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Integrating EGFR Assay with Clinical Parameters Improves Risk Classification for Relapse and Survival in Head and Neck Squamous Cell Carcinoma

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Abstract

PURPOSE—Epidermal growth factor receptor (EGFR) overexpression has been consistently found to be an independent predictor of local-regional relapse (LRR) after radiotherapy. We assessed the extent by which it can refine risk classification for overall survival (OS) and LRR in patients with head and neck squamous cell carcinoma (HNSCC).

METHODS AND MATERIALS—EGFR expression in locally advanced HNSCC was measured by immunohistochemistry (IHC) in a series of patients randomized to receive accelerated or conventional radiation regimens in a phase III trial. Subsequently, data of the two series were pooled (N=533) for conducting a recursive partitioning analysis that incorporated clinical parameters (performance status, primary site, T- and N-categories, etc.) and four molecular markers (EGFR, p53, Ki-67, and microvessel density).

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RESULTS—This study confirmed that patients with higher than median levels of tumor EGFR expression had a lower OS (RR: 1.90, $P=0.0010$) and a higher LRR (RR: 1.91, $P=0.0163$). Of the four markers analyzed, only EGFR was found to contribute to refining classification of patients into three risk classes with distinct OS and LRR outcomes. The addition of EGFR to three clinical parameters could identify patients having up to a 5-fold difference in the risk of LRR.

CONCLUSIONS—Adding pretreatment EGFR expression data to known robust clinical prognostic variables improved the estimation of the probability for OS and LRR after radiotherapy. Its use for stratifying or selecting patients with defined tumor feature and pattern of relapse for enrollment into clinical trials testing specific therapeutic strategy warrants further investigation.

Keywords

EGFR; Risk Classification; Prognosis; HNSCC

BACKGROUND

Clinical trials of patients with locally advanced head and neck squamous cell carcinomas (HNSCC) have shown that rational modifications of radiation regimen and combinations of radiation and chemotherapy decrease local-regional relapse (LRR) and increase overall survival (OS) rates (1-9). However, both strategies increase acute side effects and concurrent radiation-chemotherapy regimens also worsen late complications (7,10). These observations combined with better knowledge of tumor biology have increased the need to identify biomarkers for classifying tumors with different prognosis, predicting tumor response to therapy, and developing therapeutic strategies that can selectively enhance tumor response to various therapeutic modalities.

The epidermal growth factor receptor (EGFR) has emerged as both a prognostic-predictive biomarker in HNSCC and as a target for therapeutic intervention. Initial studies have shown that high tumor EGFR expression correlates with poor survival (11,12). More recent work has demonstrated that high EGFR expression predicts for poorer tumor response to radiotherapy (13-16) or combination of chemotherapy with radiation (17). Concurrently, studies in cell lines and xenografts have shown that inhibition of EGFR by cetuximab, a monoclonal antibody against EGFR, enhances tumor response to single dose or fractionated radiation (18,19). The biological basis of these observations may be explained by the finding that ionizing radiation triggers localization of EGFR into the nucleus and promotes the repair of DNA double-strand breaks through increasing the activity of DNA-dependent kinase (DNA-PK). Blockade of EGFR by cetuximab abolishes the EGFR nuclear localization, and results in suppression of the radiation-induced activation of DNA-PK, inhibition of DNA repair and enhanced cellular radiation sensitivity (20).

Our previous study of patients enrolled into a phase III trial and treated with standard radiation therapy has shown that patients with higher EGFR-expressing HNSCC (>median value) had significantly higher LRR (HR: 1.95, $P=0.002$) and lower OS rates (HR: 1.75, $P=0.006$) relative to those with lower EGFR-expressing tumors (13). Since the methods used for EGFR assay varied amongst laboratories, we conducted this study to assess the reproducibility of image analysis-based assay and to validate its value in predicting tumor response to radiation. In addition to EGFR, three other assays, p53 expression (a surrogate marker of TP53 mutation), Ki-67 (a marker of cell proliferation) and microvessel density (MVD) have been reported to correlate with outcome in some series (21-23); therefore, we examined these three additional biomarkers with EGFR expression. To develop a model for accurate prognosis, we then assessed whether, and the extent to which, incorporation of

biological markers with clinical features can improve classification of risk for LRR and OS in a large cohort of patients enrolled into a phase III trial who received well-defined therapy.

METHODS AND MATERIALS

PATIENTS

Patients with AJCC stage III-IV, non-metastatic, squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and supraglottic larynx were enrolled into a phase III study comparing the efficacy of three modified fractionation regimens with the conventional schedule. The clinical outcome of this trial was reported earlier (24). Paraffin-embedded blocks or unstained slides submitted to the Radiation Therapy Oncology Group Tissue Repository were retrieved for the assays. The population for EGFR validation study component consisted of 267 patients randomized to receive accelerated fractionation by concomitant boost (AFX-C). Then, the previously published data of 266 patients randomized to standard fractionation (SFX) (13) were added for combined analyses (Table 1).

ASSAYS

EGFR assay—The EGFR expression was determined as previously described (13). Briefly, the slides were incubated with mouse monoclonal antibodies (Mab) that is reactive to the peptide backbone of the extracellular domain of the EGFR molecule (31G7, Zymed Laboratories, Inc.) diluted 1:50 in buffer. After incubating with a secondary antibody and counterstaining, slides were mounted for quantitative analysis. The magnitude of EGFR expression was measured by computerized quantitative image analysis using a SAMBA 4000 Cell Image Analysis System (SAMBA Technologies, Meylan, France). Parameters measured were the mean optical density (MOD: optical densities measured over the labeled areas within the structure), staining index (SI: the proportion of stained area relative to the total area of the structures), and the quick score (QS: $\text{MOD} \times \text{SI}/100$).

p53, Ki-67, and microvessel density assays—The assays were conducted as previously described (21,22,25). For the p53 IHC assay, the primary antibody was a mouse Mab directed against p53 protein (DO-7, DAKO Corp., Carpinteria, CA, dilution 1:200). For Ki-67, the slides were incubated with the Ki-67/MIB-1 Mab (DAKO Corp., Carpinteria, CA, dilution 1:100). For MVD, the sections were stained with murine Mab anti-Factor VIII-related antigen (Factor VIII-RA), subsequently treated with biotinylated second antibody and processed.

For p53 and Ki-67, the medians were chosen as cut points and the groups with equal or less than median versus greater than median value of IHC staining were compared for endpoints described below. Sections with MVD results were scanned and the number of stained microvessels within areas of invasive tumor was counted. The mean number of microvessels per field was recorded as the MVD. A prospective MVD of ≥ 60 was pre-selected as the threshold for high MVD for this study, based on previously published studies of breast and nasopharyngeal cancer (23).

STATISTICAL ANALYSIS

Three endpoints were evaluated: overall survival (OS), progression-free survival (PFS), and time to local-regional relapse (LRR). Failure for OS was death due to any cause. Failure for LRR was local or regional progression, death due to study cancer or unknown causes, non-protocol radiation therapy or chemotherapy, surgery of the primary tumor with disease present or unknown, or surgery of the regional nodes with disease present or unknown > 15 weeks from the end of radiation therapy. Distant metastasis, second primary tumor, and

death from other causes were considered as competing risks. Failure for PFS was LRR, distant metastasis, or death due to any cause. Patients were censored for second primary tumor. OS and PFS were estimated using the Kaplan-Meier method (26) and compared by the log-rank test (27). Time to LRR was estimated by the method of cumulative incidence (28) and tested with Gray's test (29).

To confirm the prognostic value of EGFR, only AFX-C patients were used. Cox proportional hazards model was used to estimate the hazard ratio between high and low EGFR after adjustment for known prognostic factors. The magnitude and distribution of pretreatment marker assays were assessed using various descriptive statistics (mean, median, etc.) along with their association with the known tumor- and patient-related prognostic variables for OS, PFS, and LRR. The distributions of EGFR for SFX and AFX-C were compared with the Kolmogorov-Smirnov test (30). To investigate the prognostic value of Ki-67, p53, and MVD, both SFX and AFX-C patients were used. Cox proportional hazards model was used to estimate the hazard ratio between high and low values after adjustment for known prognostic factors, and models were stratified by assigned treatment.

For development of risk groups, both SFX and AFX-C patients were used. The 533 patients were randomly divided into training (n=267) and test (n=266) sets stratified by assigned treatment. The training data were used to develop models and the test data to validate the results. Comparisons of baseline characteristics for the training and test sets were done using the Pearson chi-square test (31) for unordered contingency tables (gender, race, and primary site), the Kruskal-Wallis test (32) for singly ordered contingency tables (Karnofsky performance status [KPS], T- and N-categories, and AJCC Stage) and the Kolmogorov-Smirnov test of continuous distributions for EGFR expressions and age (30). Cut points for EGFR SI were limited to multiples of five from 50 to 90 and those for age were limited to multiples of five from 50 to 75. For risk classification analysis, we used a nonparametric statistical technique known as recursive partitioning analysis (RPA) with the log-rank splitting method (33). The goal was to produce three RPA classes by combining terminal nodes with similar outcome. In patients without available tumor specimens for laboratory assays, missing values were imputed 10 times and the most frequently occurring model was selected from examining all 10 separate RPA trees. The relative risks comparing the survival classes from the 10 imputations were combined using SAS software and the PROC MI and MIANALYZE procedures (34). Terminal nodes were further combined into classes based on visual inspection of Kaplan-Meier plots. Models with similar discriminatory properties and reproducibility were compared by simplicity (preference given to fewer factors). Cox regression models were utilized to determine hazard ratios (relative risks) and *P*-values between the identified classes. Only OS was used to develop the model. The resulting classes were then evaluated to see if they yielded similar classifications for PFS and LRR.

RESULTS

Patient characteristics

The 533 eligible patients (SFX arm – 266 and AFX-C arm – 267) were enrolled between 1991 and 1997 to a phase III clinical trial conducted by the Radiation Therapy Oncology Group (RTOG 90-03). Patient and tumor characteristics are summarized in Table 1. The clinical variables did not differ significantly between the training and test sets. EGFR, Ki-67, p53, and MVD values were available for 300, 223, 217, and 213 patients, respectively. The numbers of patients in each staining differed because different numbers of tissue samples and staining data were available for each marker. Detailed patient and tumor characteristics of EGFR, Ki-67 and p53 assays are presented in Supplemental Tables 1-6, and the MVD assay data were previously published (25).

EGFR assay reproducibility and correlations between EGFR expression and treatment outcome

Because the EGFR staining of the samples from SFX and AFX-C arms was performed in two separate batches, we performed comparative analyses of these two data sets to assess experimental variations and reproducibility of the EGFR assay. The continuous distributions of EGFR SI and MOD scores (Figure 1A) found in the samples from AFX-C arm were analogous to our previously reported series (13) from SFX arm ($p=0.62$ for SI; $p=0.85$ for MOD). As in our prior study, we found no correlation between EGFR expression and clinical variables including the T- and N- categories, AJCC stage grouping, KPS, age, and gender (Supplemental Table 7). Moreover, in multivariate analyses, high EGFR expression ($>$ median value for SI, MOD, or QS previously determined using samples from prior SFX arm) was also found to be a robust, independent determinant of OS, PFS, and LRR using samples from AFX-C (Supplemental Table 8). The relative risk (RR with the 95% confidence intervals) derived using EGFR-SI for these three outcome endpoints were 1.90 (1.30-2.79, $P=0.0010$), 1.74 (1.15-2.64; $P=0.0086$), and 1.91 (1.13-3.24; $P=0.0163$), respectively. Furthermore, LRR was mostly seen in patients with high EGFR expression assessed by both SI and MOD with high concordance between the sample sets from SFX and AFX-C arms (Figure 1B). Also, regardless of the treatment modalities, the magnitude of separation in the cumulative LR relapse curves depending on the level of EGFR expression were comparable (SFX- Relative Risk 1.67, $p=0.0257$; AFX-C- RR 1.91, $p=0.0163$; Figure 1C).

Determination of p53 and Ki-67 expression, and tumor microvessel density

p53 protein expression and cell proliferation as assessed by Ki-67 expression were also determined by IHC. Each marker was assessed separately and summarized in Supplemental Table 9. While the expression of p53 in $> 30\%$ (median) of the tumor cells were associated with worse PFS (RR 1.53, 95% CI 1.11-2.09, $P=0.0086$) and increased rate of LRR (RR 1.58, 95% CI 1.08-2.32, $P=0.0186$) compared to $\leq 30\%$, there was no detectable difference in OS (RR 1.24, 95% CI 0.92-1.67, $P=0.1608$). We could not detect any significant contribution of Ki-67 to outcome. The tumor MVD also did not correlate with any of the survival parameters consistent with previously published data (25).

Contribution of biological markers in refining risk classification

Initial analysis included KPS, primary site, T-category, N-category, age, gender, assigned treatment, EGFR-SI, EGFR-MOD, EGFR-QS, p53, Ki-67, and MVD. Subsequently, EGFR QS and EGFR MOD were removed due to the higher significance of EGFR-SI and the high correlation among these parameters ($r=0.71-0.99$). In addition, cut points for EGFR were limited to multiples of five so that the resulting model would have a meaningful cut off point rather than a median which may vary depending on samples analyzed. The RPA algorithm chose SI 80 as the best cut point. Of the four markers analyzed, only EGFR showed promise as an important factor in the model, so all other tumor markers were removed from further consideration. A combination of four parameters consisting of KPS, EGFR expression, primary site, and T-category (in decreasing weight) segregated the study population into three distinct, reproducible RPA classes (Class I, II and III) for OS and the final recursive partitioning tree is shown in Figure 2.

Three RPA classes have significantly different OS and LRR rates in both training and testing sets (Figure 3). The estimated 5-year OS rates for Classes I, II, and III patients were approximately 50%, 30%, and 10%, respectively, and the corresponding 5-year LRR rates were around 35%, 65%, and 85%. To quantify the contribution of EGFR expression to refining risk classification for LRR, we repeated the RPA analysis with or without this parameter. Table 2 shows that the addition of EGFR expression data improved the definition

of risk classes. The risk of patients in Class III (worst prognosis) in experiencing LRR relative to Class I (best prognosis), for example, increased from 3.4 to 5.0.

DISCUSSION

The recognition that cancers of the same site, stage and morphology have divergent natural history and respond differently to therapeutic interventions have inspired many to search for predictive markers of response to therapy. Until recently, a large body of research on HNSCC has focused on identifying surgical-pathologic features associated with poor outcome, such as the number and location of lymph node involvement, presence of extra-nodal extension, positive surgical margin, and perineural invasion (35-39). Although clustering of such surgical pathologic adverse features could identify patients who did relatively well after radical surgery alone or followed by adjuvant radiotherapy (40) with or without chemotherapy (6,7), further work is needed to guide clinical research and practice. Finding prognostic-predictive markers is particularly important for personalizing non-surgical therapy, such as radiation with or without chemotherapy or new class of agents, because surgical-pathologic data are not available in this setting.

Quantification of EGFR expression has been controversial with semi-quantitative scoring system ranging 0-3+. However, our previously study (13) on a well-defined cohort of patients with locally advanced HNSCC randomized to receive standard radiotherapy alone revealed that pretreatment EGFR expression level of the primary tumor measured by image analysis-based IHC assay was a robust predictor for tumor response to radiotherapy, more so than the T-stage. This data is consistent with other image-guided quantitative scoring systems (17). Before promoting any biomarkers for clinical application, it is crucial to determine the reproducibility of the assay, to validate its predictive value for radiation response, and to assess whether and to what extent this assay added value to the known clinical prognostic variables. This follow-up study was conducted using a well-defined group of patients enrolled into the same prospective phase III trial but randomized to receive a different radiotherapy regimen. These results establish that the EGFR assay was remarkably reproducible and confirmed the lack of correlation between the levels of EGFR expression with clinical parameters, and validated its value in predicting tumor response to radiotherapy when measured quantitatively.

Currently, there are many proposed laboratory biomarkers that are prognostic of clinical outcomes. However, there are only limited studies in HNSCC to build upon the clinical prognostic factors that are already known to be robust and often are better than expensive laboratory assays, and few that combine multiple laboratory biomarkers. To examine this issue, we pooled the current clinical and EGFR data with those of the previous series and added the results of additional three molecular assays (p53, Ki-67, and MVD) for further analysis after dividing the study population into training and test sets. Furthermore, with recent data regarding the HPV status as a strong prognostic marker and to put our results in perspective, we assayed the remaining unstained oropharyngeal carcinoma slides for p16, a surrogate marker for HPV, and determined the positivity to be approximately 33% (or approximately 20% of total number of tumor samples), probably due to lower prevalence of HPV infection in the early 1990's (unpublished data). Additional analysis incorporating the HPV data is still ongoing. Out of four markers further analyzed, only EGFR was found to have added value when combined with known robust clinical prognostic factors in identifying three classes of patients with significantly different clinical outcomes. Although p53 staining associated with worse PFS and LRR, it did not significantly contribute to the prognostic model probably because there are biological and clinical differences among the disruptive and non-disruptive mutations within the gene that are not distinguished by IHC staining (41).

This type of comprehensive prognostic data is critical for enriching patients with well defined tumor features and patterns of relapse as well as to prioritize clinically relevant laboratory biomarkers for enrollment into clinical trials and select appropriate correlative studies. Class III patients, having a 10% chance of survival and an 85% risk of developing LRR at 5 years, are ideal candidates for testing a vigorously intensified local therapy regimen. On the other hand, given the relatively favorable outcome achieved with radiation alone, it is prudent to test an agent with fairly low toxicity profile in combination with radiation for patients belonging to Class I. In addition to prognosis, this type of analyses can be applied to further examine the biomarkers for predictive potential. To ascertain EGFR expression levels as a potential predictive biomarker for either of the treatment arms (SFX versus AFX-C), we evaluated Cox models including assigned treatment (1 if SFX, 0 otherwise), EGFR (1 if > median, 0 otherwise), and treatment by EGFR interaction (as well as T stage, N stage, KPS, and primary site). The hazard ratios (95% CI for interaction) for OS, PFS, and LRR were 0.75 (0.45-1.25), 0.77 (0.45-1.31), and 0.88 (0.46-1.71), suggesting that patients with EGFR expression levels below the median may receive more benefit from AFX compared to patients with EGFR above the median. Although it did not reach statistical significance, it still generated an interesting hypothesis for future clinical trials. Thus, its use for stratifying or selecting patients for enrollment into clinical trials testing specific therapeutic strategy is recommended.

The results of the current work provide the impetus to perform other molecular marker assays using the samples in this series of patients with mature outcome data and conduct similar types of analyses to further refine risk classification. It is also desirable to incorporate the biomarkers to currently existing clinical prognostic information, and extend this approach to well defined group of patients enrolled into prospective trials testing the combination of radiation with chemotherapy or with novel agents when the clinical outcome data become available in the near future. With this goal in mind, many centers and cooperative groups have increased tissue banking efforts, including collection of samples suitable for high through put assays, for correlative studies. Such integrated investigations are changing the design of trials in the quest for realization of personalized medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EGFR assay reproducibility and correlations between EGFR expression and treatment outcome. **A:** EGFR expression measured by mean optical density (MOD - optical densities measured over the labeled areas within the structure) and staining index (SI - the proportion of stained area relative to the total area of the structures). The continuous distributions of EGFR SI and MOD scores found in the samples from AFX-C arm were analogous to our previously reported series (13) from SFX arm ($p=0.62$ for SI; $p=0.85$ for MOD). **B:** Scatter plot showing that the vast majority of tumors with SI of over 80% recurred at local-regional sites after both fractionation regimens. **C:** The rates and relative risks of local-regional

relapse in high versus low EGFR-expressing tumors after treatment with standard (SFX) or accelerated fractionation regimen (AFX-C).

A.

B

Figure 2.
Recursive partitioning analysis trees, **A**: without EGFR, and **B**: with EGFR.

Figure 3.

A: Overall survival curves, and **B:** cumulative incidences curves for local-regional relapse of three distinct outcome classes (Class I – Best, Class II – Intermediate, and Class III – Worst) derived from RPA analysis in training and test sets.

Table 1

Distribution of Patient and Tumor Characteristics

	Training Set		Test Set		Total	
	n	%	n	%	n	%
Total Patients	267	100.0	266	100.0	533	100.0
Assigned Treatment						
SFX	133	49.8	133	50.0	266	49.9
AFX-C	134	50.2	133	50.0	267	50.1
Age						
≤ 50	41	15.4	50	18.8	91	17.1
51-55	37	13.9	38	14.3	75	14.1
56-60	48	18.0	51	19.2	99	18.6
61-65	50	18.7	53	19.9	103	19.3
66-70	36	13.5	36	13.5	72	13.5
71-75	28	10.5	22	8.3	50	9.4
> 75	27	10.1	16	6.0	43	8.1
Gender						
Male	211	79.0	211	79.3	422	79.2
Female	56	21.0	55	20.7	111	20.8
KPS						
60	19	7.1	8	3.0	27	5.1
70	28	10.5	28	10.5	56	10.5
80	59	22.1	64	24.1	123	23.1
90	109	40.8	127	47.7	236	44.3
100	52	19.5	39	14.7	91	17.1
Primary Site						
Oral cavity	31	11.6	24	9.0	55	10.3
Oropharynx	158	59.2	164	61.7	322	60.4
Hypopharynx	41	15.4	32	12.0	73	13.7

	Training Set		Test Set		Total	
	n	%	n	%	n	%
Larynx	37	13.9	46	17.3	83	15.6
T - Category						
T1	14	5.2	17	6.4	31	5.8
T2	74	27.7	73	27.4	147	27.6
T3	100	37.5	98	36.8	198	37.1
T4	78	29.2	78	29.3	156	29.3
Tx	1	0.4	0	0	1	0.2
N - Category						
N0	60	22.5	56	21.1	116	21.8
N1	43	16.1	56	21.1	99	18.6
N2A	25	9.4	26	9.8	51	9.6
N2B	47	17.6	48	18.0	95	17.8
N2C	50	18.7	53	19.9	103	19.3
N3	42	15.7	27	10.2	69	12.9

Table 2

The relative risks for local-regional relapse and class features derived from analysis with or without incorporating EGFR expression data.

Analysis	Relative Risk (95% confidence intervals)	Class features
Without EGFR data	1.0	I: KPS 90-100 and T1-3 and N0-2c
	1.9 (1.5 – 2.6)	II: • KPS 60-80 and T1-3 of oral cavity, oropharynx, hypopharynx <u>or</u> • KPS 60-80 and larynx primary <u>or</u> • KPS 90-100 and T4 and N0-2c
	3.4 (2.5 – 4.6)	III: • KPS 60-80 and T4 of oral cavity, oropharynx, hypopharynx <u>or</u> • KPS 90-100 and N3
With EGFR data	1.0	I: KPS 90-100 and T1-3 and EGFR SI ≤80
	2.1 (1.4 – 2.9)	II: • KPS 60-80 and T1-3 of oral cavity, oropharynx, hypopharynx <u>or</u> • KPS 60-80 and larynx primary <u>or</u> • KPS 90-100 and T4 and EGFR SI ≤80 <u>or</u> • KPS 90-100 and EGFR SI >80
	5.0 (3.3 – 7.4)	III: KPS 60-80 and T4 of oral cavity, oropharynx, hypopharynx