VOLUME 22 · NUMBER 24 · DECEMBER 15 2004

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Modulation of Epidermal Growth Factor Receptor Status by Chemotherapy in Patients With Locally Advanced Non–Small-Cell Lung Cancer Is Rare

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Submitted February 4, 2004; accepted September 24, 2004.

Authors' disclosures of potential conflicts of interest are found at the end of this article.

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0732-183X/04/2224-4966/$20.00

DOI: 10.1200/JCO.2004.01.195

ABSTRACT

Purpose
To determine whether epidermal growth factor receptor (EGFR) expression in non–small-cell lung cancer (NSCLC) is modulated by chemotherapy and to assess the agreement of EGFR status between mediastinal nodes and the primary tumor after chemotherapy.

Patients and Methods
Patients with NSCLC stage IIIa/b pN2/3 confirmed by mediastinoscopy or mediastinostomy were treated with at least three cycles of chemotherapy before undergoing surgery. EGFR expression was evaluated on mediastinal nodes at the time of initial diagnosis and on both the primary tumor and residual metastatic nodes after treatment.

Results
EGFR expression determined on 138 of 164 patients who underwent mediastinoscopy or mediastinostomy was 0 (22 patients), 1+ (27 patients), 2+ (28 patients), and 3+ (61 patients). Fifty-four patients of 164 received chemotherapy followed by surgery. Of the 89 of 138 patients with EGFR score of 2+/3+ at the time of diagnosis, 34 patients underwent surgery after induction chemotherapy. None changed to zero EGFR immunoreactivity, with 29 patients (88%) maintaining a score of 2+/3+. Of the 22 of 138 patients with no EGFR expression at the time of diagnosis, six underwent surgical resection after induction chemotherapy. Of these six patients, four changed their EGFR expression from an EGFR score of 0 to 2+/3+. After treatment, the agreement of EGFR status between tumor and nodes in the subgroup of patients with EGFR score 2+/3+ was 89% to 92%.

Conclusion
Our data suggest a very good agreement of EGFR status before and after chemotherapy in EGFR-positive NSCLC. Induction chemotherapy can induce EGFR expression in occasional EGFR-negative tumors.

J Clin Oncol 22:4966-4970. © 2004 by American Society of Clinical Oncology

INTRODUCTION

The epidermal growth factor receptor (EGFR) is expressed in 40% to 80% of non–small-cell lung cancers (NSCLC), and expression has been associated with a poor prognosis.[1-3] Because the EGFR signaling pathway is thought to play a critical role in the growth and proliferation of NSCLC, EGFR was identified as an important target for drug development. New molecules have been designed to specifically target either the kinase domain or the interaction with its ligand.

The efficacy of this clinical approach was shown by data on the antitumor activity of gefitinib (Iressa [ZD1839]; AstraZeneca, Wilmington, DE), an orally active EGFR tyrosine kinase inhibitor, in patients with advanced NSCLC. In two phase II trials,

Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and IDEAL 2, gefitinib was given at the daily oral dose of 250 or 500 mg to 426 patients with advanced NSCLC previously treated with systemic chemotherapy. An objective tumor response rate of 10% to 19%, with a disease control rate of 42.2% to 54.4%, was observed.[4-6]

At present, no biomarker has been identified that is predictive of response to gefitinib. In preclinical studies, expression of EGFR was not an absolute requirement for tumor growth inhibition.[7,8] An analysis of EGFR membrane staining has been conducted in IDEAL 1 and 2 and has shown no evidence for a consistent relationship between EGFR expression levels and tumor response to gefitinib.[9] Similar results have been reported from the Iressa Expanded Access Program.[10]

To fully evaluate the correlation between EGFR status and response to gefitinib, it is necessary to know whether prior systemic chemotherapy could have changed the EGFR expression. Usually, the sequence of events in treating patients with advanced NSCLC is to first biopsy the tumor, then administer the chemotherapy regimen, and after, if indicated, gefitinib. Therefore, if chemotherapy can change EGFR expression, then the assessment of EGFR expression on tissue specimens collected at the time of diagnosis could be unreliable for predicting gefitinib response. Further, there are almost no data that compare EGFR status in nodes paired with primary lung tumors, an essential tool for analyzing the gefitinib data.

In this study, EGFR expression was evaluated in specimens collected at the time of diagnosis by mediastinoscopy or mediastinostomy in patients with stage IIIa/b pN2/3 NSCLC and compared with EGFR expression on both lymph nodes and tumor collected during surgery after neoadjuvant chemotherapy.

PATIENTS AND METHODS

Patients

The pathology specimens from 164 consecutive patients with clinical stage IIIa/b N2/3 were retrieved from the files of the Divisions of Pathology and Laboratory Medicine and Thoracic Surgery of the European Institute of Oncology of Milan between 1998 and 2002. New sections were cut for immunohistochemical analysis and original hematoxylin and eosin sections were reviewed; 138 lymph node specimens were tumor-positive and were analyzed for EGFR status.

Fifty-four patients underwent surgery for cancer resection after an induction chemotherapy regimen along with a complete mediastinal lymph node dissection to ensure accurate pathologic staging. Selection criteria for surgery were N2 nonbulky disease, no disease progression with induction chemotherapy, patient preference, and medical conditions precluding surgery. For the immunohistochemical study, paraffin blocks were retrieved and original hematoxylin and eosin–stained sections were reviewed.

Immunohistochemical Procedures

Mediastinal lymph nodes assessed for EGFR immunoreactivity after chemotherapy belonged to the same anatomic site as sampled during mediastinoscopy to have the most homogeneous histologic comparison of tumors. If metastatic lymph nodes in the same site were unavailable for evaluation, other pN1 or pN2 samples were chosen with the largest deposits of tumor cells. There were no significant differences in the size of analyzed lymph node fragments obtained before chemotherapy (mean ± standard deviation [SD], 1.07 ± 0.41 cm; median, 0.9 cm) by mediastinoscopy and after surgery (mean ± SD, 1.16 ± 0.34 cm; median 1) by sampling the same or similar mediastinal lymph node station. Moreover, both specimen types were comparable in terms of neoplastic cellularity, the latter being at least 60% in all tumor samples under evaluation. Primary lung tumors of resected specimens were entirely immunostained for EGFR if they were ≤ 2 cm in size, whereas at least two representative tissue blocks were evaluated in larger neoplasms. There were six tumors (five adenocarcinomas and one squamous cell carcinoma) measuring ≤ 2 cm in diameter, with the remaining ones measuring greater than 2 cm. Ten samples of nonneoplastic pulmonary parenchyma and bronchial tree at different levels, as well as peritumoral parenchyma from a representative group of 30 patients with NSCLC (18%) matched for confounding factors (age, sex, and smoking habit), were also assessed for EGFR immunoreactivity as noncarcinoma and carcinoma control groups, respectively.

In all samples, 4 micro-thin paraffin sections were incubated for 5 minutes at 37°C in 0.1% pronase solution in phosphate buffer saline 0.01 mol/L at pH 7.4, reacted with a commercially available mouse monoclonal antibody recognizing the peptide backbone of the extracellular domain of the EGFR molecule (clone 31G7; DBA, Milan, Italy) at a 50 μL/mL dilution for 1 hour at room temperature, and then incubated with a detection kit (Dako EnVision Plus-HRP; Dako, Glostrup, Denmark) according to the manufacturer's instructions. Peroxidase activity was developed with 3-3′-diaminobenzidine-copper sulfate (Sigma Chemical Co, St Louis, MO) to obtain a brown-black end product. The specificity of all immunoreactions was double-checked by substituting the primary antibody with a nonrelated mouse monoclonal antibody at a comparable dilution and with normal serum alone. Appropriate external positive controls were stained in parallel for each batch to ensure the overall immunoreactivity quality.

Scoring System for Immunohistochemistry

The slides were assessed for EGFR by one observer experienced in pulmonary pathology (G.P.) and unaware of patient identity. In all cases, the intensity (weak, moderate, or strong) and pattern (incomplete or complete) of membrane labeling and the percentage of immunoreactive neoplastic cells (by scanning at least 1,000 tumor cells in representative fields of immunostaining) were accurately recorded. Only the membrane labeling was taken into account with a 10% threshold for positivity, whereas cytoplasmic immunoreactivity was completely disregarded. Combining intensity and membrane pattern of tumor cells, a four-tier score (0 to 3+) was thus reached for each case, according to the Dako system formerly used for the HercepTest (DAKO, Carpinteria, CA). Tumors were considered negative (score 0) if cell membrane staining was completely absent or positive in up to 10% tumor cells, whereas a faintly appreciable and incomplete pattern, a weak-to-moderate but complete staining, or a strong and complete labeling of the membrane in more than 10% tumor cells were given the threshold of 1+, 2+, or 3+ scores, respectively. A

known-positive control case (NSCLC comparable in terms of fixation time and processing and overexpressing EGFR) was included in each run of staining and consistently showed the expected immunoreactivity.

Statistical Analysis

Expression of EGFR was recorded as an ordinal variable varying from 0 (no expression) to +3. Expression was also codified as negative or positive by regrouping into a single level all positive-expressing specimens independently of degree of expression. Proportion of nodal and/or tumor samples showing level-specific EGFR expression at diagnosis and after chemotherapy were provided with accompanying 95% exact binomial confidence limits. Agreement in EGFR expression between specimens before and after chemotherapy was evaluated using the Goodman and Kruskal γ statistic. When treating agreement as a two-level variable, agreement was assessed using the kappa statistic. Agreement was considered statistically significant if the *P* value associated with the corresponding estimate was $\leq .05$. Calculations were done using StatXact (Cytel Software Corp, Cambridge, MA).

RESULTS

Patients

Specimens collected by 164 consecutive mediastinoscopies or mediastinostomies performed at the time of NSCLC diagnosis were evaluated for this analysis. EGFR status was assessed in 138 of 164 cases; in other 26 patients, the lymph node samples were either completely negative for tumor colonization or showed an insufficient number of tumor cells for a reliable semiquantitative evaluation. Patient age ranged from 17 to 85 years (mean $\pm$ SD, 60.3 $\pm$ 11.1 years; median, 62 years).

Using the last WHO classification of lung tumors (1999), 51% of tumors were classified as adenocarcinomas, 40% were classified as squamous cell carcinomas, and 9% were classified as large-cell carcinomas.

EGFR expression was also evaluated in 47 tumors and 48 involved mediastinal nodes of 54 of 164 patients selected for surgery after induction chemotherapy (the remaining specimens showed no tumor or inadequate sample size). No attempt was made to obtain further biopsy samples from patients who did not undergo surgery.

EGFR Immunoreactivity in Normal Lung Tissue

All samples of normal pulmonary parenchyma and bronchial tree from patients with nonmalignant lung diseases and the non-neoplastic peritumoral lung tissue from the study patients were consistently unreactive for EGFR antibody. However, a barely appreciable to faint immunoreactivity was sometimes encountered in the basolateral layer of cell membrane of the normal bronchial epithelium ($<$ 10% of examined cells).

EGFR Status at the Time of Diagnosis

In all, 138 cases of involved mediastinal nodes were stained for EGFR status. EGFR score was 0, 1+, 2+, and 3+ in 22 (16%), 27 (20%), 28 (20%), and 61 (44%) of cases, respectively.

EGFR Status After Chemotherapy

Patients with absence of EGFR expression at the time of diagnosis. Twenty-two patients showed no EGFR immunoreactivity on specimens collected by pretreatment mediastinoscopy or mediastinostomy. Six of them underwent surgery after chemotherapy. Tumor histotypes were adenocarcinoma (three patients), squamous cell carcinoma (one patient), undifferentiated NSCLC (one patient), and large-cell carcinoma (one patient).

In all, EGFR changed to a positive status in four patients (66%) on mediastinal nodes (1+ in one case, 2+ in one case, and 3+ in two cases) and in three of five primary tumors (one tumor specimen was not available; Tables 1 and 2). Histotypes of tumors that changed to a positive EGFR status included adenocarcinoma (two patients), squamous cell carcinoma (one patient), and large-cell carcinoma (one patient). Response to induction chemotherapy was partial response and stable disease in three and one patient, respectively. Two cases (one partial response and one stable disease to induction chemotherapy) remained EGFR-negative, both on nodes and tumors.

Patients with EGFR score 1+ at the time of diagnosis. Twenty-seven patients showed EGFR score 1+ on specimens collected by pretreatment mediastinoscopy or mediastinostomy. Seven patients underwent surgery after induction chemotherapy; all had residual disease at the primary site at thoracotomy and four had residual tumor in mediastinal nodes.

One of these seven cases changed to zero EGFR immunoreactivity both on tumor and nodes. EGFR score of the remaining six primitive tumors was 1+ (two patients), 2+ (two patients), and 3+ (two patients) and on nodes was 1+ (two patients), 2+ (one patient), and not assessable in three patients (specimens not available, two patients; ypN0, one patient; Tables 1 and 2).

Patients with EGFR score 2+/3+ at the time of diagnosis. Eighty-nine patients showed EGFR score 2+/3+ on specimens collected by pretreatment mediastinoscopy or

Table 1. Epidermal Growth Factor Receptor Status on Mediastinal Nodes at Diagnosis and After Induction Chemotherapy (47 of 164 patients)

EGFR Status at Diagnosis	No. of Patients	EGFR Status After Induction Chemotherapy (No. of patients)				
		0	1+	2+	3+	NA
0	6	2	1	1	2	0
1+	7	1	2	1	0	3
2+	8	0	1	5	2	0
3+	26	0	0	7	11	8

Abbreviations: EGFR, epidermal growth factor receptor; NA, not assessable.

Table 2. Epidermal Growth Factor Receptor Status on Mediastinal Nodes at Diagnosis and on Primitive Tumor After Induction Chemotherapy (47 of 164 patients)

EGFR Status at Diagnosis	No. of Patients	EGFR Status After Induction Chemotherapy (No. of patients)				
		0	1+	2+	3+	NA
0	6	2	0	1	2	1
1+	7	1	2	2	2	0
2+	8	0	2	3	2	1
3+	26	0	2	5	15	4

Abbreviations: EGFR, epidermal growth factor receptor; NA, not assessable.

mediastinostomy. Thirty-four underwent surgery after induction chemotherapy, all but one with residual tumor in mediastinal nodes and tumor (one patient had ypN0 ypTx disease).

None of these cases changed to zero EGFR immunoreactivity. Four patients showed a minimal score (nodes or tumor) of 1+, the other 29 patients (88%) maintained a minimal score of 2+ (13 patients) or 3+ (16 patients; Tables 1 and 2).

Agreement of EGFR Status in Nodes and Tumor After Chemotherapy

The score of EGFR status in residual mediastinal nodes metastases was assessable in 48 of 164 patients who underwent surgery who had residual tumor in nodes after induction chemotherapy, and the score in the corresponding tumors was evaluated in 47 of 48 patients (one patient had pT0 disease).

In all, the score in the nodes was zero in three patients, 1+ in seven patients, 2+ in 17 patients, and 3+ in 21 patients. Analyzing the subgroup of patients with nodes scoring 2+/3+, there was an agreement in the tumor score in 34 (92%) of 37 patients. The EGFR score in the remaining three patients was zero (one patient) and 1+ (two patients). One patient was excluded because of a complete pathologic tumor response.

Analyzing the subgroup of patients with tumor score 2+/3+ (38 patients), there was an agreement in the nodes score in 34 (89%) of 38 patients. No cases showed the absence of EGFR expression, with the four remaining cases showing a score of 1+.

All the cases with absence of EGFR in mediastinal nodes (three patients) showed zero EGFR expression on the corresponding tumors. Conversely, three of five tumors with EGFR score of zero showed an agreement on nodes (remaining two cases, EGFR score of 1+ and 2+).

Statistical Analysis

Mediastinal nodes. Taking into account the ordinal nature of EGFR expression assigned by the pathologist in mediastinal node specimens obtained at diagnosis, an association was found with EGFR expression in corresponding nodes sampled during surgery after induction chemotherapy ($\gamma = 0.66$; 95% CI, 0.35 to 0.96; two-sided associated $P = .0008$). These results indicate good agreement in EGFR expression between specimens collected at diagnosis and after treatment in those patients with available paired specimens. When grouping mediastinal node specimens with positive EGFR expression into a single category, we observed that the vast majority had corresponding postchemotherapy node specimens with positive EGFR expression (29 of 30 or 96.7%; 95% exact CI, 82.8% to 99.9%). If, on the other hand, agreement is assessed, a kappa statistic of 0.375 is obtained (two-sided associated $P = .015$), indicating moderate agreement that is probably due to the discordance in the negative expressing specimens.

Tumor. An association was found between the degree of expression in mediastinal nodes sampled at diagnosis and EGFR expression in corresponding primary tumor tissue collected after induction chemotherapy (gamma = 0.51; 95% CI, 0.18 to 0.84; two-sided associated $P = .01$).

When evaluating EGFR expression as a two-level variable, the overwhelming majority of specimens showing positive expression at diagnosis had corresponding postchemotherapy primary tumor specimens with positive EGFR expression (35 of 36 or 97.2%; 95% exact CI, 85% to 99.9%). Assessing agreement, a kappa statistic of 0.449 was obtained (two-sided associated $P = .0027$), indicating moderate agreement.

DISCUSSION

This study was performed to assess whether EGFR status could be modulated by chemotherapy in patients with locally advanced NSCLC selected for surgery and to evaluate the agreement of EGFR expression between the primary tumor and mediastinal node metastases.

The incidence of EGFR positive status in this selected population of 138 patients with locally advanced NSCLC was 84%, which is consistent with the rate of expression reported by others in NSCLC.[1-3]

Our results suggest a very good overall agreement of EGFR status before and after systemic chemotherapy in EGFR-positive tumors. Overall, only one of 41 EGFR-positive tumors at diagnosis changed to EGFR-negative immunoreactivity after chemotherapy. This case scored 1+ at diagnosis.

This observation suggests that a rebiopsy in an EGFR-positive patient after chemotherapy to reassess EGFR status is not necessary. Moreover, those observations support the methodologic adequacy of the ongoing retrospective analysis of the relationship between EGFR expression and response to gefitinib for EGFR-positive patients treated in studies such as IDEAL 1 and IDEAL 2.[4,5] In these studies, according to the inclusion criteria, all patients treated with gefitinib were pretreated with systemic chemotherapy after

diagnosis. If chemotherapy has been shown to change EGFR status, all specimens collected at diagnosis would need to be ignored in correlative studies linking EGFR expression to response to gefitinib. Our data suggest that chemotherapy does not change EGFR-positive status significantly.

Our study also suggests that induction chemotherapy can induce EGFR expression in occasional patients with EGFR-negative tumors. The observation of this switch is consistent with the hypothesis that the EGFR ligand could be used as a survival factor to rescue from chemotherapy-induced damage.[11,12]

As a consequence, whenever the knowledge of EGFR status is useful for proper decision making in patients with EGFR-negative NSCLC treated with chemotherapy, a postchemotherapy rebiopsy to assess EGFR expression is recommended.

According to our data, no difference would be expected if biopsy is performed on the primary tumor or on mediastinal nodes. In this study, the analysis of the EGFR score assessed on residual mediastinal nodes at the time of surgery showed an agreement in the corresponding primary tumors of 92% and 100% in patients with a score of 2+/3+ and score of zero, respectively.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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