*Advances in Brief*

# Epidermal Growth Factor Receptor and HER2-neu mRNA Expression in Non-Small Cell Lung Cancer Is Correlated with Survival

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## Abstract

**The prognostic role of epidermal growth factor receptor (EGFR) and HER2-neu remains controversial in patients with non-small cell lung cancer (NSCLC). We studied the association between the mRNA expression of EGFR, HER2-neu, and survival in primary tumor and matching nonmalignant tissues from 83 patients with NSCLC. Analysis was performed using a quantitative real-time PCR system (Taqman). EGFR and HER2-neu mRNA expression was detectable in all (100%) specimens analyzed. Twenty-nine (34.9%) patients had high HER2-neu expression, and 28 (33.7%) patients had high EGFR expression. A high HER2-neu and EGFR coexpression was detectable in 14 (16.9%) patients. High HER2-neu expression was associated with inferior survival ($P = 0.004$), whereas high EGFR expression showed a trend toward inferior survival ($P = 0.176$). The impact of HER2-neu and EGFR coexpression on patients' survival was additive ($P = 0.003$). Multivariate analysis determined high HER2-neu expression ($P = 0.041$), and high EGFR/HER2-neu coexpression ($P = 0.030$) as significant and independent unfavorable prognostic factors. These findings indicate that HER2-neu and EGFR play a crucial role in the biological behavior of NSCLCs. Testing of molecular marker coexpression (EGFR and HER2-neu) improves the estimation of prognosis and appears to define low- and high-risk groups for treatment failure in curatively resected NSCLC.**

## Introduction

Lung cancer is the leading cause of cancer-related deaths among both males and females in Western countries. In the United States, approximately 171,000 new cases of lung cancer will be diagnosed, and 160,000 individuals will die from this disease, each year. Despite improvements in the detection and treatment of lung cancer in the past two decades, the overall 5-year survival remains <15% (1). To improve further the survival rate in patients with NSCLC,[2] their prognostic classification based on molecular alterations will be crucial. Such classifications could provide more accurate and useful diagnostic tools and, eventually, more effective therapeutic options.

EGFR (also known as *erbB-1*) and HER2-neu (also known as *erbB-2*) are members of the *erbB* gene family and encode for transmembrane receptor-type tyrosine-protein kinases (2, 3). The ligands of EGFR include epidermal growth factor, and transforming growth factor α, which, upon the binding of EGFR, transmit growth-stimulatory signals (4). High levels of EGFR expression have been detected in several human malignancies, including NSCLC (5–7). The prognostic importance of EGFR in NSCLC remains unclear. Studies using binding assays correlated increased EGFR expression with advanced stage (8) and shortened overall survival in NSCLC (9), whereas studies using less quantitative techniques for EGFR mRNA or protein expression failed to show a consistent correlation with outcome (10–12). HER2-neu protein overexpression has been demonstrated in NSCLC, including SCC, AC, and LC (7, 13–16). Earlier studies, using protein assays, reported an association with HER2-neu protein overexpression and inferior overall survival in pulmonary ACs (14, 17), whereas others did not (11).

To determine the frequency and prognostic relevance of EGFR and HER2-neu mRNA expression in NSCLC, we performed a real-time quantitative PCR (Taqman) analysis (18, 19) on surgically removed tumor specimen and adjacent nonmalignant tissue from 83 patients with curatively resected NSCLC.

## Materials and Methods

**Patients.** Eighty-three patients were included in this study, consisting of sixty-five (78.3%) men and 18 (21.7%) women with a median age of 63.5 years (range, 34–82 years). Thirty-nine (47%) patients had SCCs, 32 (38.6%) had ACs, and 12 (14.5%) had LC. The primary tumors were graded histopathologically as well-differentiated ($G_1$; one patient), moderately differentiated ($G_2$; 18 patients), and poorly differentiated

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[2] The abbreviations used are: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; UICC, Union International Contre Cancer; CI, confidence interval; AC, adenocarcinoma; SCC, squamous cell carcinoma; LC, large cell carcinoma; RT-PCR, reverse transcription-PCR.

(G3; 64 patients). Tumor staging was performed according to the UICC Tumor-Node-Metastasis classification: 41 (49.4%) had stage I tumors; 16 (19.3%) had stage II tumors; and 26 (31.3%) had stage IIIa tumors. All tumors were completely resected (R0 category) by at least a lobectomy, as quality control. Patients with histopathological stage IIIa tumors received postoperative radiotherapy. The median follow-up was 85.9 months (minimum, 63.3 months, and maximum, 105.2 months), and no patient was lost to follow-up.

**Tissue Acquisition.** Tissue for gene expression analysis was obtained immediately after lung resection before starting mediastinal lymphadenectomy and was immediately frozen in liquid nitrogen. Tissues were analyzed from the following two locations: (*a*) tumor; and (*b*) uninvolved lung tissue taken from the greatest distance to the tumor. Six-μm frozen sections were taken from blocks of tumor tissue, and starting with the first section, every fifth one was routinely stained with HE and histopathologically evaluated. Sections were pooled for RNA isolation from areas of estimated 75% malignant cells.

**RNA Extraction and cDNA Synthesis.** Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrep*Micro* mRNA Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's instructions. After RNA isolation, cDNA was prepared from each sample as described previously (20).

**PCR Quantification.** Quantitation of cDNAs and an internal reference gene (β-*actin*) was done using a fluorescence-based, real-time detection method [ABI PRISM 7700 Sequence Detection System (Taqman); Perkin-Elmer Applied Biosystems, Foster City, CA.], as previously described (18–20).

The PCR reaction mixture consisted of 600 nM of each primer, 200 nM probe; 2.5 units AmpliTaq Gold Polymerase; 200 μM of each dATP, dCTP, and dGTP; 400 μM dUTP; 5.5 mM $MgCl_2$; and 1× Taqman Buffer A containing a reference dye to a final volume of 25 μl (all reagents, Applied Biosystems, Foster City, CA). Cycling conditions were 50°C for 10 s, 95°C for 10 min, and then 46 cycles at 95°C for 15 s and 60°C for 1 min.

The primers and probe sequences used were as follows. In all cases, the first primer is the forward PCR primer, the second is the reverse PCR primer, and the third is the Taqman probe. HER2-neu: CTGAACTGGTGTATGCAGATTGC, TTCCGAGCG GCCAAGTC; 6FAM(carboxyfluorescein)5′-TGTGTACGAGCCGCACATCCTCCA-3′TAMRA(*N*,*N*,*N*′,*N*′-tetramethyl-6carboxyrhodamine); EGFR: TGCGTCTCTTGCCG GAAT, GGCTCACCCTCCAGAAGCTT; 6FAM5′-ACGCAT-TCCCTGCCT CGGCTG-3′TAMRA; β-actin: TGAGCGCGGC-TACAGCTT, TCCTTAATGTCACGCACG ATTT; 6FAM5′-ACCACCACGGCCGAGCGG-3′TAMRA.

**Statistical Analysis.** Taqman analyses yield values that are expressed as ratios between two absolute measurements (gene of interest:internal reference gene). The ratio between mRNA expression in nonmalignant lung tissue and matching tumor tissue was used as a measure of the degree of gene expression. Associations between two related variables were tested by using the Wilcoxon signed rank test. The $\chi^2$ test was used to analyze the associations between categorical clinicopathological variables. Hazards ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected number of events as calculated in the Log rank test statistic (21). The maximal $\chi^2$ method of Miller and Siegmund (22) and Halpern (23) was adapted to determine which expression value best segregated patients into poor- and good-prognosis subgroups (in terms of likelihood of surviving), with the log-rank test as the statistics used to measure the strength of the grouping. To determine a *P* that would be interpreted as a measure of the strength of the association based on the maximal $\chi^2$ analysis, 1000 boot-strap-like simulations were used to estimate the distribution of the maximal $\chi^2$ statistics under the hypothesis of no association (23). Cox's proportional hazards modeling of factors that were significant in univariate analysis was performed to identify which factors might have a significant influence on survival. The level of significance was set to $P < 0.05$.

*Table 1* Relationships with HER2-neu and EGFR mRNA expression

| Variable | EGFR | | | HER2 neu | | |
|---|---|---|---|---|---|---|
| | Low | High | *P* | Low | High | *P* |
| Sex | | | | | | |
| Male | 40 | 25 | | 45 | 20 | |
| Female | 15 | 3 | 0.84 | 20 | 9 | 0.13 |
| Smoking | | | | | | |
| Smoker | 48 | 25 | | 46 | 27 | |
| Nonsmoker | 7 | 3 | 0.79 | 8 | 2 | 0.29 |
| pT category | | | | | | |
| pT1 | 11 | 5 | | 12 | 4 | |
| pT2 | 35 | 19 | | 33 | 21 | |
| pT3 | 9 | 4 | 0.93 | 9 | 4 | 0.56 |
| pN category | | | | | | |
| pN0 | 31 | 15 | | 32 | 14 | |
| pN1 | 15 | 7 | | 15 | 7 | |
| pN2 | 9 | 6 | 0.85 | 7 | 8 | 0.25 |
| UICC stage | | | | | | |
| I | 26 | 15 | | 28 | 13 | |
| II | 13 | 3 | | 12 | 4 | |
| IIIa | 16 | 10 | 0.37 | 14 | 12 | 0.31 |
| Histology | | | | | | |
| SCC | 26 | 13 | | 28 | 11 | |
| AC | 20 | 12 | | 16 | 16 | |
| LC | 9 | 3 | 0.74 | 10 | 2 | 0.57 |
| Grading | | | | | | |
| Well differentiated | 1 | 0 | | 1 | 0 | |
| Moderately differentiated | 13 | 5 | | 13 | 5 | |
| Poorly differentiated | 41 | 23 | 0.63 | 40 | 24 | 0.57 |

## Results

HER2-neu mRNA expression was detectable by quantitative real-time RT-PCR in 83 of 83 (100%) normal lung and in 83 of 83 (100%) tumor samples. In 44 (53%) of 83 patients, the HER2-neu expression level, expressed as the ratio between HER2-neu and β-actin PCR product, was elevated in tumor compared with paired normal lung tissue (the T:N ratio was higher than 1.0). The median HER2 neu mRNA expression was 4.17 (range, 0.28–23.86) in normal lung and 4.35 (range, 0.21–68.11) in tumor tissue ($P = 0.019$; Wilcoxon test). To determine whether there was any prognostic significance attached to quantitative differences in HER2-neu mRNA expression levels, the maximal $\chi^2$ method (22, 23) was adapted to determine which HER2-neu expression level best segregated patients into poor-

*Table 2* Survival in NSCLC based on clinical and molecular parameters

| Parameter | *n* | 5-yr survival probability | RR[a] | Median survival (mo) | 95% CI | *P*[b] |
|---|---|---|---|---|---|---|
| UICC stage | | | | | | |
| I | 41 | 0.71 ± 0.07 | 1.00 | n.r.[c] | | <0.001 |
| II | 16 | 0.44 ± 0.12 | 2.11 | 33.97 ± 5.70 | 22.80; 45.14 | |
| IIIa | 26 | 0.12 ± 0.06 | 4.81 | 19.00 ± 5.14 | 8.92; 29.08 | |
| pT | | | | | | |
| $pT_1$ | 16 | 0.69 ± 0.12 | 1.00 | n.r. | | 0.016 |
| $pT_2$ | 54 | 0.46 ± 0.07 | 2.16 | 46.77 ± 18.51 | 10.49; 83.05 | |
| $pT_3$ | 13 | 0.23 ± 0.12 | 3.56 | 26.67 ± 6.09 | 14.73; 38.61 | |
| pN | | | | | | |
| $pN_0$ | 46 | 0.70 ± 0.07 | 1.00 | n.r. | | <0.001 |
| $pN_1$ | 22 | 0.32 ± 0.10 | 2.51 | 33.71 ± 6.86 | 20.22; 47.12 | |
| $pN_2$ | 15 | 0.00 ± 0.00 | 6.59 | 16.70 ± 4.01 | 8.84; 24.56 | |
| HER2-neu | | | | | | |
| Low | 54 | 0.59 ± 0.07 | 1.00 | n.r. | | 0.004 |
| High | 29 | 0.24 ± 0.08 | 2.27 | 31.10 ± 4.66 | 21.96; 40.24 | |
| EGFR | | | | | | |
| Low | 55 | 0.53 ± 0.07 | 1.00 | n.r. | | 0.176 |
| High | 28 | 0.36 ± 0.09 | 1.50 | 32.37 ± 12.22 | 8.43; 56.31 | |
| Double marker (HER2/EGFR) | | | | | | |
| Low/Low | 40 | 0.63 ± 0.08 | 1.00 | n.r. | | 0.003 |
| Low/High | 14 | 0.50 ± 0.13 | 1.39 | 45.47 | | |
| High/Low | 15 | 0.27 ± 0.11 | 2.34 | 31.10 ± 8.33 | 14.77; 47.73 | |
| High/High | 14 | 0.21 ± 0.11 | 2.65 | 22.03 ± 10.07 | 2.30; 41.76 | |

[a] RR, relative risk. RR can be thought of as the average increased risk of dying at any point in time for patients in the second group compared with those in the first group.
[b] Based on median survival (log-rank test).
[c] n.r., not reached, cannot be calculated.

and good-prognosis subgroups. This method found that segregation was best achieved by using a T:N HER2-neu expression ratio of 1.8 as a cutoff value. By this criterion, 29 (34.9%) patients had a high HER2-neu expression and 54 (65.1%) had a low HER2-neu expression. Table 1 shows associations between clinicopathological data and *HER2-neu* gene expression status. There were no statistical significant differences detectable. HER2-neu mRNA expression was significantly associated with patient survival. The median survival (51.4 months; range, 3.8–105.3 months) was not reached in the low HER2 neu-expression group compared with 31.1 months (95% CIs, 21.96; 40.24) in the high HER2-neu expression group. To determine a *P*, we used bootstrap-like simulations to estimate the distribution of a maximal $\chi^2$ statistic, because the cutoff point of 1.8 had been chosen after examining the data. The resulting adjusted *P* was .004 (log-rank test). The importance of HER2-neu as a prognostic factor was next determined by the Cox's proportional hazards model analysis. In a univariate analysis of potential prognostic factors, high HER2-neu expression as well as advanced pT classification, pN classification, and tumor stage were significant unfavorable prognostic factors (Table 2). In a multivariate analysis of prognostic factors (Table 3, *Models A* and *B*), high HER2-neu expression was a significant and independent unfavorable prognostic factor, as was advanced pN classification and tumor stage.

*Table 3* Cox proportional hazards regression models

| Model | Parameter[a] | Hazards ratio | 95% CI | *P* |
|---|---|---|---|---|
| A | Stage | | | 0.0001 |
| | I/IIIa | 0.219 | 0.11–0.44 | 0.0001 |
| | II/IIIa | 0.524 | 0.23–1.17 | 0.177 |
| | HER2-neu | 1.894 | 1.02–3.51 | 0.043 |
| B | pT | | | 0.127 |
| | $pT_1/pT_3$ | 0.311 | 0.10–0.97 | 0.044 |
| | $pT_2/pT_3$ | 0.692 | 0.32–1.50 | 0.354 |
| | pN | | | 0.0001 |
| | $pN_0/pN_2$ | 0.143 | 0.07–0.31 | 0.0001 |
| | $pN_1/pN_2$ | 0.333 | 0.14–0.75 | 0.008 |
| | HER2-neu | 1.890 | 1.03–3.48 | 0.041 |
| C | Stage | | | 0.0001 |
| | I/IIIa | 0.554 | 0.11–0.44 | 0.0001 |
| | II/IIIa | 0.554 | 0.24–1.26 | 0.159 |
| | Double marker | 1.331 | 1.03–1.73 | 0.030 |
| D | pT | | | 0.168 |
| | $pT_1/pT_3$ | 0.335 | 0.11–1.05 | 0.061 |
| | $pT_2/pT_3$ | 0.704 | 0.32–1.55 | 0.384 |
| | pN | | | 0.0001 |
| | $pN_0/pN_2$ | 0.143 | 0.07–0.31 | 0.0001 |
| | $pN_1/pN_2$ | 0.333 | 0.14–0.74 | 0.007 |
| | Double marker | 1.280 | 1.00–1.63 | 0.046 |

[a] Parameter section: e.g., stage I/IIIa means stage I compared with stage IIIa.

EGFR mRNA expression was detectable by quantitative real-time RT-PCR in 83 of 83 (100%) normal lung and in 83 of 83 (100%) tumor samples. In 42 (51%) of 83 patients, the EGFR expression level was elevated in tumor compared with paired normal lung tissue (the T:N ratio was >1.0). The median EGFR mRNA expression was 8.17 (range, 0.31–46.26) in normal lung and 7.22 (range, 0.27–97.49) in tumor tissue (*P* = not significant). The maximal $\chi^2$ method (22, 23) determined a T:N EGFR expression ratio of 1.8 as a cutoff value to best segregate patients into low- and high-EGFR expressors. By this criterion,

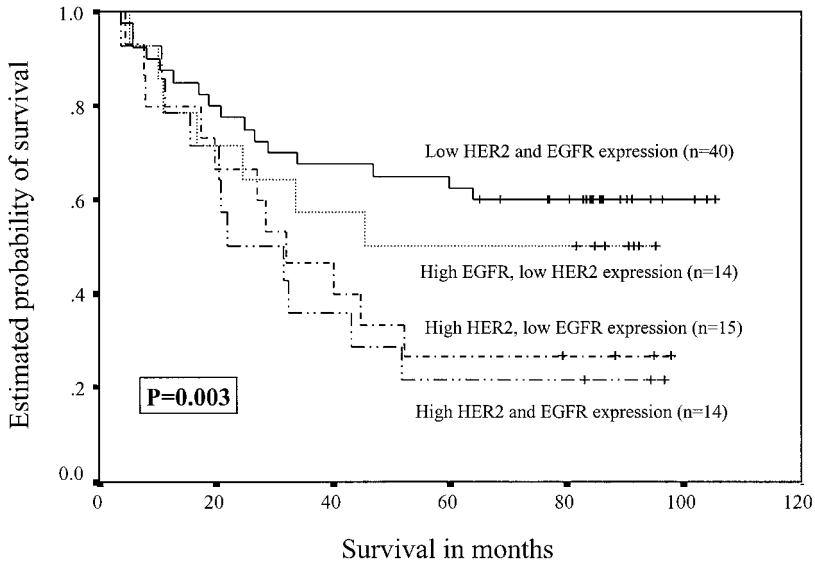


*Fig. 1* Estimated probability of survival of curatively resected non-small cell lung cancer patients *versus* combined patterns of EGFR and HER2-neu coexpression in NSCLC. The median survival was not reached in the group that showed low HER2-neu and EFGR expression, compared with 45.47 months in the high EGFR-expression group, 31.10 months (95% CI, 14.77–47.43) in the high HER2-neu-expression group, and 22.03 months (95% CI, 2.30; 41.76; $P = 0.003$) in the high HER2-neu- and EGFR-expression group.

28 (33.7%) patients had a high EGFR-expression status and 55 (66.3%) had a low EGFR-expression status. There were no statistical significant differences between clinicopathological variables and EGFR mRNA expression status detectable (Table 1). A trend toward inferior overall survival was observable for the high EGFR expression group but did not reach statistical significance (Table 2). The median survival was not reached in the low EGFR-expression group compared with 32.37 months (95% CI, 8.43–56.31) in the high-EGFR expressor group ($P = 0.176$).

High expression levels of HER2-neu and EGFR were found in 14 of 83 (16.9%) patients. Forty of 83 (48.2%) patients showed a low expression status for HER2-neu and EGFR, whereas 14 of 83 (16.9%) showed a high expression for EGFR only, and 15 of 83 (18.1%) patients displayed a high expression for HER2-neu. The median survival was not reached in the group that showed low HER2-neu and EFGR expression, compared with 45.47 months in the high EGFR-expression group, 31.10 months (95% CI, 14.77–47.43) in the high HER2-neu-expression group, and 22.03 months (95% CI, 2.30; 41.76; $P = 0.003$; log-rank test; Table 2 and Fig. 1) in the high-HER2-neu and -EGFR expression group. Univariate analysis displayed high HER2-neu and high EGFR coexpression as a significant unfavorable prognostic factor (Table 2). In a multivariate analysis of prognostic factors (Table 3, *Models C* and *D*), high HER2-neu and high EGFR coexpression was a significant and independent unfavorable prognostic factor, as was advanced pN classification and tumor stage.

## Discussion

In this study, we measured the mRNA expression of HER2-neu and EGFR in 83 tumor tissues and matched specimen of nontumor tissues from NSCLC patients to test the hypothesis that the quantity of expression of these genes by themselves or in combination are of prognostic importance in this disease. Expression of both genes was detectable in all specimen analyzed. However, the intratumoral content of mRNA expression varied among the tumors over a range of 324-fold for HER2-neu and 361-fold for EGFR. This observation of seemingly variable amounts of mRNA implies heterogeneity of expression patterns within individual tumor cells and may be reflected in the biological behavior of these tumors.

Previous studies of HER2-neu and EGFR expression in NSCLC reported enormous variations in frequencies of NSCLC tumors scored positive for both EGFR and HER2-neu expression. Overexpression of HER2-neu, defined as positive staining, were reported in 13–80% in ACs (11, 14, 17, 24–27), in 2–45% in SCCs (11, 14, 24, 25, 28), and in 0–20% in LCs, (11, 14) by using paraffin-embedded slides (14, 24, 28) or HER2-neu antisera (11, 14, 17, 24, 27) for immunohistochemistry. Consequently, different results have been reported concerning the impact of HER2-neu overexpression in NSCLC. Kern *at al.* (14) and Tateishi *et al.* (24) reported significantly inferior survival in patients overexpressing HER2-neu, whereas Pfeiffer *et al.* (11) did not detect differences in survival in HER2-neu-overexpressing ACs. It is noteworthy that the latter studies refer to AC only. Thus far, an association of HER2-neu overexpression and survival in SCCs and LCs of the lung has not been reported. In this study, we used a new approach to detect HER2-neu expression in NSCLC. First, we used a real-time RT-PCR (Taqman) method with high sensitivity and specificity for mRNA quantitation (18, 19, 29). Secondly, mRNA expression status was determined by a method used by Mafune *et al.* (30), who calculated individual tumor:normal (T:N) expression ratios in matching tissues obtained from patients with SCCs of the esophagus. This method of analysis leads to a precise expression value for each patient, because it is based on the individual background expression obtained from matching nonmalignant tissue. Using this method, we detected a high HER2-neu expression status in 34.9% of NSCLC patients analyzed. In addition, multivariate analysis revealed high HER2-neu expression as an

independent prognostic factor for survival ($P = 0.041$) in NSCLC.

Expression of EGFR in NSCLC has been reported extensively in studies using immunohistochemical methods for expression analysis (7, 8, 9, 10, 12, 24, 31–34), with frequencies for EGFR overexpression between 32% and 47% in NSCLC (10, 12, 24, 32, 33). Significant differences in EGFR expression have been reported among histological subtypes, generally with higher EGFR expression in SCC compared with AC and LC (9, 7, 12, 33, 34). Most of the studies reported no correlation of EGFR overexpression with patients' survival (12, 10, 24, 32, 33). Only Ohsaki *et al.* (34) correlated EGFR protein expression with poor prognosis in NSCLC patients with p53 overexpression ($P = 0.024$). Consistent with most previous reports, we found that EGFR mRNA expression was not prognostically significant for patients with curatively resected NSCLC.

One of the critical questions is the evaluation of interrelationships between factors reported to be of prognostic importance, and only a few studies have addressed this question regarding HER2-neu and EGFR coexpression in NSCLC. Tateishi *et al.* (24), measuring EGFR and HER2-neu protein expression, reported cooverexpression in 13% of ACs analyzed, and found that cooverexpression of these two genes was associated with inferior 5-year survival. The latter finding is consistent with the results obtained in our study and confirms that high HER2-neu and EGFR coexpression have a role in the biological behavior of NSCLC and the prognosis of patients with NSCLC.

HER2-neu expression may be linked to a chemoresistant phenotype in human NSCLC. Tumors cell lines overexpressing the HER2-neu protein product $p185^{neu}$ have been shown to be more resistant to cisplatin (35–38). Recently, the United States Food and Drug Administration approved the use of the monoclonal antibody trastazumab (Herceptin) for the treatment of HER2-neu-overexpressing metastatic breast cancers (16). The possibility that adjuvant chemotherapy might improve the survival in patients with NSCLC makes this drug, among others, an attractive target for patients with NSCLC. Demonstration of HER2-neu overexpression in NSCLC, using a standardized method, is essential for establishing clinical trials for this or other drugs. A recent report indicates nonspecificity when using the HercepTest for measurement of HER2-neu expression in invasive breast cancers, concluding that the HercepTest may have a high false positivity (39). The highly sensitive and specific Taqman technology used in this study might prove useful as an alternative measurement for HER2-neu quantitation.

In summary, we have demonstrated the considerable heterogeneity in EGFR and HER2-neu expression of individual NSCLCs, a heterogeneity likely to be reflected in the biological behavior of the tumors, and then identified high HER2-neu expression and high EGFR/HER2-neu coexpression as potential prognostic factors of NSCLCs. EGFR and HER2-neu may have a great value in identifying NSCLC patients at high risk of early disease recurrence after surgery and in selecting patients who will benefit from intensive adjuvant therapy.

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