Significance of EGFR Protein Expression and Gene Amplification in Non–Small Cell Lung Carcinoma

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Abstract

We evaluated epidermal growth factor receptor (EGFR) protein expression by immunohistochemical analysis and EGFR *gene amplification by fluorescence in situ hybridization in 199 consecutive newly diagnosed and surgically treated patients with primary non–small cell lung carcinoma (NSCLC) and correlated results with clinicopathologic findings. EGFR protein expression was more common in squamous cell carcinoma (SCC; 17 [26.2%]) than in adenocarcinoma (14 [11.1%];* P = *.0076) and more frequently associated with* EGFR *amplification (8 [14.5%] vs 4 [3.6%] cases;* P = *.0208). Poor differentiation was associated with a higher average number of* EGFR *gene copies per cell (mean, 4.18;* P = *.0322) and a higher* EGFR*/chromosome 7 ratio (mean, 1.84;* P = *.0324). N0 disease showed a higher number of* EGFR *gene copies (mean, 4.196;* P = *.0163). SCCs demonstrated a higher* EGFR*/chromosome 7 ratio than adenocarcinomas (mean, 1.95 vs 1.47;* P = *.0324), particularly T1 tumors (mean, 1.79;* P = *.0243). Statistical analysis failed to show correlation between outcome and EGFR protein expression and gene amplification in early NSCLC. EGFR protein expression was uncoupled from gene amplification in most cases, although good correlation occurred in a subset of SCCs.*

Despite improved surgical techniques and systemic chemotherapy treatments, patients with non–small cell lung carcinoma (NSCLC) have only 5% to 15% 5-year survival after initial diagnosis. The epidermal growth factor receptor (EGFR) that was discovered almost 3 decades ago has emerged as a leading target for the treatment of patients with NSCLC.[1] Inhibition of the EGFR pathway by a monoclonal antibody against the receptor or a small molecular inhibitor of the receptor tyrosine kinase is being evaluated as therapy for various malignant neoplasms. Recently, it has been shown that specific mutations in the *EGFR* gene may identify lung cancer patients with a good response to the tyrosine kinase inhibitor gefitinib.[2-4] These specific mutations most frequently occur in a subset of well- to moderately differentiated adenocarcinomas of the lung, particularly in the Asian, nonsmoker, female population.[5]

Because the response to other targeted agents such as trastuzumab and tamoxifen depends mainly on the level of expression of the target in the tumor, several trials have focused on EGFR protein expression but have failed to predict gefitinib sensitivity by EGFR protein levels as determined by immunohistochemical analysis or immunoblotting.[6,7] On the other hand, the prognostic significance of EGFR gene overexpression in NSCLC is uncertain, although its overexpression has been linked to poor prognosis in various solid tumors, including prostate, breast, colon, ovary, and head and neck.[8-11]

The significance of EGFR protein overexpression is still controversial.[12-15] Furthermore, mechanisms other than mutation and amplification might have a role in regulation of EGFR gene expression in NSCLC. Even though 2 EGFR inhibitors have been approved for the treatment of advanced NSCLC and there is increasing knowledge of underlying

molecular mechanisms for targeted lung cancer therapies, there are no validated patient selection criteria for therapy with these agents. The focus of our study was to analyze the relationship between EGFR protein expression and gene amplification and to determine the potential prognostic significance of these results.

Materials and Methods

This prospective study was conducted at the University of Pittsburgh Medical Center, Pittsburgh, PA, from August 2000 to January 2002 after obtaining approval from the institutional review board to conduct the study on an anonymized basis. Tumor samples from 199 consecutive patients with NSCLC (128 adenocarcinoma, 67 squamous cell carcinoma, and 4 large cell carcinomas) who underwent surgical resection were analyzed for EGFR protein expression by immunohistochemical analysis and for *EGFR* gene amplification by fluorescence in situ hybridization (FISH) **Image 1** and **Image 2**.

All H&E-stained histologic sections of the tumors were reviewed by 2 pathologists (S.D. and S.A.Y.). The histologic type was determined according to World Health Organization criteria.[16] Bronchioloalveolar carcinomas and large cell neuroendocrine carcinomas were excluded from the study. Large cell carcinomas were not included in further statistical analysis because of the small number of cases. Cases in which preoperative chemotherapy or radiation had been given were excluded from analysis. All patients were followed up to determine outcome and survival.

The immunohistochemical study was performed and graded using anti-EGFR mouse monoclonal antibody at a dilution of 1:200 (DakoCytomation, Carpinteria, CA) according to the manufacturer's instructions. Membrane expression was graded from 0 to 3 (in the style of DakoSource for HER-2/neu). For the purpose of the analysis, tumors with a score more than 2 were regarded as showing overexpression.[17] Only complete clear staining of the tumor cell membrane was interpreted as positive. Cytoplasmic or granular staining was considered negative. Sections of lung adenocarcinoma previously identified as positive for EGFR protein served as positive control samples. Negative control experiments included omitting the primary antibody and replacing it with normal serum.

FISH analysis of *EGFR* amplification was performed by using the standard method with the dual-color EGFR SpectrumOrange/CEP7 SpectrumGreen probe and paraffin pretreatment reagent kit (Vysis, Downers Grove, IL).[18] In brief, paraffin sections were deparaffinized, dehydrated in ethanol, and air dried. Sections were digested with protease K (0.5 mg/mL) at 37°C for 28 minutes. The slides were denatured at 75°C for 5 minutes and dehydrated in ethanol. The probes were denatured for 5 minutes at 75°C before hybridization. Slides were hybridized overnight at 37°C and washed in 2× standard saline citrate/0.3% Nonidet P-40 at 72°C for 2 minutes. Nuclei were counterstained with 4'6-diamidino-phenylindole/antifade (Vysis). Each FISH assay included normal lung tissue sections as negative control samples and sections of lung non–small cell carcinoma previously identified as carrying *EGFR* gene amplification as

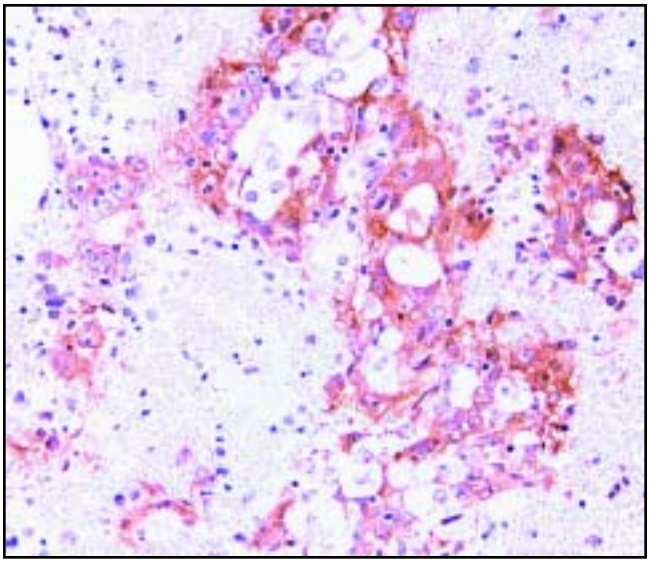

Image 1 Immunohistochemical analysis of epidermal growth factor receptor (EGFR) protein expression. Squamous cell carcinoma of the lung with strong, complete membrane staining for EGFR (×20).

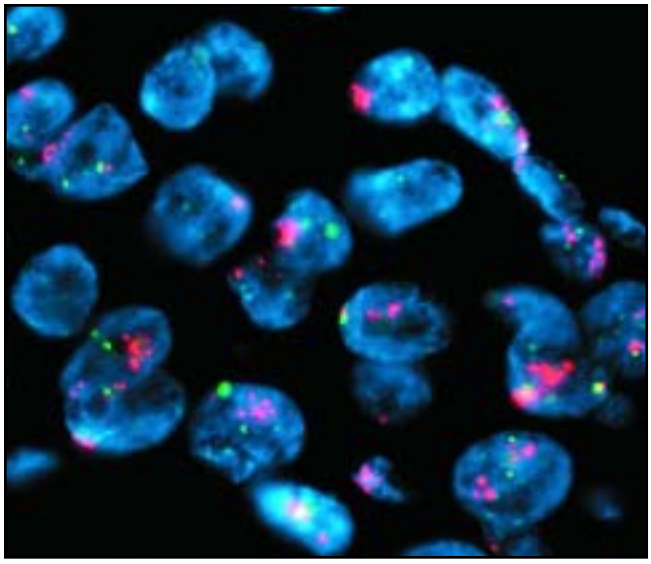

Image 2 Epidermal growth factor receptor (*EGFR*) gene amplification in non–small cell lung carcinoma using fluorescence in situ hybridization with EGFR-specific (red) and chromosome 7–specific (green) probe (×630).

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positive control samples. Analyses were performed using a fluorescence microscope (Nikon Optiphot-2 and Quips Genetic Workstation) equipped with Chroma Technology 83000 filter set with single-band exitors for Texas red/rhodamine, fluorescein isothiocyanate, and 4'6-diamidinophenylindole (UV 360 nm).

The histologic areas previously selected on the H&E-stained sections were identified on the FISH-treated slides. Only individual and well-delineated cells were scored. Overlapping cells were excluded from the analysis. At least 60 cells were scored for each case and control sample.

Each tumor was assessed by the average and the maximum numbers of copies of *EGFR* genes per cell and the average *EGFR*/chromosome 7 copy number (CEP7) ratio. *Amplification* was defined as a ratio of *EGFR* signals to chromosome 7 centromere signals of 2.0 or more.

Comparisons of the proportions of variables within clinicopathologic characteristics were performed by using the Fisher exact test or the χ^2 test, as appropriate. *EGFR* gene copy number, chromosome 7 number, and ratio of gene to chromosome were analyzed categorically and considered positive at greater than the median (2.60, 2.14, and 1.1147, respectively). Survival estimates were calculated with the Kaplan-Meier method and compared using the log-rank test. All statistics were performed using SAS statistical software, version 8.02 (SAS Institute, Cary, NC). Statistical significance was defined as a *P* value of .05 or less, and all tests were 2-tailed. *EGFR* gene amplification and protein expression were compared by using κ, using the Landis and Koch criteria.

Results

Of 195 patients, 93 (47.6%) were women and 102 (52.3%) were men, with ages ranging from 35 to 85 years (mean, 68 years). There were 128 adenocarcinomas (65.6%) and 67 squamous cell carcinomas (34.4%). The majority of the tumors were moderately differentiated (110 [56.4%]) and stage I (115 [59.0%]). All patients were smokers. Complete follow-up until April of 2004 or death was obtained for all patients. At last follow-up, 35 patients had died of lung cancer and 160 patients were alive. The mean follow-up period was 18 months from surgery.

The results of EGFR protein expression and FISH analysis together with clinicopathologic data are shown in **Table 1**. EGFR protein overexpression was more frequent in squamous cell carcinoma (17 cases [25.4%]) than in adenocarcinoma (14 cases [10.9%]; $P = .0076$), but overexpression was independent of tumor differentiation ($P = .5737$). EGFR protein expression was not associated with age, sex, pathologic stage, tumor or lymph node status, or angiolymphatic or pleural invasion.

In 20 cases (10 squamous cell carcinomas, 10 adenocarcinomas), *EGFR* gene amplification ($P = .1200$) was demonstrated by FISH analysis. *EGFR* gene amplification was more frequently seen in women than in men (14 vs 6 [13.5% vs 6.3%]), but the difference did not reach statistical significance ($P = .094$). The presence of amplification did not correlate with any of the analyzed clinicopathologic variables.

The average number of *EGFR* gene copies per cell was higher in poorly differentiated carcinomas (mean, 4.18) than

Table 1
EGFR Status as Determined by Immunohistochemical and FISH Analysis in 195 Cases of NSCLC*

Variable	EGFR Expression	*P*	*EGFR* Gene Copies (Mean)	*P*	Ratio of *EGFR* Gene/ Chromosome 7 (Mean)	*P*	*EGFR* Amplification	*P*
Histologic type		.0076†		.8287		.0049†		.12
ADC (n = 128)	14 (10.9)		3.63		1.47		10 (7.8)	
SCC (n = 67)	17 (25.4)		4.19		1.18		10 (14.9)	
Tumor grade		.5737		.0322†		.0324†		.2485
Well-differentiated (n = 14)	1 (7.1)		2.53		1.10		0.0 (0)	
Moderately differentiated (n = 110)	19 (17.3)		3.69		1.54		12.73	
Poorly differentiated (n = 73)	11 (15.1)		4.18		1.84		8.0	
Lymph node status		.2255		.0163†		.0725		.8778
N0 (n = 117)	22 (18.8)		4.196		1.75		11.29	
N1 (n = 35)	7 (20.0)		3.415		1.57		8.33	
N2 (n = 23)	1 (4.3)		3.307		1.40		12.50	
Tumor status		.9422		.2941		.0243†		.2193
T1 (n = 54)	9 (16.7)		4.075		1.79		8.47	
T2 (n = 105)	17 (16.2)		3.979		1.68		14.02	
T3 (n = 9)	2 (22.2)		3.027		1.31		0	
T4 (n = 19)	3 (15.8)		2.677		1.09		0	

ADC, adenocarcinoma; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; NSCLC, non–small cell lung carcinoma; SCC, squamous cell carcinoma.
* Data are given as number (percentage) unless otherwise indicated.
† Statistically significant.

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in moderately (mean, 3.69) and well-differentiated tumors (mean, 2.53; $P = .032$). A higher number of *EGFR* gene copies per cell also was seen in cases without lymph node metastases (N0) than in cases with N1 or N2 metastatic disease (4.196 vs 3.415 and 3.307, respectively; $P = .0163$). Squamous cell carcinomas demonstrated a statistically significant higher ratio of *EGFR* gene to chromosome 7 copy number than adenocarcinoma (mean ratio, 1.96 vs 1.47; $P = .0049$). A higher ratio of *EGFR* gene to chromosome 7 copy number also was seen in poorly differentiated carcinomas (mean ratio, 1.84) than in well- (mean ratio, 1.10) and moderately differentiated (mean ratio, 1.54) tumors ($P = .0324$). The ratio of *EGFR* gene to chromosome 7 correlated with tumor status; specifically, it was higher in T1 tumors (mean ratio, 1.79) than in T2 (mean ratio, 1.68) and T3 tumors (mean ratio, 1.31; $P = .0243$). Similar to the *EGFR* gene copy number per cell, there was a trend toward a higher ratio in the tumors without evidence of lymph node metastases (1.75 vs 1.57 and 1.40; $P = .0725$).

As shown in **Table 2**, the level of agreement for EGFR protein expression determined by immunohistochemical analysis and *EGFR* gene amplification by FISH analysis demonstrated a κ of 0.436, which by the Landis and Koch criteria indicates a "moderate" level of agreement. Of 31 cases with protein overexpression (18 squamous cell carcinomas and 13 adenocarcinomas) 12 cases (39%) (8 squamous cell carcinomas and 4 adenocarcinomas) showed *EGFR* gene amplification by FISH, and 5 cases (1 squamous cell carcinoma and 4 adenocarcinomas) that were negative by immunohistochemical analysis had gene amplification. Coexistence of *EGFR* gene amplification and protein expression was seen more often in squamous cell carcinomas than in adenocarcinomas ($P = .0208$).

There was no significant difference in survival between patients with EGFR protein overexpression and those without by univariate or multivariate analysis adjusting for potential confounders. Similarly, there was no difference in the univariate survival curves between patients with *EGFR* gene amplification and those without by univariate or multivariate analysis. Mean survival times for patients with and without EGFR protein overexpression were 9.97 and 12.24 months, respectively ($P = .1259$). Patients with *EGFR* gene amplification tended to have shorter survival than patients without gene amplification (10.7 and 12 months, respectively). Mean survival time for patients with protein overexpression and gene amplification was 10.58 and was 12.33 months for patients without EGFR protein overexpression and normal gene status. These results suggest that survival in the early course of NSCLC is not affected by EGFR protein expression or gene amplification.

Table 2
Comparison of EGFR Protein Expression by Immunohistochemical Analysis and *EGFR* Gene Amplification by FISH*

	Gene Amplification	
Protein Expression	**Negative**	**Positive**
Negative	160	5
Positive	19	12

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization.
* κ, 0.436.

Discussion

EGFR recently has attracted clinical attention because of the development of targeted therapies. Lynch et al[2] and Paez et al[3] reported that clinical responsiveness to gefitinib was associated with somatic mutations in the TK domain of the *EGFR* gene in NSCLC. In addition, Paez et al[3] found that the mutations were more frequent in adenocarcinomas, particularly in women of Asian origin. The role of EGFR protein overexpression, which is regulated by very complex and not clearly understood mechanisms, is uncertain.

In the present study, similar to the findings of Reissmann et al,[17] we were able to detect EGFR protein expression in only 16% of the analyzed tumors. This is lower than the 45% to 67% frequency of EGFR protein expression in NSCLC reported by others.[19] This discrepancy could be explained in part by possible variations in protocols and lack of standardized interpretation criteria because immunohistochemical analysis may be influenced by length and method of fixation and the degree of antigen retrieval.[20,21] Furthermore, the results clearly depend on specificity and sensitivity of available antibodies.[22] Interpretation of immunohistochemical results relies on subjective judgment. With EGFR, some authors have reported only membrane staining as opposed to cytoplasmic staining, whereas others did not report any preferential location of the receptor.[23-25] It seems that techniques for EGFR protein overexpression and their interpretations should be standardized because immunohistochemical analysis remains an option for routine clinical use, as recently suggested by Cappuzzo et al.[26]

The observation that EGFR protein expression in squamous cell carcinoma of the lung is higher than in adenocarcinoma clearly was confirmed in our study.[5,27] Consistent with most previous reports, we found that EGFR protein expression did not correlate with clinicopathologic variables such as sex, tumor grade or stage, lymph node status, or angiolymphatic and pleural invasion.

There are conflicting data in the literature about EGFR protein expression and outcome and survival of patients. Our study validates the findings of previous reports in which no

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correlation of EGFR expression and outcome was identified.[14,28] The discrepancy between results again could be explained by the lack of standardized interpretation criteria.

Another potential bias is patient selection criteria. In our study, EGFR protein expression was determined on tumor tissue at the time of primary diagnosis. Many other studies did not address presurgical treatments; therefore, it is possible that some discrepancies between studies may be explained by tumor changes acquired after chemotherapy or radiotherapy.

Some studies suggested that the mechanisms of EGFR protein overexpression depend on tumor type. For example, gene amplification has been indicated as a major cause of EGFR protein expression in glioblastomas.[29,30] The relationship between EGFR protein expression and gene amplification is not clearly understood in lung carcinoma.

FISH has been established as the reference method for assessment of gene amplification for many years. There are several ways to quantitate gene expression by FISH, including average or maximum gene copy numbers, the ratio of gene copy number to chromosome number, and the percentage of cells with unbalanced gene copy numbers.

Our study demonstrated that a higher ratio of *EGFR* gene copies to chromosome 7 number frequently is associated with T1 and T2 poorly differentiated squamous cell carcinomas. There was a trend toward a higher ratio in tumors without evidence of lymph node metastases that was not statistically significant; however, the number of *EGFR* gene copies was significantly higher in N0 disease. These findings suggest that alterations of the *EGFR* gene occur relatively early in the clinical course of squamous cell carcinomas and are associated with its pathogenesis rather than progression. The therapeutic significance of these observations is uncertain, but potentially these may help define the role of targeted agents after definitive treatment for early-stage NSCLC.

EGFR gene amplification was seen in only about 10% of the tumors in our study, which is similar to the findings of Hirsch et al.[14] Despite conflicting results from earlier studies, we showed that *EGFR* gene amplification did not influence survival. In our study, we have shown that some parameters such as number of *EGFR* gene copies per cell and ratio of *EGFR* gene to chromosome 7, determined by FISH, may correlate with tumor differentiation, tumor stage, and lymph node status. Although these results were statistically significant, our findings are based on a single institution experience with a relatively small number of patients, and our data need to be verified in a larger cohort of patients.

In our study, gene amplification correlated with protein expression, and it seems that gene amplification is a mechanism for protein overexpression in a subset of squamous cell carcinomas of the lung. In contrast with the study by Hirsch et al,[14] who found EGFR protein overexpression in all tumors with gene amplification, we demonstrated that protein expression does not necessarily require gene amplification, suggesting that other mechanisms such as gene mutation and transcriptional or posttranscriptional factors might have a role.

Our study confirms and extends the previously reported findings regarding EGFR protein overexpression and gene amplification. In addition, our findings suggest that *EGFR* gene amplification indicates the importance of molecular analysis of tumor specimens to establish the appropriate molecular targeted treatment. Further studies of patients treated with EGFR inhibitors other than gefitinib would be necessary to estimate the influence of *EGFR* gene amplification on response to therapy and survival.

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