer and higher EGFR intensity (OS $P = .001$, DSS $P = .0002$), lower p16 and higher EGFR intensity (OS $P = .001$, DSS $P = .002$), and low p53 and high Bcl-xL (OS $P = .003$, DSS $P < .0001$) was maintained after adjustment for age, sex, T class, N class,

smoking status, and primary site. Although Karnofsky performance status (KPS) influenced OS, it was not significant in the multivariate analysis after other variables were considered.

Discussion

We prospectively examined pretreatment biopsies from patients with advanced SCCOP enrolled on a clinical trial in which all patients were treated with IC; responders were given CRT, and nonresponders underwent surgery.[43] This allowed us to determine how molecular factors and patient characteristics affected response to chemotherapy, CRT, and survival in a group of homogeneously treated patients.

In our cohort, EGFR, HPV16,[1] and p16 each independently predicted survival and, as combined markers, identified the patients with the best and worst survival. If a patient's tumor had high EGFR expression and contained HPV or expressed high p16, the survival probability was improved compared with those without HPV or p16. HPV titer and p16 expression were inversely related to EGFR expression. Kim et al[45] reported an association between HPV and p16 expression and an inverse relationship between HPV and EGFR expression but did not report any association with outcome in a series of patients with tonsillar cancer (73% HPV positive). In patients with oropharyngeal cancer (28% HPV positive), Reimers et al[46] found only a trend for EGFR to predict outcome, but the combination of p16 and EGFR expression stratified patients with better and worse OS and DSS. They also found a tendency for lower EGFR expression in p16-positive tumors. Prior reports in patients with laryngeal cancer[47] and with laryngeal papillomatosis[48] suggested that the HPV-E5 protein increased EGFR expression. However, because 15 of 25 HPV-positive patient cases in our study expressed low EGFR, it is unlikely that high EGFR expression is secondary to HPV in most instances. This is the first report that has combined HPV titer, expression of EGFR, and p16 in pretreatment SCCOP biopsies and has analyzed these markers in relation to smoking, sex, response to therapy, and outcome.

To understand why women in our study had worse outcomes than men, we examined differences in biomarker expression and clinical factors. As a group, the women were older than the men, were either current or past smokers, and were less likely to be HPV positive. Similarly, there was a strong trend for higher EGFR expression among the tumors from women. Thus, for each category, the women had less favorable statuses. We and others[2,49,50] found an inverse correlation between smoking and HPV. We also observed that EGFR expression was significantly higher in current smokers than in past smokers, who in turn had higher EGFR levels than those who never smoked. This suggests that smoking may contribute to increased EGFR expression, possibly through increased hypoxia in the tumor tissue of smokers.[22] When patients were stratified according to smoking status and EGFR expression, those who were current smokers with high tumor EGFR expression had poorer DSS than patients in all other groups.

In this cohort, p53 and Bcl-xL together are markers of outcome and survival that identify tumors with good and poor outcome, independent of HPV. This observation is consistent with in vitro studies, in which we observed that HNSCC tumor cells with low (wild-type) p53 and low Bcl-xL undergo apoptosis in response to cisplatin and that those with low (wild-type) p53 and high Bcl-xL are cisplatin resistant.[41] Interestingly, in our current study, the cisplatin-sensitive phenotype translated into improved DSS and OS. Of the patients whose tumors had low p53 and low Bcl-xL expression, eight of nine were alive at last follow-up, whereas five of seven with low p53 and high Bcl-xL had died as a result of their cancers. The predictive value of these markers may be explained by high expression of the apoptosis-blocking protein, Bcl-xL, which inhibits apoptosis in tumors with wild-type *p53*. Our in vitro studies showed that cells with this phenotype arrest, induce DNA repair, and begin growing again within 24 to 48 hours

after cisplatin treatment (Bauer et al, submitted for publication). This behavior would be consistent with poor outcome in patients. In contrast, the favorable outcome of patients whose tumors have low p53 and low Bcl-xL is likely a result of p53-mediated apoptosis secondary to treatment-induced DNA damage, as we have observed in vitro.[41] We recently observed a similar association of the low p53 and low Bcl-xL phenotype with larynx preservation and with a trend for improved survival in patients on the chemotherapy arm of the VA larynx cancer trial.[51,52]

Intermediate survival results were obtained with the tumors that overexpressed p53 irrespective of Bcl-xL expression. In our in vitro studies, tumor cells that overexpressed mutant p53 generally were more sensitive to cisplatin than those with wild-type *p53* and high Bcl-xL,[53] presumably because cells with mutant *p53* lack p53-mediated arrest and repair. In our cohort, only a subset of patients with SCCOP that overexpressed p53 harbored *p53* mutations. Many of the tumors that overexpressed p53 were positive for HPV, which, in itself, influences outcome and response to therapy. Others also observed p53 overexpression in HPV-positive tumors.[54,55] The mechanism responsible for overexpression of wild-type p53 in the context of HPV is not known and is the subject of continuing study in our lab.

Smoking cessation strategies are warranted in patients with SCCOP, because current smokers benefited far less than past smokers from this successful therapeutic approach. Pretreatment biopsies to identify those nonsmoking patients with high HPV loads and low EGFR expression for the least aggressive therapy also may be indicated to minimize treatment morbidity. Targeted use of EGFR inhibitors in patients with high tumor EGFR expression, either as single agents or in combination with current therapy, could improve the outcome for the group of patients with the poorest survival. Likewise, for patients who have the low p53 and high Bcl-x combination, inhibitors of cell survival proteins have promise as targeted agents.[56-58] Our results showed highly significant associations between biomarkers and outcome and identified patients that would or would not benefit from current treatments. However, ours is a relatively small sample size; these findings must be validated in a larger cohort of patients so that individualized targeted treatments can be designed to improve survival in the patients at greatest risk.

Acknowledgments

We thank our patients, who graciously consented to be a part of this study, and Tamara H. Miller, Nancy E. Wallace, and Heidi L. Mason, who provided outstanding nursing care and support for the patients.

Supported by Grants No. R01 DE13346 from the National Institutes of Health (NIH) NIDCR, P30 DC 05188 from the NIH NIDCD, and R01 CA83087 from the NIH National Cancer Institute; Head and Neck Cancer SPORE Grant No. P50 CA97248 from the NIH-NCI NIDCR; NCI National Cancer Center Support Grant No. P30 CA46592 from the Cancer Center Support; and Grant No. MEDC-410, and Loan No. MEDC-239 (to Sensigen) from the state of Michigan.

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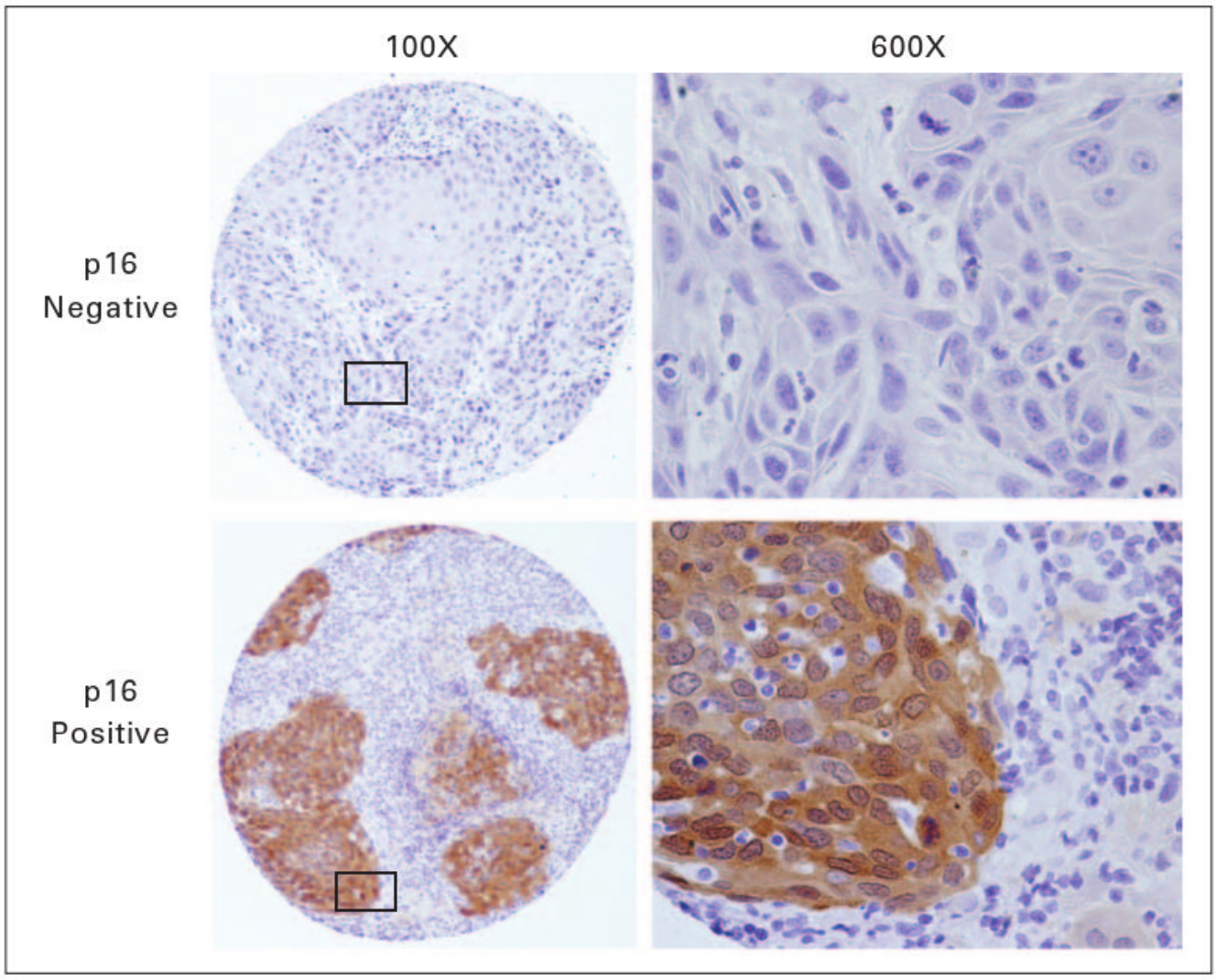

Fig 1.
Representative images of p16 staining. A core showing no p16 expression score = 1 and a core showing high p16 stain proportion score = 4.

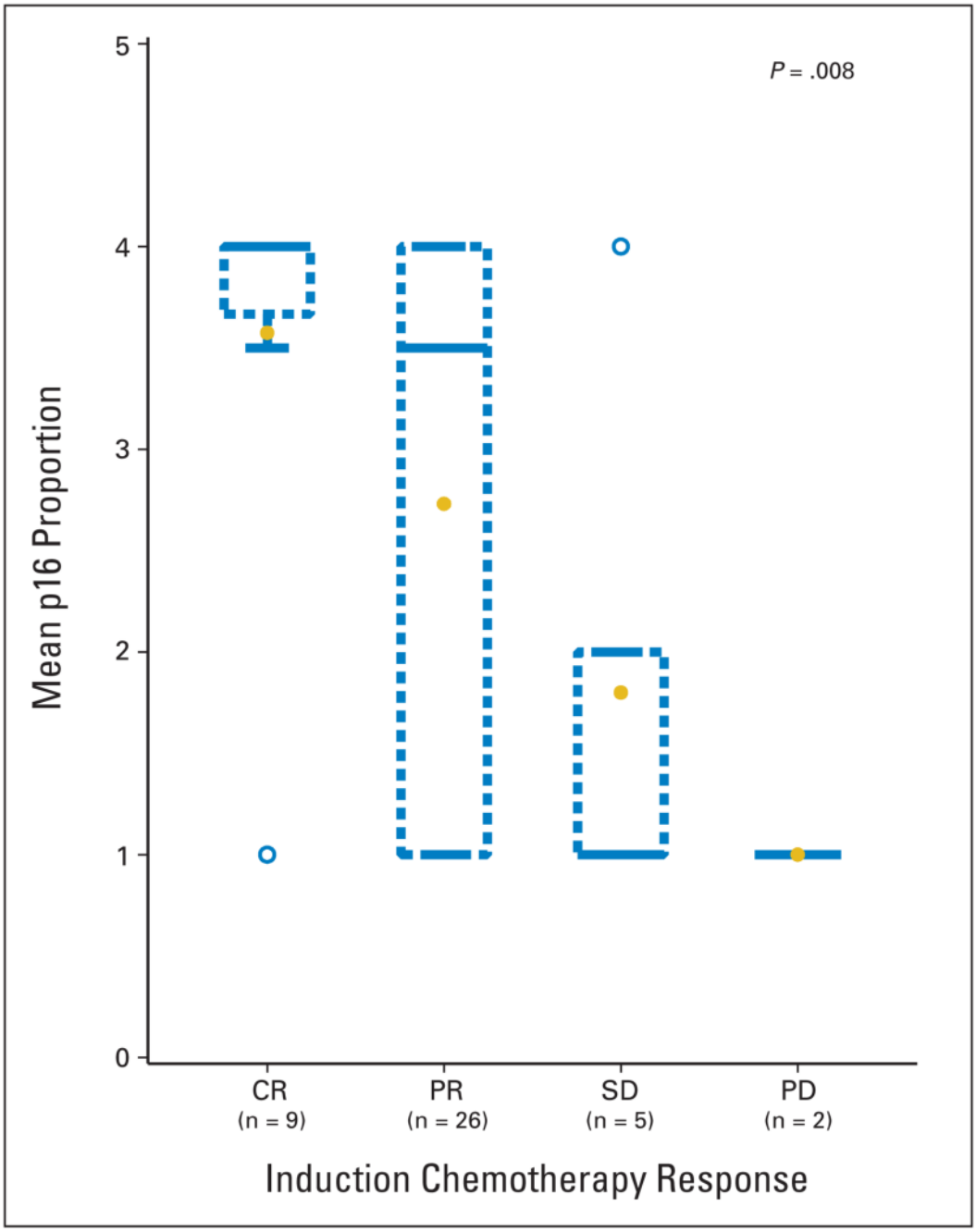

Fig 2.
Response to induction chemotherapy on the basis of mean p16 stain proportion. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Blue box, interquartile range (25th to 75th percentile), not including outliers; yellow dot, mean; blue circle, outlier; blue horizontal bar, median.

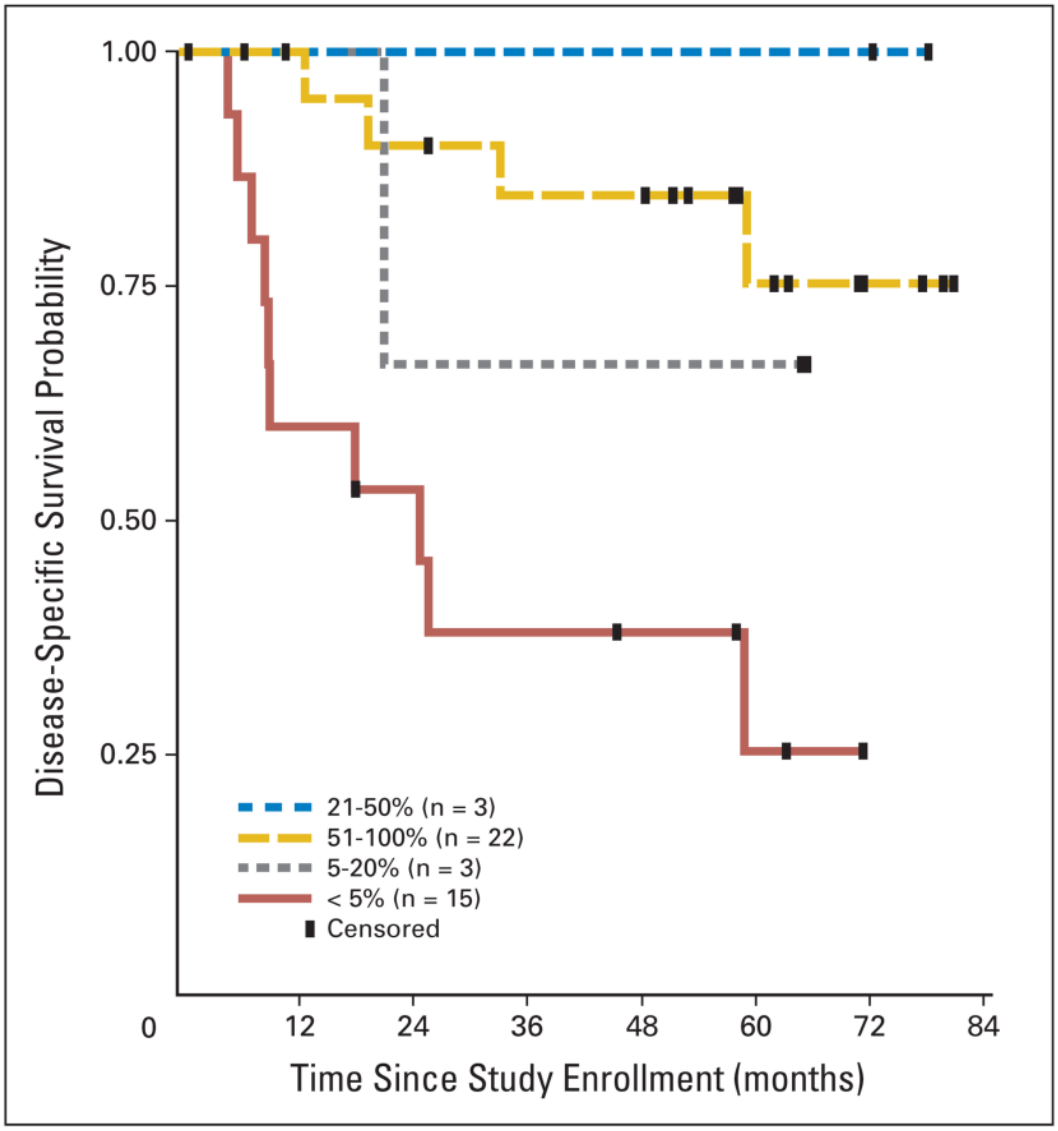

Fig 3.
Disease-specific survival of patients according to p16 stain proportion.

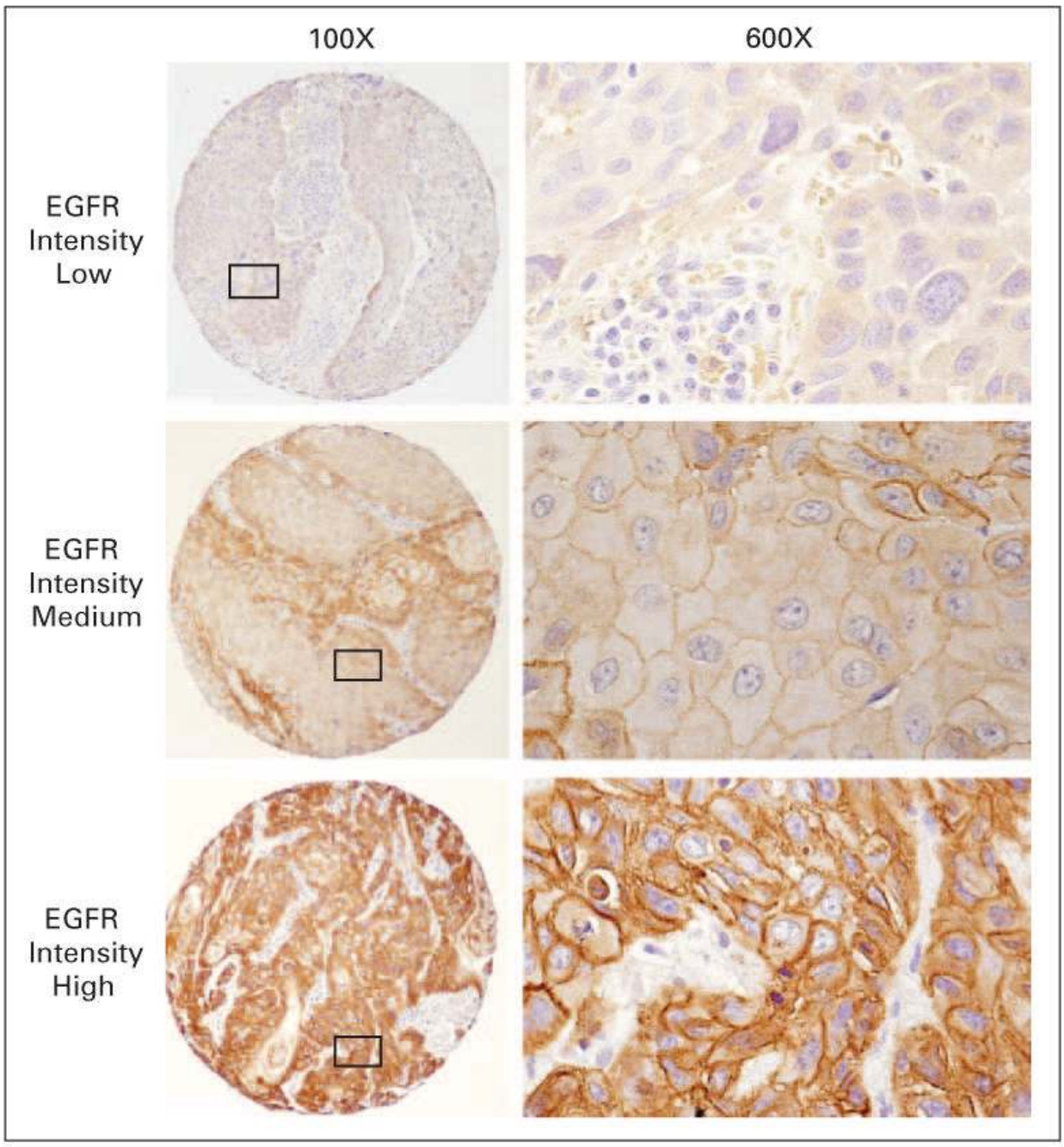

Fig 4.
Representative images of epidermal growth factor receptor (EGFR) staining. Cores showing low score = 2; medium score = 3; and high score = 4; EGFR stain intensities (used in all analyses) correlated strongly with proportion of EGFR-positive cells ($P < .0001$).

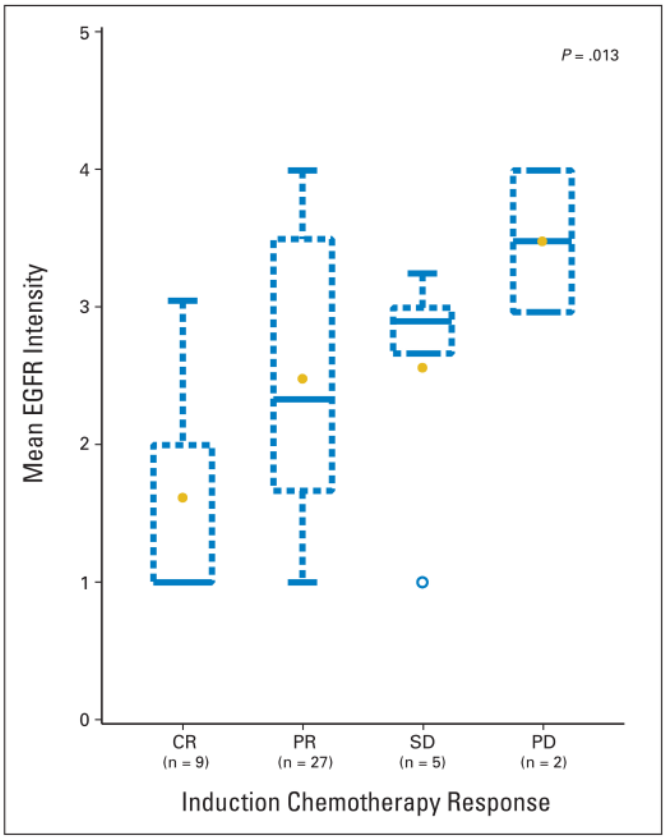

Fig 5.

Response to induction chemotherapy on the basis of mean epidermal growth factor receptor intensity. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Blue box, interquartile range (25th to 75th percentile), not including outliers; yellow dot, mean; blue circle, outlier; blue horizontal bar, median.

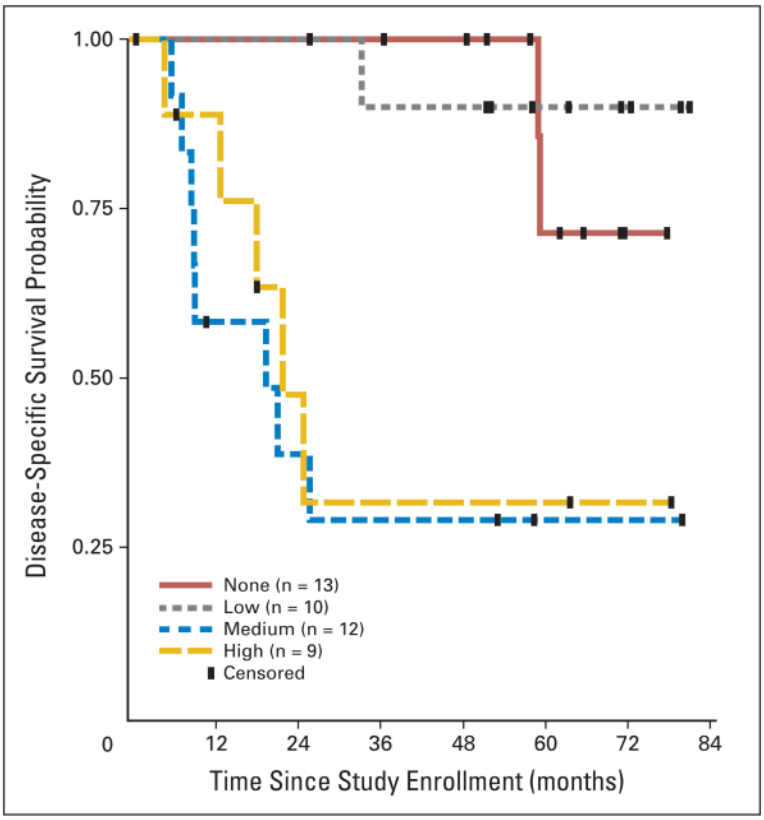

Fig 6.
Disease-specific survival of patients according to epidermal growth factor receptor intensity.

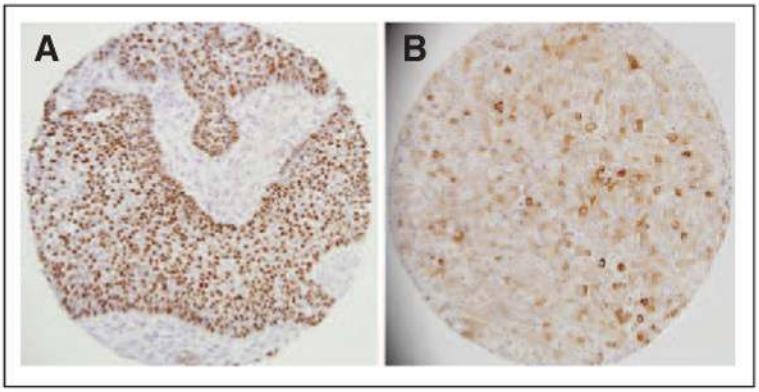

Fig 7.
(A) Representative core showing p53 staining. (B) Representative core showing Bcl-xL staining.

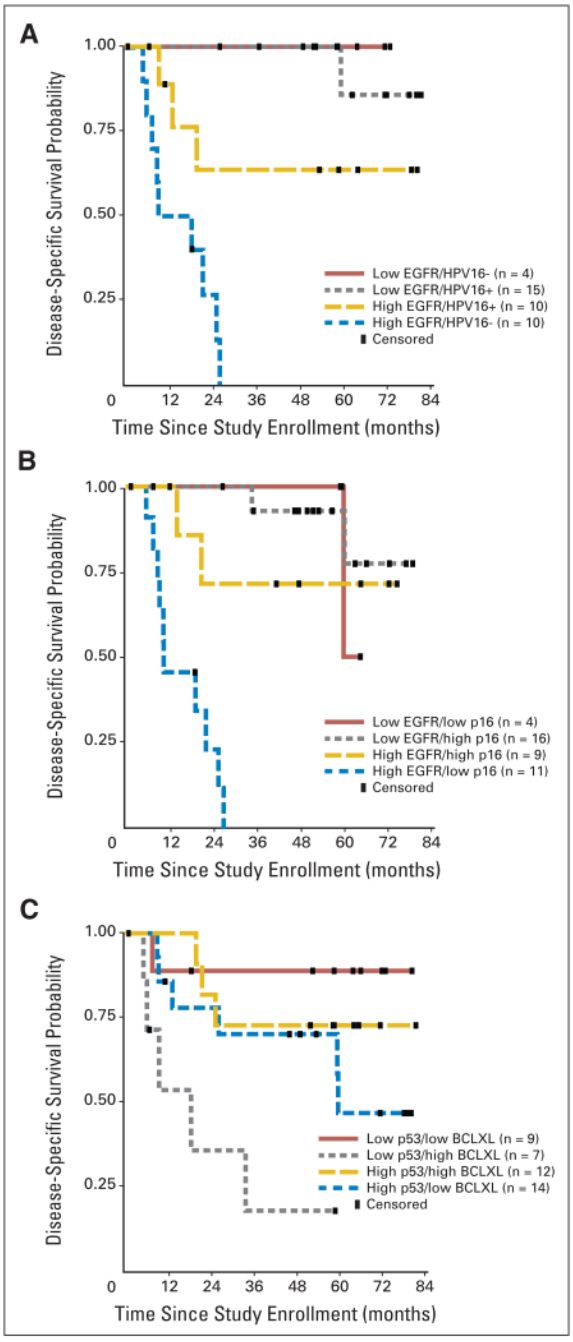

Fig 8.

(A) Disease-specific survival according to epidermal growth factor receptor (EGFR) intensity and HPV16. (B) Disease-specific survival according to EGFR intensity and p16 proportion. (C) Disease-specific survival of patients on the basis of tumor p53 (stain intensity) and Bcl-xL (stain intensity) expression.

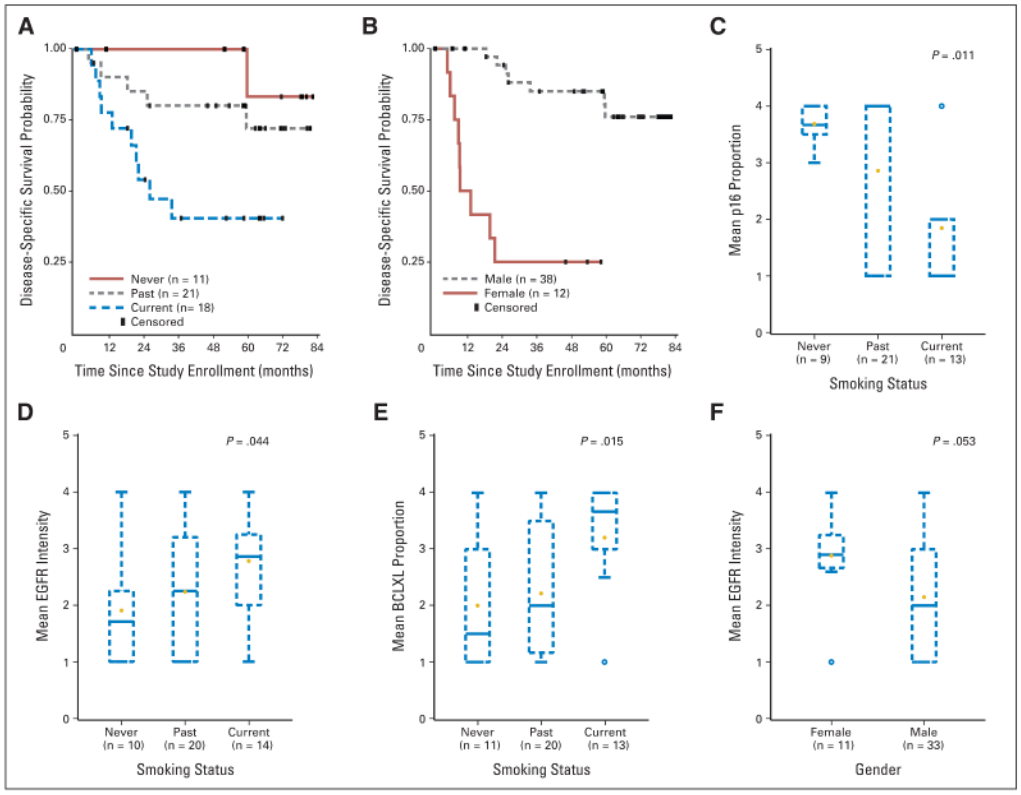

Fig 9.

(A) Disease-specific survival on the basis of smoking status. (B) Disease-specific survival on the basis of sex. Smoking status of patients and (C) p16 proportion, (D) epidermal growth factor receptor (EGFR) intensity, and (E) Bcl-xL proportion. (F) Mean EGFR intensities on the basis of sex. (C, D, E, F) Blue box, interquartile range (25th to 75th percentile), not including outliers; yellow dot, mean; blue circle, outlier; blue horizontal bar, median.

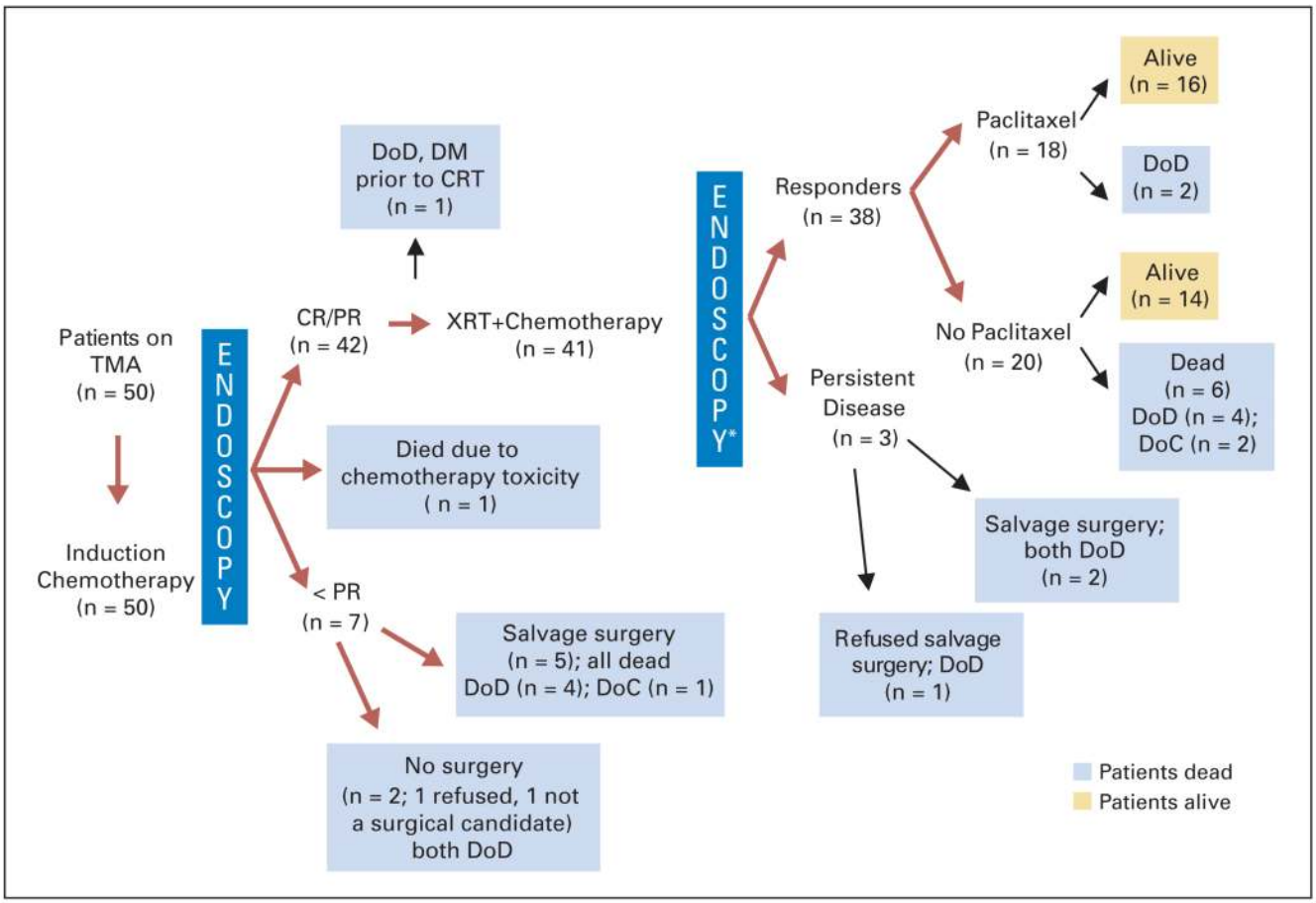

Fig A1.

Schema of patients represented on tissue microarray (TMA) according to treatment. Endoscopy*, endoscopy with biopsy. CRT, chemotherapy/radiotherapy; CR, complete response; PR, partial response; IC, induction chemotherapy; CR/PR, complete response/partial response (> 50% response to induction chemotherapy); < PR, stable disease or progressive disease (≤ 50% response to induction chemotherapy); DoC, died of other causes; DoD, died of disease; DM, distant metastases; XRT, radiation therapy.

Table 1

Characteristics of Patients on Tissue Microarray With Biomarkers, Response to Therapy, and Survival Outcomes

			Analyses							
			Therapy Response				Survival Outcomes			
			C*		CRT		OS		DSS	
Characteristic	**Female**	**Male**	**Spearman ρ**	***P***	**OR**	**95% CL**	**HR**	**95% CL**	**HR**	**95% CL**
Sex†	12	38		**.050‡§**	**0.11**	**0.02, 0.48‖**	**7.54**	**2.92, 19.47¶**	**11.12**	**3.64, 33.93¶**
Age, years#	62**; 53-74††	55**; 40-77††	0.16	.27	0.95	0.88, 1.02	**1.05**	**1.00, 1.10‡**	**1.06**	**1.01, 1.12‡**
Median										
Range										
Smoking status‡‡			**0.47**	**.0008‖**	0.50	0.19, 1.29	**2.04**	**1.05, 3.95‡**	**3.08**	**1.35, 7.03‡**
Never	0	11								
Former	5	16								
Current	7	11								
T class‖‖			**0.43**	**.0023‖**	0.43	0.18, 1.02	**1.95**	**1.12, 3.40‡**	**2.71**	**1.35, 5.40‖**
1	1	4								
2	0	11								
3	5	15								
4	6	8								
N class			−0.05	.72	1.52	0.73, 3.17	0.67	0.4, 1.13	0.58	0.33, 1.03
0	5	4								
1	1	10								
2	5	21								
3	1	3								
KPS			−0.21	.16	1.05	0.95, 1.16	**0.94**	**0.89, 0.99‡**	0.95	0.89, 1.02
80	1	3								
90	6	3								
100	5	32								
Primary site¶¶				.84§	0.41	0.10, 1.76	**3.48**	**1.16, 10.43‡**	**3.8**	**1.08, 13.38‡**

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			Analyses							
			Therapy Response				Survival Outcomes			
			C*		CRT		OS		DSS	
Characteristic	**Female**	**Male**	**Spearman *ρ***	***P***	**OR**	**95% CL**	**HR**	**95% CL**	**HR**	**95% CL**
Tonsil	3	17								
Base of tongue	9	21								
p16 proportion (n = 43)##	1.0**	3.7**	**−0.41**	**.008**‖	**2.18**	**1.24, 3.84**‡	**0.56**	**0.39, 0.80**‖	**0.53**	0.35, 0.81‖
EGFR intensity (n = 44)***	2.9**	2.0**	**0.38**	**.01**‡	0.52	0.26, 1.04	**2.13**	**1.35, 3.35**‖	**2.22**	**1.35, 3.65**‖
p53 mutation status (n = 42)				.54§	0.67	0.10, 4.27	2.14	0.75, 6.12	2.27	0.69, 7.44
Mutant	2	4								
Wild type	10	26								
p53 intensity (n = 45)	2.83**	2.67**	0.07	.65	1.41	0.71, 2.82	0.89	0.56, 1.41	0.86	0.51, 1.44
Bcl-xL intensity (n = 44)	2.25**	2.0**	0.06	.68	0.92	0.47, 1.82	1.08	0.7, 1.66	1.11	0.68, 1.79
p53 and Bcl-xL combined (n = 42) †††				.76‡‡‡		0.09‡‡‡				
p53							**4.98**	**1.39, 17.83**‡	**7.65**	**1.74, 33.5**‡
Bcl-xL							**10.47**	**2.12, 51.67**‖	**10.47**	**3.05, 123.08**‖
Interaction *P*								**.005**		**.002**

NOTE. Bold text indicates a significant finding.

Abbreviations: IC, induction chemotherapy; CRT, chemotherapy/radiotherapy; OS, overall survival; DSS, disease-specific survival; OR, odds ratio; CL, confidence limit; HR, hazard ratio; KPS, Karnofsky performance status; EGFR, epidermal growth factor receptor.

*Analyzed as ordinal data, in which the coding for complete response at the primary site, partial response (> 50%) at the primary site, stable disease, and progressive disease are 1, 2, 3, and 4, respectively.

†Female sex was associated with poor outcome.

‡$P < .05$.

[§] Cochran-Armitage trend test.

[‖] $P < .005$.

[¶] $P < .0001$.

[#] Older age was associated with poor outcome.

[**] Median.

[††] Range.

[‡‡] Current smoking status was associated with poor outcome.

[‖‖] Higher T class was associated with poor outcome.

[¶¶] Base-of-tongue subsite was associated with poor outcome.

[##] Lower p16 expression was associated with poor outcome.

[***] Higher EGFR expression was associated with poor outcome.

[†††] Low p53/high Bcl-xL expression was associated with poor outcome.

[‡‡‡] Likelihood ratio test.

Table 2

P Values of Marker, Smoking, and Sex Effects for Survival from Cox Models

	***P* for Survival by Effect**													
	A: Single Marker After Smoking		**B: Smoking After Marker**		**C: Single Marker After Sex**		**D: Sex After Marker**		**E: Sex After Smoking and Marker**		**F: Smoking After Sex and Marker**		**G: Marker After Sex and Smoking**	
Marker	**OS**	**DSS**	**OS**	**DSS**	**OS**	**DSS**	**OS**	**DSS**	**OS**	**DSS**	**OS**	**DSS**	**OS**	**DSS**
p16[*]	.012	.058	.41	.071	.002	.005	.0005	.0002	.0007	.0002	.60	.113	.006	.02
EGFR[†]	.003	.006	.019	.001	.002	.003	.0006	< .0001	.003	.0004	.14	.014	.005	.009
p53 mutated[‡]	.41	.57	.059	.005	.51	.58	.0003	<.0001	.0009	.0003[§]	.23	.025	.62	.76
p53 and Bcl-xL[¶]	.028	.011	.009	.0009	.002	.0003	< .0001	<.0001	.0001	< .0001	.16	.018	.003	.0004

NOTE. Current smoking status was associated with poor survival outcomes, and female sex was associated with poor survival outcomes.

Abbreviations: OS, overall survival; DSS, disease-specific survival; EGFR, epidermal growth factor receptor.

[*] Lower p16 expression was associated with poor survival outcomes.

[†] Higher EGFR expression was associated with poor survival outcomes.

[‡] p53 mutation status was not associated with survival outcomes.

[§] Interaction of sex and p53 mutation status was significant (*P* = .007). Women with wild-type p53 had poorer prognoses than men with wild-type p53.

[¶] Low p53 and high Bcl-xL as combined markers were associated with poor survival outcomes.

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Table A1

Primers Used for PCR of Exons 4 Through 9 of p53

Exon	Primer
4.1F	5′-CCCATCTACAGTCCCCCTTG-3′
4.1R	5′-GGTGTAGGAGCTGCTGGTG-3′
4.1F	5′-CTGAAGACCCAGGTCCAGATGAA-3′
4.2R	5′-AACTGACCGTGCAAGTCACA-3′
5F	5′-CTTGTGCCCTGACTTTCAACTCTGTCTC-3′
5R	5′-TGGGCAACCAGCCCTGTCGTCTCTCCA-3′
6F	5′-CCAGGCCTCTGATTCCTCACTGATTGCTC-3′
6R	5′-GCCACTGACAACCACCCTTAACCCCTC-3′
7F	5′-GCCTCATCTTGGGCCTGTGTTATCTCC-3′
7R	5′-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3′
8F	5′-GTAGGACCTGATTTCCTTACTGCCTCTTGC-3′
8R	5′-ATAACTGCACCCTTGGTCTCCTCCACCGC-3′
9F	5′-CACTTTTATCACCTTTCCTTGCCTCTTTCC-3′
9R	5′-AACTTTCCACTTGATAAGAGGTCCCAAGAC-3′

Abbreviations: PCR, polymerase chain reaction; F, forward; R, reverse.