# Synchronous Overexpression of Epidermal Growth Factor Receptor and HER2-*neu* Protein Is a Predictor of Poor Outcome in Patients with Stage I Non-Small Cell Lung Cancer

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## ABSTRACT

***Purpose:*** **Despite maximal therapy, surgically treated patients with stage I non-small cell lung cancer (NSCLC) are at risk for developing metastatic disease. Histopathologic findings cannot adequately predict disease progression, so there is a need to identify molecular factors that serve this purpose. Because the ErbB receptors play an important role in lung cancer progression, we analyzed the expression of epidermal growth factor receptor (EGFR), phosphorylated EGFR, transforming growth factor α (TGFα), and HER2-*neu* as potential prognostic factors in stage I NSCLC.**

***Experimental Design:*** **Using immunohistochemical techniques, we retrospectively analyzed formalin-fixed, paraffin-embedded samples from 111 patients with resected pathological stage I NSCLC. Then we correlated these data with patient clinical outcome.**

***Results:*** **Median follow-up was 69.3 months. EGFR overexpression (defined as >10% membranous staining) was found in 66 tumors (59.5%). It was significantly more common in $T_2$ tumors than in $T_1$ tumors ($P = 0.001$), and in more squamous cell carcinomas than in adenocarcinomas ($P = 0.07$). HER2-*neu* overexpression was found in 19 tumors (17.1%) and was significantly more common in adenocarcinomas than in squamous cell carcinomas ($P = 0.035$). Synchronous overexpression of EGFR and HER2-*neu* was found in 11 tumors (9.9%). Patients with these tumors had a significantly shorter time to recurrence ($P = 0.006$) and a trend toward shorter overall survival ($P = 0.093$). Phosphorylated EGFR and transforming growth factor α were detected but were not related to prognosis.**

***Conclusions:*** **Synchronous overexpression of EGFR and HER2-*neu* at the protein level predicts increased recurrence risk and may predict decreased survival in patients with stage I NSCLC. This suggests that important interactions take place among the different members of the ErbB family during tumor development and suggests a method for choosing targeted therapy. A prospective study is planned.**

## INTRODUCTION

The overall 5-year survival rate in patients with lung cancer is <15% (1). Although the prognosis is best for patients with stage I surgically treated lung cancer, the 5-year survival rate among these patients is only 57–67% (2). Histopathologic findings alone are insufficient to predict disease progression and clinical outcome, so molecular prognostic factors are critically needed that can identify patients who have a high risk of disease progression and are, thus, most likely to benefit from adjuvant therapy.

Several factors that predict poor outcome in patients with stage I disease have been proposed, including the presence of the K-*ras* oncogene (3), a high ratio of type IV collagenases to E-cadherin (4), a low level of bcl-2 protein (5), a high level of retinoic acid receptor-β protein (6), and the loss of blood-group antigen A (7). None of these factors are routinely and clinically detected, and no medical intervention has been developed to target these molecules. Other factors that have been evaluated for their potential prognostic role in lung cancer are the ErbB family of receptors, for which therapeutic approaches do exist. These receptors play a pivotal role in tumor cell proliferation, survival, adhesion, migration, and differentiation, and also play a role in tumor angiogenesis (8). In addition, in many cancers, the expression of these receptors may be related to patient survival (9). The ErbB family comprises four structurally related receptors: ErbB1 [more commonly known as EGFR (epidermal growth factor receptor) and also called HER1], ErbB2 (HER2-neu), ErbB3 (HER3), and ErbB4 (HER4). On ligand stimulation, the receptors form either homodimers or heterodimers, which activate their cytoplasmic domain. This tyrosine-autophosphorylated region functions as a docking site for messenger proteins, which initiate cascades of cytoplasmic and nuclear mitogenic pathways (10). Inhibition of these pathways is facilitated by several newly developed compounds that have shown promising results in preclinical and clinical trials (11).

High-level expression of EGFR is one of the earliest oc-

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*Table 1* Characteristics of 111 Patients with Stage I NSCLC[a]

| Characteristic | No. of patients (%) |
|---|---|
| Sex | |
| Male | 54 (49.0) |
| Female | 57 (51.0) |
| Age | |
| Range | 28–88 years |
| Mean ± SD | 64.7 ± 10 years |
| Median | 65 years |
| History of smoking[b] | 76 (72.3) |
| Histologic subtype | |
| Adenocarcinoma | 36 (32.5) |
| SCC | 40 (36.0) |
| Bronchioalveolar carcinoma | 20 (18.0) |
| Other[c] | 15 (13.5) |
| Pathologic stage | |
| IA ($T_1$, tumor < 3.0 cm in diameter) | 40 (36.0) |
| IB ($T_2$, tumor > 3.0 cm in diameter) | 71 (64.0) |
| Type of surgery[d] | |
| Wedge resection | 10 (10.3) |
| Segmentectomy | 10 (10.3) |
| Lobectomy | 70 (72.2) |
| Pneumonectomy | 7 (7.2) |
| Duration of follow-up, median | 69.3 months |

[a] NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.
[b] Data available for 105 of 111 patients.
[c] Includes patients with large-cell lung cancer, adenosquamous carcinoma, and NSCLC not otherwise specified.
[d] Data available for 97 of 111 patients.

curring and most consistently detected abnormalities in the bronchial epithelium of heavy smokers, and is pronounced in 65–84% of all non-small cell lung cancers (NSCLCs), especially squamous cell carcinomas (SCCs; Refs. 12, 13). HER2-*neu* overexpression is less common; it is found in <35% of patients with NSCLC, mainly in those with adenocarcinoma (14). EGFR overexpression has been associated with lower relapse-free and overall survival rates in several malignancies, mainly head and neck cancer, whereas HER2-*neu* has been implicated as a prognostic factor in breast cancer. However, the relationship between EGFR or HER2-*neu* overexpression and NSCLC prognosis is controversial (9). The heterodimer EGFR/HER2-*neu* has been shown to have a stronger proliferative effect than the corresponding homodimers (15, 16). Amplification of EGFR and HER2-*neu* mRNA (17) or overexpression of their proteins (18) has been found to relate to survival in patients with NSCLC, although contradictory results have also been reported (19, 20). Comparison of studies that have evaluated ErbB receptors as prognostic indicators is complicated by the variety of histological findings, stages, and immunohistochemical techniques, including the use of different antibodies and scoring methods. On the basis of the results from the studies cited above, we hypothesized that expression of ErbB receptors is prognostic in a subset of patients with lung cancer. To test this hypothesis, we used immunohistochemical techniques to measure expression of transforming growth factor (TGF) α, a potent ligand for EGFR, EGFR, phosphorylated EGFR, and HER2-*neu*, in tumor samples from patients with pathologically determined stage I NSCLC, and we then examined the relationship between these findings and disease-free and overall survival as determined from the medical records of the patients.

## PATIENTS AND METHODS

**Study Population.** We retrospectively examined the records of all 1093 patients with NSCLC who had undergone surgery from 1987 through 1994 at The University of Texas M. D. Anderson Cancer Center. The patients were identified through a search of the database maintained by the Department of Thoracic and Cardiovascular Surgery. Three hundred seventy five patients had stage I disease, 179 (48%) with stage IA and 196 (54%) with stage IB. From this group, 120 patients for whom tissue samples were available and who had had a follow-up period of >5 years were identified. This group was representative of the entire database. Nine patients were eliminated from the final analysis because examination of medical records revealed that they had had stage II or IIIA disease ($n = 6$) or because we did not have survival data ($n = 3$), resulting in a final cohort of 111 patients. We verified and updated the survival data in the patient records through October 2001 using the database. Clinical end points for the study were overall survival (time from surgery to death) and disease-free survival (time from surgery to diagnosis of local or distant recurrence).

**Reagents.** Primary antibodies used for immunohistochemical staining were mouse monoclonal anti-EGFR (clone 31G7; Zymed, South San Francisco, CA), mouse monoclonal anti-TGFα (Ab-2; Oncogene Research Products, San Diego, CA), and rabbit monoclonal anti-phosphorylated EGFR (Tyr 845; Cell Signaling Technology, Beverly, MA). A HercepTest kit (DakoCytomation, Carpinteria, CA) and a Multi-Link kit (BioGenex, San Ramon, CA) were also used.

**Immunohistochemical Techniques.** Formalin-fixed, paraffin-embedded tissue sections (4–6 μM) were obtained from the Surgical Pathology Laboratory at M. D. Anderson

*Table 2* Results of immunohistochemical analysis

| | No. (%) of tumors | | | | | |
|---|---|---|---|---|---|---|
| Score | EGFR[a] (Membranous) | Phospho-EGFR (Nuclear) | Phospho-EGFR (Membranous) | Phospho-EGFR (Cytoplasmic) | TGF-α (Cytoplasmic) | HER2-*neu* (Membranous) |
| 0 | 27 (24.3) | 16 (14.4) | 104 (93.7) | 58 (52.2) | 81 (73.0) | 51 (45.9) |
| 1 | 18 (16.2) | 21 (18.9) | 1 (0.9) | 26 (23.4) | 27 (24.3) | 41 (36.9) |
| 2 | 36 (32.4) | 40 (36.0) | 4 (3.6) | 18 (16.2) | 1 (0.9) | 13 (11.7) |
| 3 | 30 (27.0) | 34 (30.6) | 2 (1.8) | 9 (8.1) | 2 (1.8) | 6 (5.4) |
| Total | 111 (100) | 111 (100) | 111 (100) | 111 (100) | 111 (100) | 111 (100) |

[a] EGFR, epidermal growth factor receptor; phospho-EGFR, phosphorylated epidermal growth factor receptor; TGF, transforming growth factor.

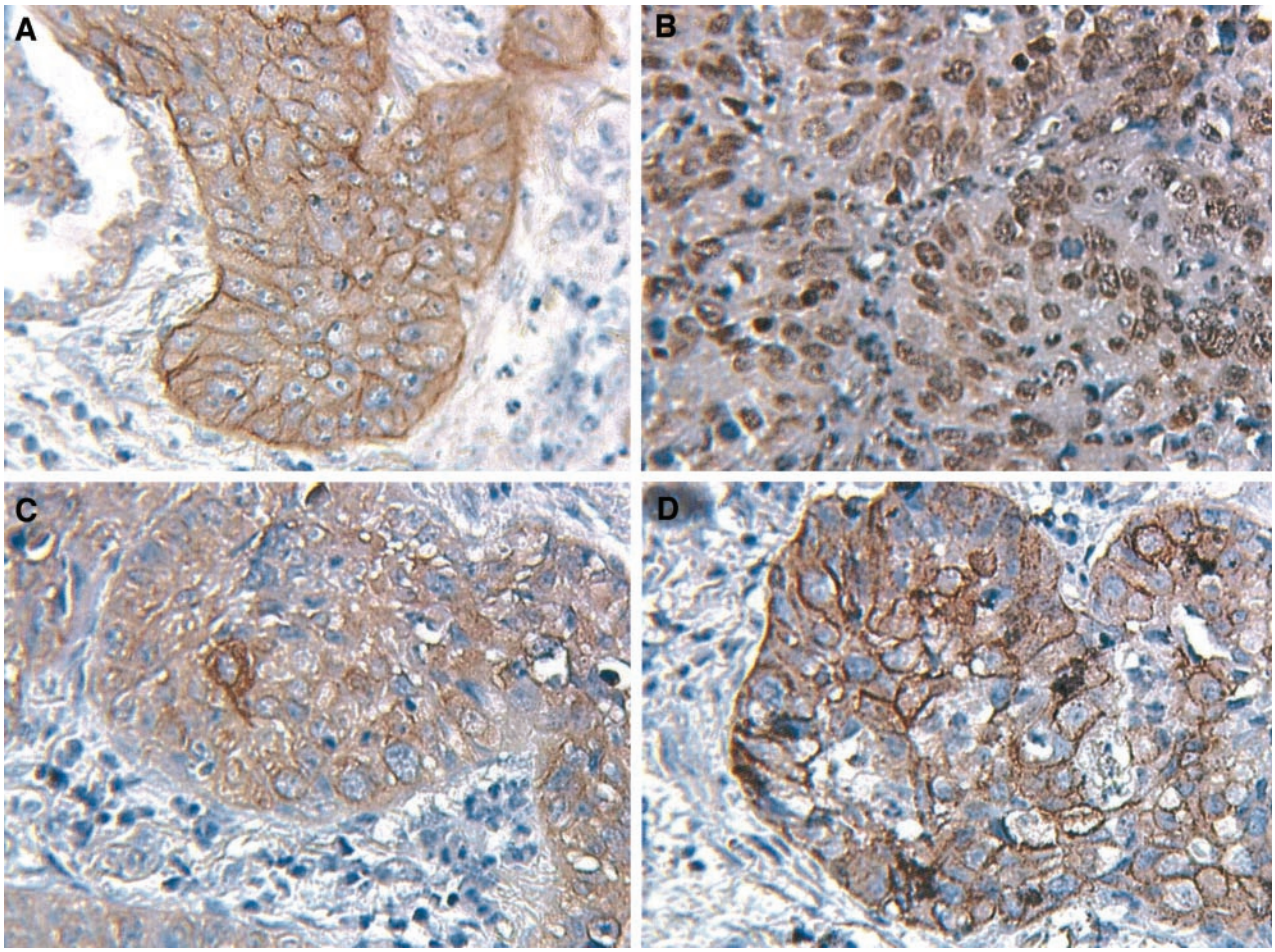


*Fig. 1* Representative findings on immunohistochemical staining for (*A*) epidermal growth factor receptor, (*B*) phosphorylated epidermal growth factor receptor, (*C*) transforming growth factor α, and (*D*) HER2-*neu*.

Cancer Center. The sections were mounted on silane-coated ProbeOn slides (Fisher Scientific, Houston, TX). They were deparaffinized in xylene and then treated with a graded series of alcohol [100%, 95%, and 80% ethanol (v/v) in double-distilled $H_2O$] and rehydrated in PBS (pH 7.5). For antigen retrieval, sections analyzed for EGFR were treated with pepsin (Biomeda, Foster City, CA) for 20 min at 37°C, and sections analyzed for phosphorylated EGFR were treated with 10 mM sodium citrate in water (pH 6.0) and heated in a microwave (full power for 2 min, medium power for 2 min, and low power for an additional 6 min). An endogenous peroxidase was blocked by immersing the sections for 12 min in 3% $H_2O_2$ (Fisher Scientific) in PBS (for TGF-α and EGFR) or in methanol (Fisher Scientific; for phosphorylated EGFR). Samples were incubated overnight at 4°C with primary antibodies to EGFR, TGF-α, and phosphorylated EGFR at a 1:100 dilution. We used the Multi-Link kit to detect antigen-antibody reaction. Sections were visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with Gill's hematoxylin. Slides analyzed for EGFR were not counterstained. Staining for HER2-*neu* was performed using the HercepTest kit (21).

**Review and Scoring of Tissue Sections.** A positive control sample was evaluated with each batch of slides. Two researchers blinded to clinical follow-up data (M. G. and A. O.), including a thoracic pathologist (M. G.), reviewed the stained sections. EGFR and HER2-*neu* expression were evaluated by examination of membrane staining, and TGF-α expression was evaluated by examination of cytoplasmic staining. Staining for phosphorylated EGFR was categorized as membranous, cytoplasmic, or nuclear according to the dominant pattern. Each slide was assigned a score using a semiquantitative four-category grading system: 0 (no tumor expression), 1 (1–10% tumor expression), 2 (>10% to 20% tumor expression), or 3 (>20% tumor expression). Samples with a score of 2 or 3 were considered to overexpress that particular protein.

**Statistical Analysis.** The Kaplan-Meier method (22) was used to estimate the cumulative survival functions and the cumulative recurrence hazard functions. The log-rank test was used to test for equality of the cumulative functions between expressions of a given protein. The $\chi^2$ test was used to test for equality of proportions of $T_1$ *versus* $T_2$ and adenocarcinoma *versus* SCC in cases with overexpression of EGFR, HER2-*neu*,

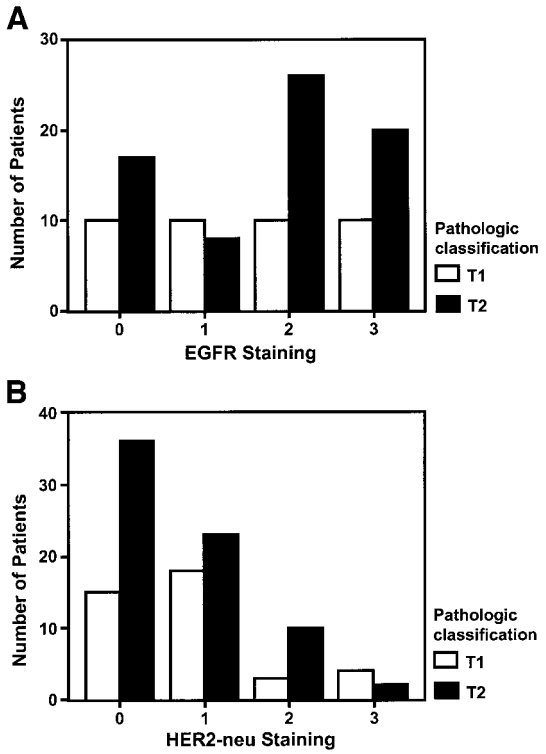


*Fig. 2* Membranous overexpression of epidermal growth factor receptor (*A*; $P = 0.001$) but not HER2-*neu* (*B*; $P = 0.251$) was more common in tumors of higher pathological stages.

and both proteins. The significance level was set at $P < 0.05$. The prognostic effect of putative covariates on disease-free survival and overall survival was evaluated using the univariate and multiple-covariate Cox models. To compensate for multiple tests of combinations between receptors and prognosis, we defined the significance level at $P < 0.01$. SPSS statistical software (SPSS Inc., Chicago, IL) was used to perform the analyses and create graphs.

## RESULTS

**Patients.** Patient characteristics and demographics are summarized in Table 1. At the time of the last follow-up (median follow-up, 69.3 months), 70 patients had died.

**Immunohistochemical Analysis.** All 111 of the samples exhibited a positive immunohistochemical reaction compared with a negative control reaction in which the primary antibody was omitted. The semiquantitative data are summarized in Table 2, and representative samples are shown in Fig. 1.

Sixty-six tumors (59.5%) overexpressed EGFR, and 19 tumors (17.1%) overexpressed HER2-*neu*. EGFR overexpression was more common in $T_2$ tumors than in $T_1$ tumors (Fig. 2). More SCCs than adenocarcinomas overexpressed EGFR, but this difference was not statistically significant (Fig. 3). HER2-*neu* overexpression was not associated with pathological stage (Fig. 2) but was more common in adenocarcinomas than in SCCs (Fig. 3).

Several patterns of phosphorylated EGFR staining were observed (Table 2). Six tumors (5.4%) had membranous overexpression of phosphorylated EGFR, 27 tumors (24.3%) had cytoplasmic overexpression of phosphorylated EGFR, and 74 tumors (66.6%) had nuclear overexpression of phosphorylated EGFR. In some cases, more than one pattern was observed, but statistical analysis was based on the most abundant pattern. No relationship was found between any pattern of staining for phosphorylated EGFR and expression of EGFR.

Only 3 tumors (2.7%) expressed TGF-α. For these, we found no relationship between any pattern of staining for phosphorylated EGFR.

**Disease-Free Survival.** The median time from surgery to a diagnosis of recurrence was 117.2 months (mean, 104.9 months). No single patient characteristic or immunohistochemical staining result (regardless of whether a positive score was defined as 1–3, or as 2 or 3) related to disease-free survival. The 11 patients (9.9%) whose tumors overexpressed both EGFR and HER2-*neu* had a significantly shorter median time to recurrence than did the other patients (39.9 months *versus* undeterminable time for other patients; $P = 0.006$; Fig. 4).

**Overall Survival.** The median time from surgery to death was 81.4 months (mean, 80.6 months). Survival rates did not change throughout the years studied (data not shown). No single immunohistochemical staining result (regardless of whether a positive score was defined as 1–3, or as 2 or 3) was related to overall survival. The only patient factor associated

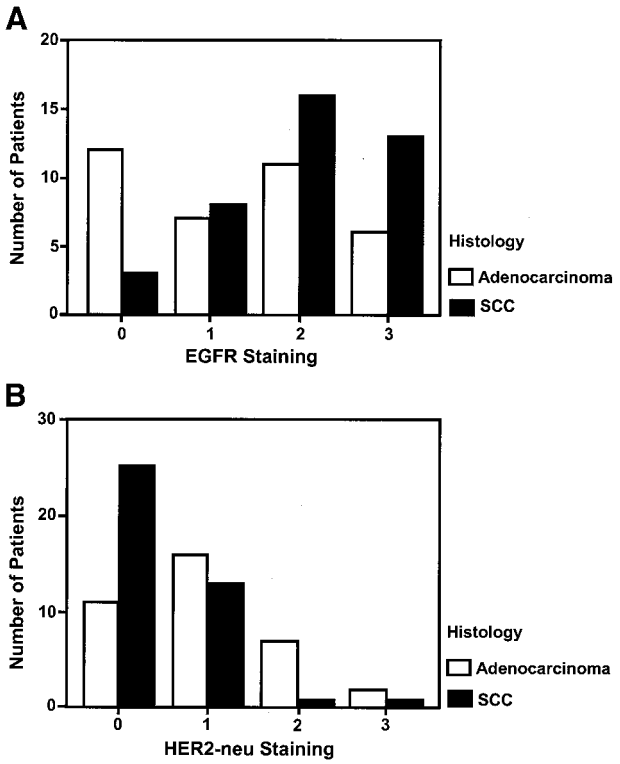


*Fig. 3* More patients with squamous cell carcinoma (*SCC*) than with adenocarcinoma had epidermal growth factor receptor overexpression (*A*), but this difference was not statistically significant ($P = 0.07$). HER2-*neu* (*B*) was significantly more common in patients with adenocarcinoma than in patients with SCC ($P = 0.035$).

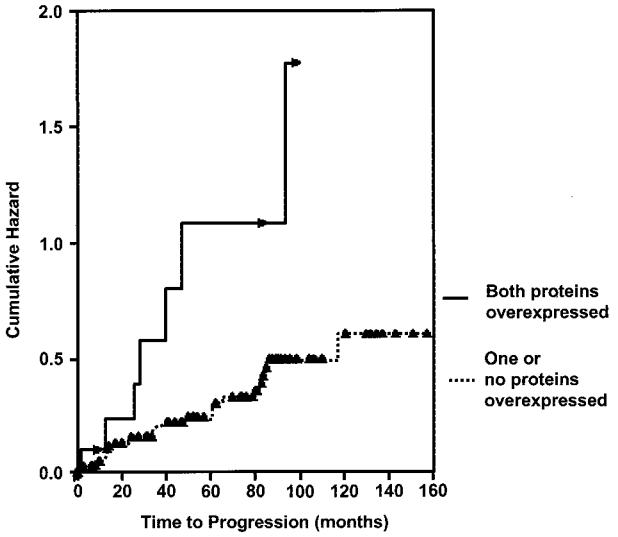


*Fig. 4* The 11 patients (9.9%) whose tumors synchronously overexpressed epidermal growth factor receptor and HER2-*neu* (———) had a significantly shorter recurrence-free interval than did the other patients (····; $P = 0.006$).

with overall survival was age: patients in the youngest quartile (age 28–60 years) had significantly longer overall survival than did patients in the oldest quartile (age 72–88 years; $P = 0.046$). Histological characteristics and pathological stage were found to affect overall survival to a lesser degree: patients with adenocarcinoma survived longer than did patients with SCC ($P = 0.053$), and as expected, patients with $T_1$ tumors survived longer than did those with $T_2$ tumors ($P = 0.052$). Smoking history did not relate directly to overall survival ($P = 0.6161$), but more smokers had SCC than had adenocarcinoma ($P = 0.002$).

Using a multivariate Cox regression model, no relationship was found among these factors, and smoking history did not contribute to the overall lower survival rate in patients with SCC. The 11 patients (9.9%) in whom both EGFR and HER2-*neu* were overexpressed had a median overall survival of 44.2 months compared with 84.4 months in the other patients ($P = 0.093$; Fig. 5). Representative immunohistochemical determination of EGFR and HER2-*neu* for a patient with synchronous overexpression of the proteins (case 1) and a patient with no expression of the proteins (case 2) are presented in Fig. 6.

Multivariate Cox regression using the backward elimination method was used for 74 patients with adenocarcinoma or SCC. It revealed four factors associated with shorter survival: synchronous overexpression of EGFR and HER2-*neu* ($P = 0.027$), SCC ($P = 0.015$), higher pathological stage ($P = 0.05$), and poorly differentiated tumor grade ($P = 0.019$).

No relationship was found between TGF-α or phosphorylated EGFR and clinical outcome on either univariate or multivariate analyses.

## DISCUSSION

In our analysis of 111 cases of stage I NSCLC, 59.5% of the tumors overexpressed EGFR. EGFR expression was found more often in $T_2$ tumors than in $T_1$ tumors ($P = 0.001$), and there was a trend toward EGFR overexpression being more common in SCCs than in adenocarcinomas ($P = 0.07$). No relationship was found between expression of EGFR as a single factor and overall or disease-free survival. Fewer tumors (17.1%) overexpressed HER2-*neu*, which was found more often in patients with adenocarcinoma than in patients with SCC ($P = 0.035$). No relationship was found between HER2-*neu* overexpression and disease stage or survival. These findings are consistent with previous studies that describe the important role of the ErbB family in lung cancer development (12, 14).

We found a trend toward shorter overall survival ($P = 0.093$) and significantly shorter disease-free survival ($P = 0.006$) in patients with lung cancer overexpressing both EGFR and HER2-*neu*. These results are similar to those of other studies that determined EGFR-HER2-*neu* mRNA or protein coexpression. Brabender *et al.* (17) recently analyzed 83 surgically resected NSCLC tumors from patients with stage I-IIIa disease and reported that both high HER2-*neu* gene expression and high EGFR-HER2-*neu* mRNA coexpression were significantly related to poor survival rates. Tateishi *et al.* (18) studied 119 cases of primary human lung adenocarcinoma and found that coexpression of EGFR and HER2-*neu* protein was more common in patients with metastasis. As far as we know, our report is the first to demonstrate a significant relationship between synchronous coexpression of EGFR and HER2-*neu* at the level of protein and patient prognosis in a large cohort of surgically treated patients with pathological stage I NSCLC. Our clinical observations are also supported by preclinical studies of receptor heterodimerization. Pinkas-Kramarski *et al.* (15) showed that the heterodimer EGFR-HER2-*neu* has a higher proliferative index than the corresponding homodimers have. Graus-Porta *et al.* (16) suggested that HER2-*neu* acts as a common receptor subunit of other ErbB proteins because it enhances ligand-induced receptor activation, potentiates and

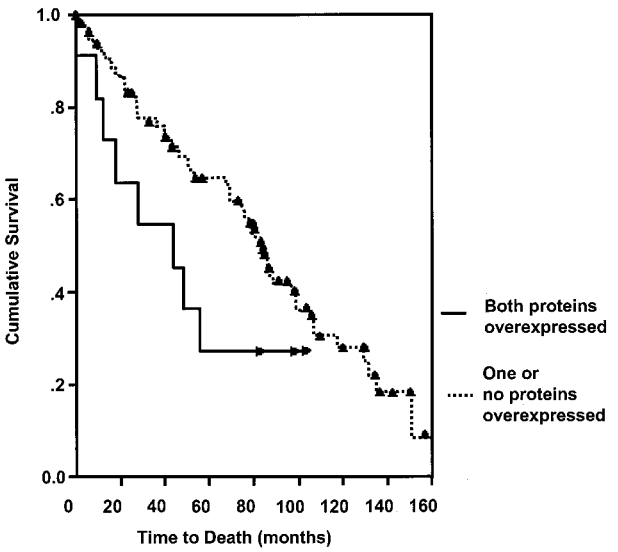


*Fig. 5* The 11 patients (9.9%) whose tumors synchronously overexpressed epidermal growth factor receptor and HER2-*neu* (———) had a shorter overall survival time than did the other patients (····; $P = 0.093$).

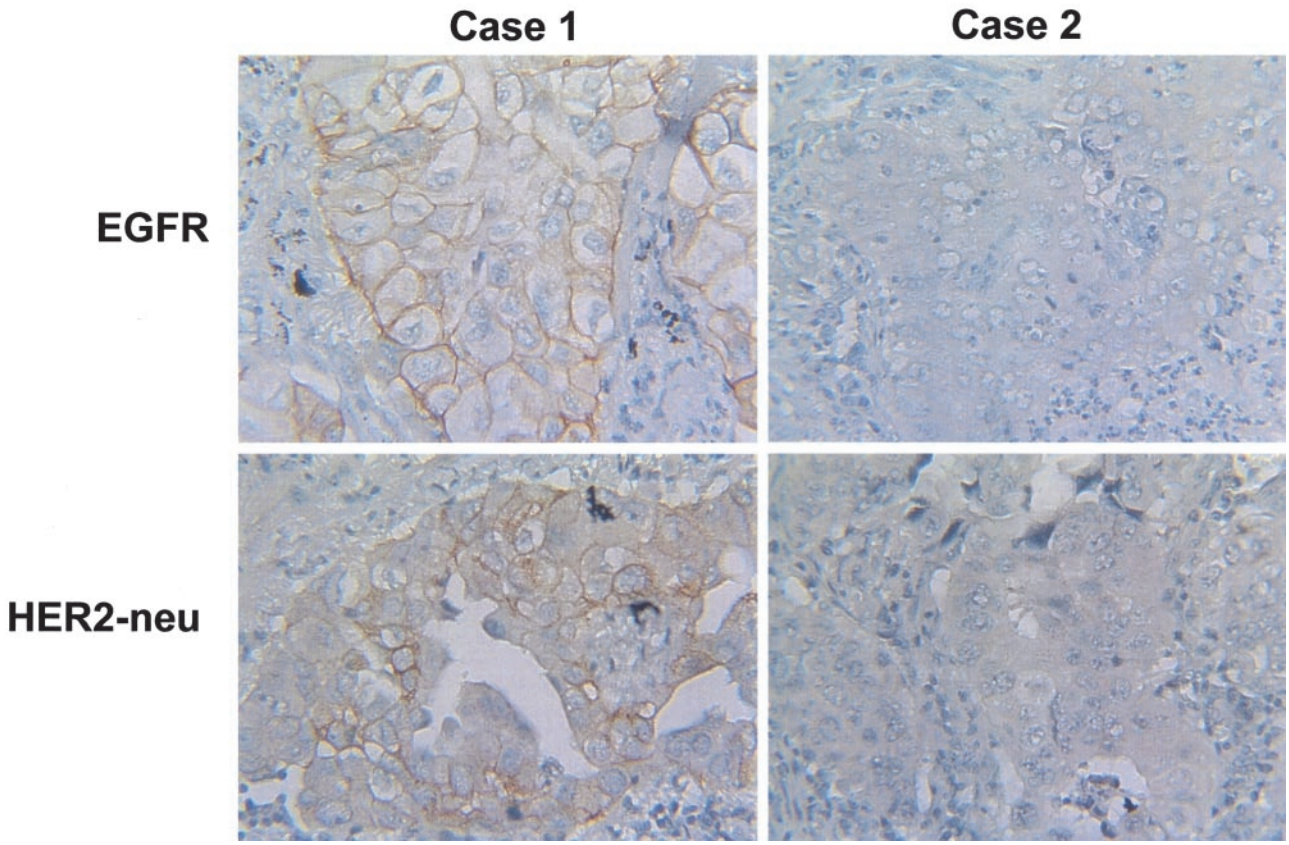


*Fig. 6* Representative immunohistochemical findings for both proteins (case 1) or no protein (case 2) overexpression. Both had adenocarcinoma and $T_2$ disease. Case 1 recurred with 1.6 months; the patient died 11.9 months after surgery. In case 2, the patient died 105.6 months after surgery.

prolongs the signal transduction pathways, and increases the affinity of the receptors to their ligands. Moreover, Brandt *et al.* (23) showed that the EGFR/HER2-*neu* heterodimer determines a motogenic phenotype in human breast cancer cell lines, and Moasser *et al.* (24) showed that the best results with ZD1839, an EGFR tyrosine kinase inhibitor, are achieved in cell lines overexpressing both EGFR and HER2-*neu*. We suggest that synchronous overexpression of EGFR and HER2-*neu* by NSCLC tumors may indicate heterodimerization, leading to higher proliferation potential of the tumor, which translates into shorter overall survival and substantially shorter disease-free survival.

Different patterns of staining have been noted for phosphorylated EGFR: membranous, cytoplasmic, and nuclear. The nuclear pattern of immunohistochemical staining for phosphorylated EGFR has been used to determine the biological response to EGFR tyrosine kinase inhibitor (TKI) in human (25, 26) and animal studies (27). These reports clearly showed that the signal of phosphorylated EGFR, as determined by nuclear localization, is diminished after EGFR-TKI administration. Holt *et al.* (28) showed that EGFR can be found in the nucleus of tumor cells, and Lin *et al.* (29) reported that EGFR may function as a transcription factor to activate genes required for proliferating activities. However, in our study, nuclear staining of phosphorylated EGFR was also observed in other cells, such as lymphocytes and macrophages. In addition, we found no relationship between tumors expressing phosphorylated EGFR and their total receptor level or expression of TGF-α, the receptor ligand, regardless of whether a positive score was defined as 1–3, or as 2 or 3. Additional studies will be needed to confirm the importance of these findings on phosphorylated EGFR.

In our study, more patients had adenocarcinoma or bronchioloalveolar carcinoma (32.5% and 18%, respectively) than had SCC (36%). This result is compatible with the trend seen in recent years of an increase in the proportion of patients with NSCLC who have adenocarcinoma (30). Histological subtype was found to affect overall survival; patients with adenocarcinoma survived longer than did those with SCC ($P = 0.053$).

Our data support the importance of biological staging (*i.e.*, determination of molecular markers) in lung cancer (31–35) and suggest that targeting ErbB-mediated signaling may benefit patients with lung cancer. Indeed, several approaches that focus on EGFR and HER2-*neu* are being evaluated. These approaches include monoclonal antibodies against the ligand, ligand-toxin conjugates, receptor monoclonal antibodies (humanized, murine, or chimeric), and TKIs (11). Small-molecule EGFR-TKIs are the furthest along in clinical development for NSCLC. Results of treatment with ZD1839 as a single agent in >400 patients with advanced NSCLC showed response rates of 11.8–18.4% (36–38). In addition, trastuzumab, a recombinant humanized monoclonal antibody that targets HER2-*neu*, has been

studied in the treatment of NSCLC. Preliminary results suggest that the combination of chemotherapy and trastuzumab is well tolerated (39). We suggest that the use of biological staging to classify lung cancers would identify the patients likely to benefit from this combined therapy.

Of special interest in our study is that the ligand TGF-α was detected in only 3 tumors (2.7%). This finding and the lack of an association with phosphorylated EGFR tumors is perturbing and should be studied additionally, because receptor activation is theoretically associated with ligand stimulation. However, the low rate of expression of this ligand may be another explanation for the relatively low rate of response of NSCLC to EGFR-TKIs. Baker *et al.* (40) analyzed recently the response of several tumor cell lines and the corresponding orthotopic tumors in nude mice to EGFR-TKIs. They reported that the best results were achieved when both tumor cells and tumor-associated endothelial cells expressed phosphorylated EGFR. Furthermore, the authors suggested that tumor-associated endothelial cells express the receptor by a mechanism that is dependent on TGF-α secretion by the tumor. We suggest that EGFR-TKIs could be beneficial in treating NSCLC tumors that overexpress both EGFR and HER2-*neu* and may be most efficient in treating the subpopulation of patients whose tumors also overexpress TGF-α. The correlation between EGFR and HER2-*neu* expression, clinical outcome, and response to molecular targeted therapy with EGFR TKIs has been studied recently in bronchioloalveolar by Franklin *et al.* (41) and Miller *et al.* (42), where this dual overexpression is seen and therapeutic benefit observed. We conclude that the use of adjuvant therapy in treating surgically treated patients with stage I NSCLC whose tumors express high levels of EGFR and HER2-*neu* is appealing and should be considered for a clinical trial.

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