

Quantitative Determination of Nuclear and Cytoplasmic Epidermal Growth Factor Receptor Expression in Oropharyngeal Squamous Cell Cancer by Using Automated Quantitative Analysis

Amanda Psyrri,¹ Ziwei Yu,² Paul M. Weinberger,² Clarence Sasaki,² Bruce Haffty,³ Robert Camp,⁴ David Rimm,⁴ and Barbara Ann Burtness¹

Abstract **Background:** Several lines of evidence support the epidermal growth factor receptor (EGFR) as a molecular target for therapy in head and neck squamous cell carcinomas (HNSCC). Determination of tumor EGFR levels by conventional immunohistochemistry has not always predicted antitumor efficacy. Quantitative assays may provide more accurate assessment of the level of EGFR receptor in the tumor, which may thus provide more reliable prognostic and predictive information. We studied the prognostic value of quantitative assessment of EGFR in oropharyngeal squamous cell cancers treated with radiotherapy.

Experimental Design: We studied EGFR protein expression on a tissue microarray composed of 95 oropharyngeal cancer cases using an *in situ* molecular-based method of quantitative assessment of protein expression (AQUA) and correlated those with clinical and pathologic data. Automated, quantitative analysis uses cytokeratin to define pixels as cancer (tumor mask) within the array spot and measures intensity of EGFR expression using a Cy5-conjugated antibody within the mask. A continuous index score is generated, which is directly proportional to the number of molecules per unit area, and cases were defined as high expressing if they were above the median expression level.

Results: The mean follow-up time for survivors was 44.9 months, and for the entire cohort was 34.8 months. Patients with high tumor EGFR expression levels had a local recurrence rate of 58% compared with 17% for patients with low EGFR tumor expression ($P < 0.01$). Similarly, patients with high nuclear EGFR expression had a local recurrence rate of 54% compared with 21% for patients with low EGFR nuclear expression ($P < 0.05$). Additionally, patients with high tumor and nuclear EGFR levels had inferior disease-free survival compared with low expressors (19% versus 43% and 19% versus 45%, respectively. $P < 0.05$ for each). In multivariate analysis adjusting for well-characterized prognostic variables, high tumor and nuclear EGFR expression levels retained their prognostic significance.

Conclusion: The AQUA system provides a continuous measurement of EGFR on paraffin-embedded tissue and was able to reveal the association between EGFR expression and outcome expected from the biological role of EGFR. In the future, EGFR AQUA score may be useful in predicting response to EGFR-targeted therapies.

Mortality rates of patients with head and neck squamous cell cancer (HNSCC) have not improved substantially over the past 30 years, despite advances in diagnosis and therapy (1).

Although a curable malignancy if diagnosed at an early stage, patients often present with advanced locoregional (stages III and IV) poor prognosis disease (1). Following standard therapy, only ~45% of the latter group of patients will be alive after 2 years (2). Advances in molecular biology have led to identification of molecular targets in cancers and the development of agents that reverse the abnormal function of these proteins.

Several lines of evidence support the epidermal growth factor receptor (EGFR) as a molecular target for therapy of HNSCC. First, overexpression of EGFR is one of the most common molecular alterations in HNSCC (3); the level of EGFR expression on head and neck cancers is elevated relative to expression on normal adjacent squamous mucosa in 83% to 100% of cases (3). Second, increased receptor content is often associated with increased production of ligands, such as transforming growth factor- α , by the HNSCC (3). Furthermore,

Authors' Affiliations: Departments of ¹Medical Oncology, ²Otolaryngology, ³Radiation Therapy, and ⁴Pathology, Yale University School of Medicine, New Haven, Connecticut

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Requests for reprints: Barbara Burtness, Fox Chase Cancer Center, Department of Medical Oncology, 333 CoHman Ave., Philadelphia, PA 19111.

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treatment with EGFR-targeted therapy such as the chimeric monoclonal antibody cetuximab (C225) or the quinazoline gefitinib inhibits EGFR signaling and potentiates the effects of chemotherapy or radiation (4–7).

A critical issue in EGFR-targeted therapy has been patient selection because the intensity of EGFR staining by immunohistochemistry has not been consistently associated with efficacy (7–9). In addition, the pathologist scores EGFR status based on the percentage of cells with membranous and/or cytoplasmic staining. However, there is recent evidence to suggest that EGFR, a plasma membrane receptor tyrosine kinase, may enter the nucleus and directly function as a transcriptional factor (10, 11). Thus, nuclear EGFR staining should not be overlooked in the assessment of the EGFR status.

Resistance to EGFR inhibition may be related to abnormalities in redundant receptor or in signaling molecules downstream from EGFR. Nonetheless, the prognostic value of EGFR expression within many tumor types, and the relatively nonquantitative methods used to determine EGFR expression in specimens from clinical trials of EGFR inhibitors, suggest that further study of the predictive worth of expression of the primary target of these therapies—EGFR itself—with more quantitative methods, is warranted. Because activation of EGFR pathway leads to transcription of genes responsible for cell cycle progression, we hypothesized that elevated EGFR protein levels would have an adverse impact on prognosis. A fully quantitative method of analysis for tissue microarrays that allows calculation of expression ratios has been developed. This method allows measurement of protein expression within subcellular compartments that results in a number directly proportional to the number of molecules expressed per unit area (the concentration). This novel technology uses molecular methods to define subcellular compartments. It then quantifies the amount of protein expressed within the compartment by colocalization. Therefore, this technology permits preservation of tissue morphology, while quantifying protein expression in paraffin-embedded tissue. Here, we show that measurement of EGFR protein levels on paraffin-embedded tissue using this method is feasible and results in more reliable assessment of EGFR protein expression than conventional immunohistochemistry.

Materials and Methods

Tissue microarray construction. The cohort was assembled from patients with primary oropharyngeal cancer of squamous cell histology treated at Yale-New Haven Hospital between 1980 and 1999. Patients were treated with primary external beam radiotherapy (EBRT) or gross total surgical resection and postoperative radiotherapy. Exclusion criteria included presentation with metastatic disease or failure to receive a full course of radiation therapy. Following institutional review board approval, the tissue microarray was constructed as previously described (12), including 95 cases that met inclusion criteria. Tissue cores were obtained from paraffin-embedded formalin-fixed tissue blocks from the Yale University Department of Pathology archives. Slides from all blocks were reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have shown that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for

analysis by immunohistochemistry. Addition of a duplicate histospot, whereas not necessary, provides marginally improved reliability (12). The tissue microarray was then cut to yield 5 μ m sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Quantitative immunohistochemistry. Tissue microarray slides were deparaffinized and stained as previously described (12). In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in distilled water, antigen retrieval was accomplished by application of proteinase K and incubation for 30 minutes. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin for 30 minutes at room temperature. Following these steps, slides were incubated with primary antibody at 4°C overnight. Primary monoclonal antibody to EGFR (clone H11; DAKO, Carpinteria, CA) was used at 1:50 dilution in 0.3% bovine serum albumin/TBS. This antibody has been validated in previous studies using immunohistochemistry and Western blot analysis of normal and neoplastic tissue (13, 14). Subsequently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase–decorated dextran polymer backbone (Envision; DAKO) for 1 hour at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit antipancytokeratin antibody z0622; DAKO) with subsequent goat anti-rabbit antibody conjugated to Alexa 546 fluorophore (A11035, Molecular Probes, Eugene, OR). We added 4',6-diamidino-2-phenylindole to visualize nuclei. Target (EGFR) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin-Elmer Corp., Wellesley, MA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol–containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Vernon Hills, IL).

Automated image acquisition and analysis. Automated image acquisition and analysis using AQUA has been described previously (15). In brief, monochromatic, high-resolution (1,024 \times 1,024 pixel; 0.5 μ m) images were obtained of each histospot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. The 4',6-diamidino-2-phenylindole signal within this mask was then used to identify tumor nuclei. The EGFR signal (AQUA score) was scored on a normalized scale of 1 to 255 expressed as pixel intensity divided by the target area (tumor mask or tumor nuclei). AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

Statistical analysis. Histospots containing <10% tumor, as assessed by mask area, were excluded from further analysis. Local recurrence was defined as time from day of diagnosis to development of locally recurrent disease. Overall survival was defined as time from day of diagnosis to death from any cause. Disease-free survival was defined as time from day of diagnosis to the first of either death from any cause or disease progression. For survival analysis, automated AQUA scores were converted to binomial variables of high versus low expression around the median. The decision to break at the median was the joint result of ease of interpretation and maximizing the efficiency of the comparison between EGFR groups. In previous investigations, the median has been used as a cut point (16). Disease-free survival and local recurrence were subsequently assessed by Kaplan-Meier analysis with log-rank for determining statistical significance. All survival analysis was done at 5-year cutoffs. Relative risk was assessed by the multivariate Cox proportional hazards model. Prognostic variables included in this model were EGFR expression class, management (EBRT versus postoperative radiotherapy), histologic grade, subsite within the oropharynx, and tumor-node-metastasis (TNM) stage. Comparison of EGFR expression class with the clinical and pathologic variables, such as gender, TNM stage, histologic grade, treatment method (primary EBRT versus primary surgical excision plus radiotherapy), chemotherapy treatment, and oropharyngeal subsite, were made using χ^2 analysis.

Comparison of EGFR expression and patient age was made by Spearman correlation. All calculations and analyses were done with SPSS 11.5 for Windows (SPSS, Inc., Chicago IL) and where appropriate were two-tailed.

Results

Clinical and pathologic variable analysis

There were 95 patients with primary oropharyngeal carcinoma that met inclusion criteria for this study. Mean follow-up time for survivors was 44.9 months, and 34.8 months for the entire cohort. Seventy-four were male, 21 female, with age ranging from 41 to 79 years old. Eleven patients (11.6%) were TNM stage II, 27 (28.4%) stage III, and 57 (60.0%) stage IV. Oropharyngeal subsites included 41 (43.2%) tonsillar fossae, 49 (51.6%) base of tongue, and 5 (7.4%) other oropharynx. Fifty-eight (61.1%) patients were managed with primary EBRT, 35 (36.8%) with surgical excision followed by postoperative EBRT, and 2 (2.9%) not recorded. Twenty-one patients (22.1%) were treated with

chemotherapy. Clinical response to initial therapy was complete response in 65 (68.4%) patients, partial or no response in 10 (10.5%) patients, with 20 (21.1%) not recorded. For histologic grade, 6 (6.3%) tumors were well differentiated, 44 (46.3%) were moderately differentiated, 34 (35.8%) were poorly differentiated, and 11 (11.6%) not recorded. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Quantitative immunohistochemistry (AQUA) for epidermal growth factor receptor protein expression

Of the 95 patients included in this study, 67 (70.5%) had sufficient tissue for analysis of EGFR protein expression by AQUA. The tumors deemed insufficient for analysis had <10% tumor area represented on the tissue microarray and were excluded from analysis. As visualized by fluorescent immunohistochemistry, EGFR displayed both cytoplasmic and nuclear expression pattern (Fig. 1). EGFR expression followed a skewed distribution as expected for a cancer tissue

Table 1. Demographic, clinical, and pathologic data

	n	EGFR tumor expression class*			EGFR nuclear expression class*		
		Low	High	P	Low	High	P
Age							
41-79 (median 61)	95	33	34	>0.5	34	33	>0.5
Gender							
Male	74	23	26	>0.5	25	24	>0.5
Female	21	10	8		9	9	
Site							
Tonsils	41	15	16	>0.5	15	16	>0.5
Base of tongue	49	16	16		16	17	
Other/not recorded	5						
TNM stage							
II	11	3	4	>0.5	4	3	>0.5
III	27	9	10		8	11	
IV	57	21	20		22	19	
Grade							
Well differentiated	6	2	2	0.24	2	2	0.19
Moderately differentiated	44	18	14		19	13	
Poorly differentiated	34	8	14		8	14	
Not recorded	11						
Management							
Primary radiotherapy	58	21	21	>0.5	21	21	>0.5
Postoperative radiotherapy	35	11	13		12	12	
Not recorded	2						
Response							
Complete response	65	26	21	0.051	27	20	0.025 [†]
Partial/no response	10	1	7		1	7	
Not recorded	20						
Chemotherapy							
Yes	21	7	9	>0.5	7	9	>0.5
No	74	26	25		27	24	

* Comparison data by EGFR expression class does not include the 27 to 28 cases for which EGFR expression could not be determined because of insufficient tissue representation in the tissue microarray.

[†] Significant at the 0.05 level.

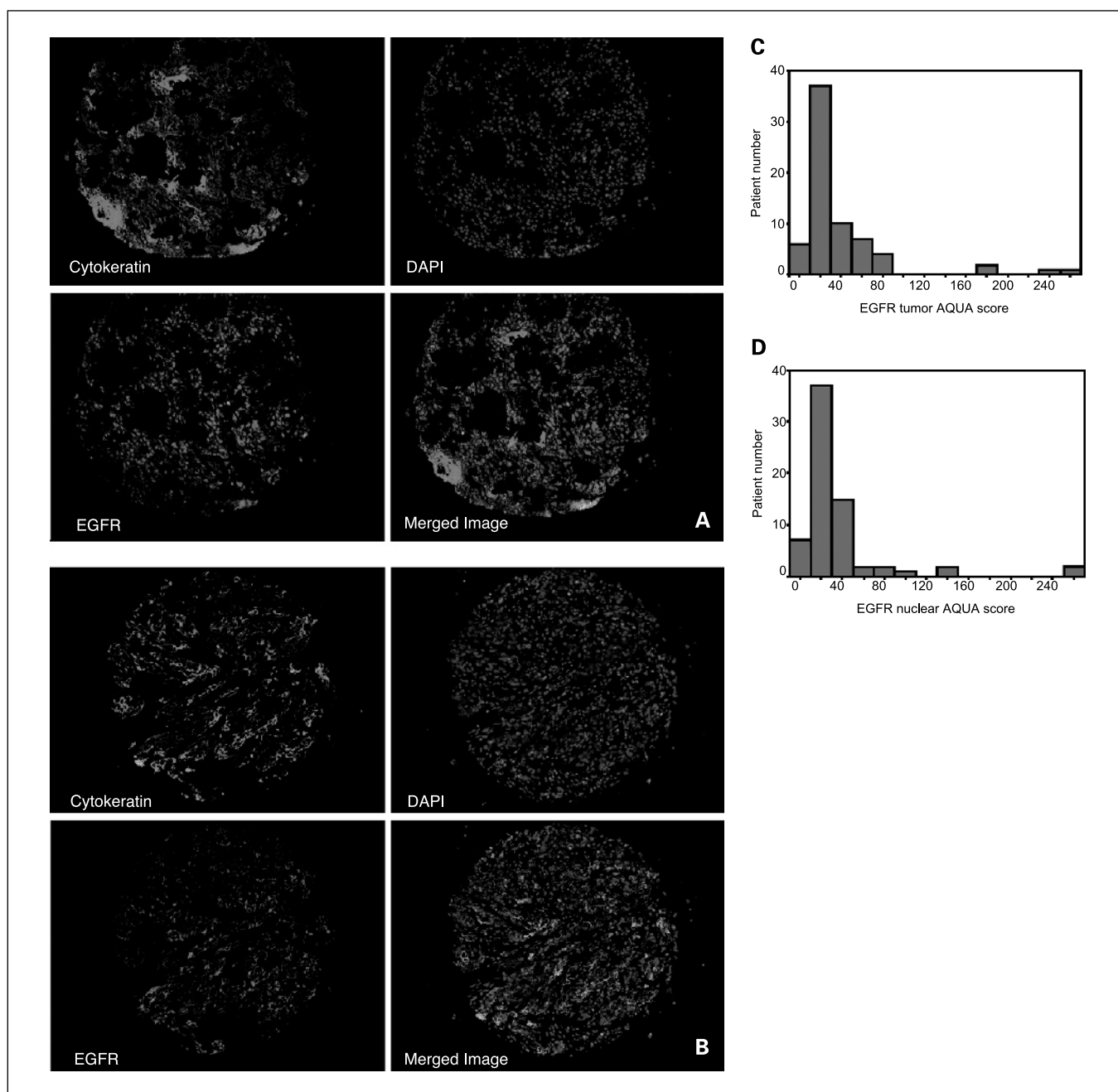


Fig. 1. EGFR immunofluorescence and AQUA analysis. The majority of tumors displayed mixed membranous (A) and nuclear (B) staining. AQUA analysis showed a left skewed distribution for EGFR tumor (C) and nuclear (D) expression, respectively.

biomarker (Fig. 1). AQUA scores for each compartment were split at the median (20.8 for tumor EGFR and 20.4 for nuclear EGFR) to yield binomial (high versus low) variables. Patients with tumor EGFR expression <20.8 were classified as low expressors ($n = 33$), and patients with tumor EGFR expression >20.8 were classified as high expressors ($n = 34$). Similarly, patients with nuclear EGFR expression <20.4 were classified as low expressors ($n = 34$), and patients with nuclear EGFR expression >20.4 were classified as high expressors ($n = 33$).

Correlation of clinical/pathologic data and epidermal growth factor receptor expression

Patients who were recorded as having partial or no response to therapy were significantly more likely to have high nuclear EGFR expression, with 87.5% of partial/nonresponders being classified as high nuclear EGFR, versus 12.5% low EGFR ($P = 0.025$). There was a similar trend noted for tumor EGFR, but this did not reach statistical significance ($P = 0.051$). There was no association between tumor or nuclear EGFR expression and any of the other clinicopathologic variables (Table 1).

Univariate survival analysis

Local recurrence. The expression status of EGFR was evaluated for association with local recurrence using Kaplan-Meier survival analysis (Fig. 2A and D) with log-rank statistic for determining significance. This analysis showed that high tumor and nuclear EGFR expression are associated with increased 5-year local recurrence rates. Patients with high tumor EGFR expression had a local recurrence rate of 57.8% compared with 17.2% for patients with low EGFR tumor expression ($P = 0.0078$). Patients with high nuclear EGFR expression had a local recurrence rate of 54.4% compared with 21.4% for patients with low EGFR nuclear expression ($P = 0.0277$).

Disease-free survival. The expression status of EGFR was also evaluated for association with disease-free survival. Kaplan-Meier analysis (Fig. 2B and E) showed that patients with high tumor EGFR expression had inferior 5-year disease-free survival (19.0%) compared with those with low tumor EGFR expression (43.4%; $P = 0.0298$). High EGFR nuclear expressors also had inferior 5-year disease-free survival (19.0%) compared with those with low nuclear EGFR expression (44.8%; $P = 0.0386$).

Overall survival. The expression status of EGFR was also evaluated for association with overall survival. Kaplan-Meier analysis (Fig. 2C and F) showed that patients with high tumor EGFR expression tended toward inferior 5-year overall survival (21.1%) compared with those with low tumor EGFR expression (40.2%), but this did not reach statistical significance ($P = 0.064$). High EGFR nuclear expressors also tended toward inferior 5-year overall survival (23.5%)

compared with those with low nuclear EGFR expression (35.9% ; $P = 0.11$). Results for univariate analysis are summarized in Table 2.

Multivariate survival analysis

Using the Cox proportional hazards model, we did multivariate analysis to assess the independent predictive value of tumor EGFR expression groups (high versus low) for local recurrence and disease-free survival. Multivariate analysis adjusting for well-characterized prognostic variables was also done to assess the independent predictive value of nuclear EGFR (high versus low expressors) for local recurrence and disease-free survival. The other prognostic variables included in the two multivariate models were subsite within oropharynx, TNM stage, management (EBRT versus primary surgical excision plus radiotherapy), and histologic grade. In multivariate model 1, only high tumor EGFR expression status remained an independent prognostic factor for local recurrence ($P = 0.027$). For disease-free survival, high tumor EGFR expression trended toward significance ($P = 0.07$). In multivariate model 2, nuclear EGFR retained its prognostic value for local recurrence ($P = 0.041$), along with management ($P = 0.047$). High nuclear EGFR trended toward significance for disease-free survival ($P = 0.07$). Results for multivariate survival analysis are summarized in Table 3.

Discussion

Studies evaluating EGFR expression in tumor tissues have used numerous methods and, in general, have provided a

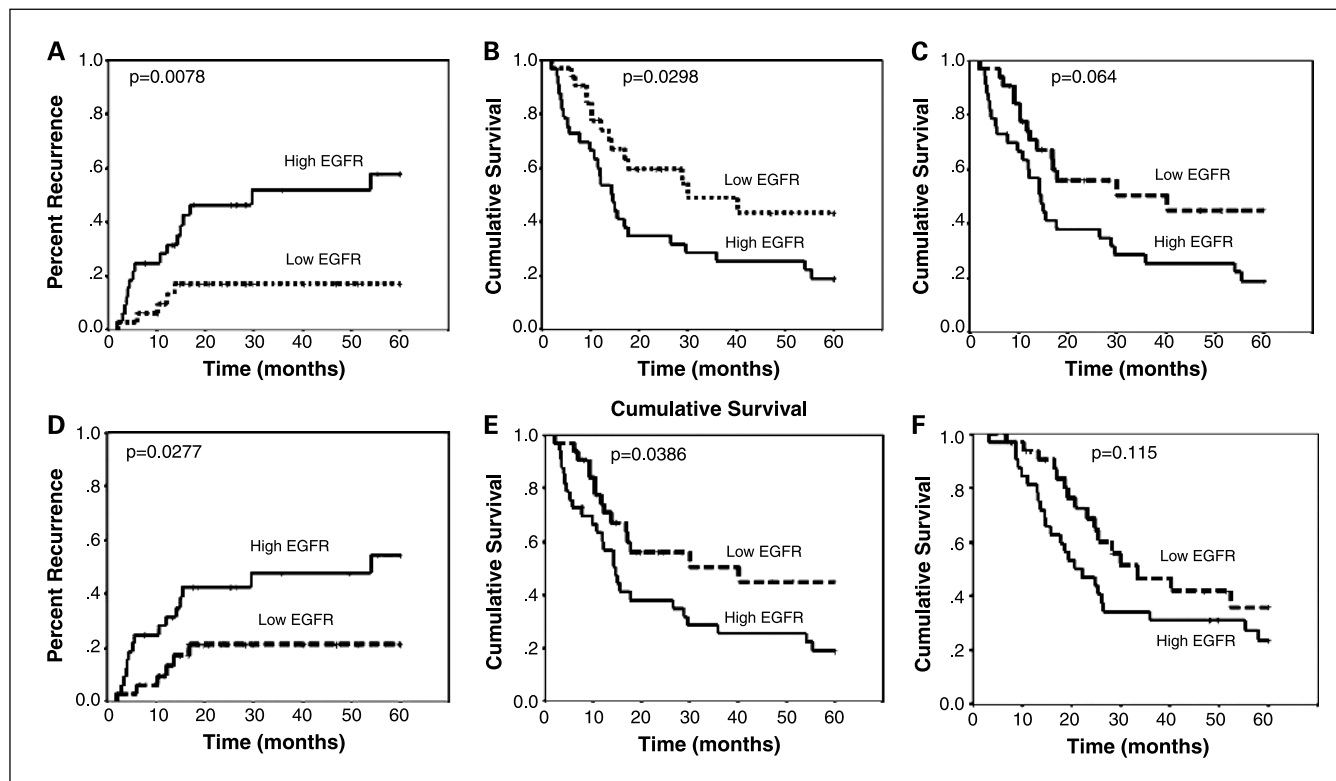


Fig. 2. Kaplan-Meier survival analysis by EGFR expression. For local recurrence, patients with high EGFR tumor (A) and high EGFR nuclear expression (D) showed markedly increased 5-year recurrence rates. For disease-free survival, patients with high EGFR tumor (B) and high EGFR nuclear expression (E) displayed inferior disease-free survival. For overall survival, there was a trend toward worse survival in both high EGFR tumor (C) and high EGFR nuclear (F) groups, but this did not reach statistical significance.

Table 2. Univariate 5-year survival analysis (Kaplan-Meier log rank)

	Mean survival or recurrence time (mo)	Cumulative survival or recurrence (\pm SE)	P
EGFR tumor expression			
Local recurrence*			
Low tumor EGFR	51.2	17.2% (7)	0.0078 [†]
High tumor EGFR	34.2	57.8% (10)	
Disease-free survival*			
Low tumor EGFR	35.8	43.4% (10)	0.0298 [†]
High tumor EGFR	42.2	19.0% (7)	
Overall survival			
Low tumor EGFR	39.3	40.2% (11)	0.0642
High tumor EGFR	29.4	21.1% (7)	
EGFR nuclear expression			
Local recurrence*			
Low nuclear EGFR	49.4	21.4% (8)	0.0277 [†]
High nuclear EGFR	35.9	54.4% (11)	
Disease-free survival*			
Low nuclear EGFR	35.8	44.8% (10)	0.0386 [†]
High nuclear EGFR	24.3	19.0% (7)	
Overall survival			
Low nuclear EGFR	38.0	35.9% (10)	0.1147
High nuclear EGFR	30.1	23.5% (8)	

*One patient lacked recurrence data and was excluded from local recurrence and disease-free survival calculations.
[†]Significant at the 0.01 level.
[‡]Significant at the 0.05 level.

rather loose definition of overexpression without an accurate determination of receptor levels. Studies with the humanized EGFR antibody cetuximab (C225) and the EGFR tyrosine kinase inhibitor gefitinib have shown responses in human tumors and cell lines expressing a wide range of EGFR levels, from very low to very high (17, 18). One implication of these data is that low EGFR-expressing but still inhibitor-sensitive cells may not score as positive with the widely used immunohistochemical method. An important limitation with standard immunohistochemistry is that low antibody concentrations lack sensitivity at the low end of protein expression and high antibody concentrations fail to differentiate between mid and high levels of protein expression because of saturation combined with higher background and nonspecific staining. With the availability of EGFR inhibitors, the need for assays that will appropriately select patients for EGFR-targeted therapy becomes more urgent.

We had previously examined the present cohort for EGFR overexpression by conventional immunohistochemistry and found, surprisingly, that EGFR levels are protective against local recurrence (19). This paradoxical result, although consistent with two other studies, challenges the validity of pathologist-based scoring. It was postulated that a more quantitative assessment of expression would be necessary. In the present study, using quantitative immunohistochemistry, we were able to show that EGFR expression levels are inversely

correlated with outcome in oropharyngeal squamous cell cancers. In multivariate analysis, adjusted for well-recognized prognostic indicators, EGFR maintained its independent prognostic value. This analysis shows the power of continuous automated assessment to define subclasses of tumors not achievable using standard pathologist-based assessment. Using this technology, we were able to show an association between EGFR expression levels and outcome consistent with the biological role of EGFR in tumor behavior. The use of AQUA has been validated in several tumor types, including HNSCC (15, 20–22), where the application of this technology revealed associations between biomarkers and outcome not discernable with conventional immunohistochemistry. Additionally, we found an association between elevated nuclear EGFR and lack of clinical response to initial therapy. A similar

Table 3. Multivariate 5-year survival analysis by Cox regression

Variable	Hazard ratio (95% confidence interval)	P
EGFR tumor expression		
Local recurrence*		
Management	0.314 (0.1-1.0)	0.057
Histologic grade	1.212 (0.5-2.8)	>0.5
TNM stage	0.947 (0.5-1.9)	>0.5
Subsite within oropharynx	1.424 (0.5-3.8)	0.48
High tumor EGFR expression	3.632 (1.2-11.4)	0.027 [†]
Disease-free survival*		
Management	0.598 (0.3-1.3)	0.19
Histologic grade	1.229 (0.7-2.2)	>0.5
TNM stage	1.050 (0.6-1.8)	>0.5
Subsite within oropharynx	1.169 (0.6-2.3)	>0.5
High tumor EGFR expression	1.975 (1.0-4.1)	0.07
EGFR nuclear expression		
Local recurrence*		
Management	0.297 (0.1-1.0)	0.047 [†]
Histologic grade	1.165 (0.5-2.7)	>0.5
TNM stage	0.872 (0.4-1.8)	>0.5
Subsite within oropharynx	1.192 (0.5-3.1)	>0.5
High nuclear EGFR expression	3.212 (1.1-9.8)	0.041 [†]
Disease-free survival*		
Management	0.583 (0.3-1.3)	0.17
Histologic grade	1.183 (0.6-2.2)	>0.5
TNM stage	1.037 (0.6-1.8)	>0.5
Subsite within oropharynx	1.108 (0.6-2.2)	>0.5
High nuclear EGFR expression	1.953 (0.9-4.1)	0.07

*One patient lacked recurrence data and was excluded from local recurrence and disease-free survival calculations.
[†]Significant at the 0.05 level.

trend was noted for tumor EGFR but did not reach statistical significance. As predictors of response to therapy are desperately needed, it is possible that AQUA analysis of EGFR may be an attractive candidate marker.

The discrepancy between the results we obtained with AQUA and conventional immunohistochemistry shows one of the problems with by-eye scoring of immunohistochemical stains (i.e., the difficulty humans have in translating a continuous marker into a nominal 4-point scale). Specifically, the pathologist tends to group things as positive or negative whereas the automated device results in continuous full-scale scores.

Our finding that not only tumor EGFR expression, but also nuclear EGFR levels, strongly independently predict for outcome deserves mention. EGFR is generally known as plasma membrane receptor tyrosine kinase, which sends signals to the nucleus via the mitogen-activated protein kinase, the phospholipase C γ -protein kinase C δ s, and the phosphatidylinositol 3-kinase pathways. However, recently, data are accumulating to imply that nuclear localization and action of EGFR may occur as well (10, 11). EGFR may enter

the nucleus and directly act as transcriptional factor, bypassing the protein phosphorylation cascades. Lin et al. (10) showed that nuclear EGFR is associated with the promoter region of cyclin D1 *in vivo* and activates transcription. To our knowledge, an association of nuclear EGFR with poor prognosis has not been reported previously. Nuclear localization and action of EGFR are worthy of study as they constitute a potential mechanism of resistance to EGFR-targeted therapies: Because nuclear EGFR directly activates transcription bypassing the protein phosphorylation cascades, EGFR-rich tumors may not respond to EGFR inhibitors blocking only receptor-mediated signaling.

Our findings require validation in a second cohort. These results, if confirmed, have important clinical implications. AQUA may be used in pharmacodynamic studies to identify patient cancers sensitive to EGFR inhibitors. Because effective preclinical models are not available, pharmacodynamic end points in phase I/II clinical trials with anti-EGFR agents, examining the effects of EGFR-directed therapies on the molecular targets and the downstream pathways, become critically important.

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