Abstract

Purpose: Epidermal growth factor receptor (EGFR) mRNA expression and EGFR gene dosage by quantitative PCR in tumor samples obtained from patients with gefitinib-treated non–small cell lung cancer were analyzed in order to determine the association with treatment outcome, clinical, and biological features [EGFR copy number by fluorescent in situ hybridization (FISH), EGFR tyrosine kinase mutations, and EGFR protein expression].

Experimental Design: EGFR mRNA expression was measured by real-time quantitative reverse transcription-PCR in 64 patients, and EGFR gene dosage was analyzed by real-time quantitative PCR in 82 patients from paraffin-embedded specimens.

Results: EGFR mRNA expression was higher in responders to gefitinib as compared with non-responders (P = 0.012). Patients with high EGFR mRNA expression (5.01) had 43% response probability, whereas patients with low EGFR mRNA expression had 8% response probability (P = 0.006). Patients with high EGFR mRNA expression had longer median progression-free (5.3 versus 2.8 months, P = 0.028) but not overall survival (13.8 versus 10.9 months, P = 0.87). EGFR mRNA expression was higher in FISH-positive patients (P = 0.001) and in patients with positive EGFR immunostaining (P < 0.001) but not in patients with EGFR mutations (P = 0.19). EGFR gene dosage did not predict response (P = 0.54), progression-free (P = 0.73), or overall survival (P = 0.89). EGFR gene dosage was not associated with FISH positivity (P = 0.15), relative mRNA expression (P = 0.27), EGFR mutation status (P = 0.39), and EGFR protein expression (P = 0.35).

Conclusion: EGFR mRNA expression is a predictive biomarker for response to gefitinib and to progression-free survival after gefitinib treatment. EGFR gene dosage is neither predictive for response nor progression-free nor overall survival.

Novel therapeutic options in non–small cell lung cancer (NSCLC) include epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib (1, 2). Erlotinib produced a significant survival advantage compared with placebo in a randomized phase III trial, but 30% of patients in each arm died in the first 3 months with no evidence for benefit in at least this fraction of patients (3). Thus, the search for an optimal selection of patients who will benefit from these treatments remains important. Patient selection based on clinical favorable variables is not sufficient, as some survival benefit was observed in groups with low response rates such as males and squamous cell histologies as some survival benefit was observed in groups with low response rates such as males and squamous cell histologies (3, 4). Thus, selection based on molecular markers may be more promising.

EGFR gene copy number, EGFR mutation status, and protein expression have been evaluated for their prediction of response to EGFR TKIs. In previous studies from our group, high EGFR copy number evaluated by fluorescent in situ hybridization (FISH) correlated with improved response and survival of NSCLC patients treated with gefitinib (5, 6). These findings were confirmed in subgroup analyses of two phase III clinical studies, comparing erlotinib (BR.21 trial; ref. 7) or gefitinib (ISEL trial; ref. 8) with placebo in second- or third-line treatment of advanced NSCLC. In the NCIC BR.21 trial, the positive treatment effect of erlotinib was confined to the FISH-positive group (FISH) correlated with improved response and survival of NSCLC patients treated with gefitinib (5, 6). These findings were confirmed in subgroup analyses of two phase III clinical studies, comparing erlotinib (BR.21 trial; ref. 7) or gefitinib (ISEL trial; ref. 8) with placebo in second- or third-line treatment of advanced NSCLC. In the NCIC BR.21 trial, the positive treatment effect of erlotinib was confined to the FISH-positive group as compared with the FISH-negative group of patients, both in terms of response rate (20% versus 2%, respectively) and survival (hazard ratios of 0.44 versus 0.85,
respectively; ref. 7). Biomarker analyses from the ISEL trial confirmed these results (response rates of 17.5% versus 3.4% and hazard ratios of 0.61 versus 1.14 in EGF FISH-positive versus FISH-negative patients, respectively; ref. 8).

Mutations of EGF TK domain are associated with increased responsiveness to EGFR TKIs (5, 9–15). There are conflicting data reported on the association of EGF TK activating mutations and survival advantage from gefitinib or erlotinib, especially in Western NSCLC populations. In the BR.21 study, the hazard ratio of death was almost identical for patients with mutated and wild-type EGF (0.73 and 0.77, respectively; ref. 7). In contrast to this prospective trial, several retrospective studies showed prolonged survival in gefitinib-treated patients, mostly in Asian populations (12–15).

There are also conflicting data on whether EGF gene dosage evaluated by quantitative PCR (qPCR) is associated with outcome following gefitinib or erlotinib therapy, and there are no data reported on a direct comparison of predictive value of FISH versus qPCR for clinical outcome in lung cancer. Bell and colleagues recently published data on a large number of gefitinib-treated patients participating in IDEAL and INTACT studies (16). They did EGF gene dosage analysis by qPCR and compared the results with mutation status, protein expression, and patient outcome. No predictive significance of EGF gene dosage for treatment benefit was found. Another study noted increased response rate and prolonged progression-free but not overall survival in patients with high EGF gene dosage who were treated with gefitinib (14).

Apart from the present cohort of patients, we are aware of only one study published on EGF mRNA expression in relation to other biomarkers and outcome of gefitinib-treated patients (15). In this study, EGF mRNA expression analysis was limited to 28 patients. EGF-mutant patients had a tendency towards higher mRNA expression, but response and survival data according to EGF mRNA expression were not evaluated.

In the present report, we evaluated EGF mRNA expression and gene dosage by qPCR technique and compared these results with our previously published data on EGF mutations, EGF copy number by FISH, and EGF protein expression by immunohistochemistry (5). We also analyzed these data with regard to patients’ clinical characteristics, objective response rate, disease control rate, and progression-free and overall survival.

**Materials and Methods**

**Patient selection, tissue sample collection, and previously analyzed molecular markers.** The cohort included 102 patients with advanced NSCLC previously evaluated for EGF status (5). Eligibility included histologically confirmed NSCLC with measurable, locally advanced or metastatic disease, progressing or relapsing after chemotherapy, or medical contraindications for chemotherapy. Gefitinib was given at a dose of 250 mg daily and patients were evaluated by computer tomography for tumor response after 2 months according to the Response Evaluation Criteria in Solid Tumors guidelines (17). The methodology of FISH, EGF mutation analysis, and protein expression assessment has been previously described in detail (5). Briefly, FISH-negative samples were defined as those with no or low gene copy number (≥four copies of the gene per cell in >40% of cells) and FISH-positive samples were defined as those with high gene copy number (≥four copies of the gene per cell in ≥40% of cells) or amplification (tight gene clusters and a ratio of gene/chromosome per cell ≥2 or ≥15 gene copies per cell in ≥10% of the cells). For EGF mutation analysis, genomic DNA was amplified by touchdown heminested PCR for exons 18, 19, and 21 and sequenced in both sense and antisense directions (–90% of EGF mutations are located in these exons; ref. 18). Protein expression was defined by multiplying the percentage of positive cells by staining intensity. The total protein expression was scored by two independent pathologists and ranged from 0 to 400. For statistical analysis, scores of 0 to 19 were regarded as immunohistochemistry negative, whereas scores of 20 to 100 were regarded as immunohistochemistry positive.

**Tissue preparation, laser capture microdissection, EGF RNA and DNA extraction, qPCR, and quantitative reverse transcription-PCR.** Paraffin-embedded tumor blocks were reviewed for quality and tumor content and 5-μm-thick sections were obtained. Sections were mounted on uncoated glass slides deparaffinized in xylene, hydrated and stained with nuclear fast red (American MasterTech Scientific Inc., Lodi, CA) for tumor cell visualization. Microdissection was not done in 22 cases in which the proportion of tumor cells was abundant. The remaining samples were isolated by laser capture microdissection (PALM Microsystems; Leica, Wetzlar, Germany) according to the standard procedure (19). RNA isolation after dissection was done according to a proprietary procedure of Response Genetics, Inc., (U.S. patent no. 6248535). Complementary DNA was prepared as described previously (20). Quantification of the genes of interest and an internal reference gene (β-actin) was conducted using a fluorescence-based real-time detection method [ABI PRISM 7700 Sequence Detection System (TaqlMan); Perkin-Elmer Applied Biosystems], as previously described (21). Cycling conditions were 50°C for 10 seconds and 95°C for 10 seconds, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. EGF expression was analyzed using forward primer EGF-1753F, 5′-TGGCCTGTCCTTGTCCCGAAT-3′; reverse primer EGF-1823R, 5′-GGCTACACCCCTCAGAAGGGTT-3′; and TaqMan probe EGF-1773Tc, 5′-ACGCACTTCCCTGCCCTGCTG-3′. EGF mRNA expression levels (also referred to as relative mRNA expression) were calculated as ratios (differences between the Ct values) between EGF and β-actin, that provides a normalization factor for the amount of RNA isolated from a specimen. β-Actin primers were: forward primer 5′-AGGCCGCGGCTCA- CACCTT-3′; reverse primer 5′-TCCTTAAATGTGCAGCAAGATT-3′; and TaqMan probe, 5′-ACACACGCGGCGCGACGCT-3′. For EGF gene dosage evaluation, DNA was extracted using the QiAamp kit (Qiagen, Valencia, CA). Selected EGF regions were evaluated by real-time PCR using the following primers: forward primer dEGFR-144394F, 5′-CGTCTCTTGCCGGAATGT-3′; reverse primer dEGFR-144479R, 5′- GGATTAAGAAATACCTCCTAACCC-3′; and TaqMan probe, dEGFR- 144412Tc, AGCCATCTTCCCTGCCTGCTG (GenBank accession no. AV588246).

**Statistical analyses.** All continuous variables were not normally distributed, as assessed by Kolmogorov-Smirnov test. Qualitative variables were compared by Pearson’s χ² test or Fisher's exact test, where appropriate. Groups of continuous variables were compared using Mann-Whitney U test or Kruskal-Wallis test, where appropriate. Median values of mRNA expression and EGF gene dosage by qPCR were used to compare these biomarkers with clinical patient characteristics. Because we found a difference between mRNA expression in responding vs nonresponding patients, we subsequently used the response data to select the best cutoff value and dichotomize patients into groups with low and high EGF mRNA expression using a receiver-operating characteristic curve and the Youden index (±5.01, low EGF expression; ±5.01, high EGF expression; ref. 22). Because there was no relation between EGF gene dosage and response, we used the median value of 0.36 (±0.36, low EGF gene dosage) in all comparisons. To assess the association between quantitative variables, nonparametric Spearman’s correlation was used. The Kaplan-Meier method was used for survival analysis and
the log-rank test for univariate comparisons. Additionally, univariate Cox proportional model was fit to assess the influence of relative EGFR expression and gene dosage as continuous variables on progression-free and overall survival. Multivariate analysis was done with backward manual elimination based on likelihood-ratio statistics. All variables met the assumption of proportional hazards using the supremum test (23). The SPSS 13.0 statistical package (Chicago, IL) was used for calculations. SAS V9.3 was used to assess the proportional hazard assumption. A significance level of 0.05 was used for hypothesis testing. All reported P values are two-sided.

Results

**Patient population.** From the original cohort of 102 patients, 10 pretreatment paraffin-embedded biopsies were not available, 23 samples did not contain enough tumor tissue for accurate measurement, and 5 samples had sufficient area of tumor, but failed to provide enough mRNA after extraction for EGFR mRNA expression analysis. Thus, paraffin-embedded samples yielded sufficient mRNA quantity in 64 patients who were included in the analysis of EGFR mRNA expression (70% of available samples and 93% of samples of acceptable quality for mRNA testing).

For EGFR gene dosage assessment by qPCR, two biopsies were not available and 18 had an insufficient amount of tumor cells from the original cohort of 102 samples, leaving 82 specimens. This group included 56 males (68%) and 26 females (32%), 70 patients with stage IV (85%) and 12 patients with stage III NSCLC (15%), 37 patients with performance status 0 according to WHO (45%), 32 patients with WHO performance status 1 (39%) and 13 patients with WHO performance status 2 (16%). There were 69 current or former smokers (“ever-smokers”, 84%) and 13 never-smokers (16%), the median age was 62 years (range, 25-83 years). Adenocarcinoma was the most prevalent histologic diagnosis (53 patients, 65%; including 7 patients with bronchioalveolar features), followed by squamous cell carcinoma (20 patients, 24%), undifferentiated NSCLC (8 patients, 10%), and large cell carcinoma (1 patient, 1%). Eighty patients (98%) were evaluable for response and 9 (11%) were classified with response.

Clinical characteristics and survival of subgroups analyzed for relative mRNA expression and for EGFR gene dosage were similar to the initial population (data not shown).

**EGFR mRNA expression assessed by quantitative reverse transcription-PCR.** Relative EGFR mRNA expression was detectable by qRT-PCR (quantitative reverse transcription-PCR) in all 64 samples that were available for the analysis. The median EGFR mRNA expression was 1.98 (range, 0.17-28.27). Patient characteristics according to median relative EGFR mRNA expression are shown in Table 1. There was no association between EGFR mRNA expression and gender, histology, performance status, smoking history, stage or age.

Evaluable patients who responded to gefitinib (n = 10) had significantly higher median relative EGFR mRNA expression as compared with nonresponders (n = 52; 5.14 versus 1.68, \( P = 0.012 \)). Patients with EGFR mRNA expression \( >5.01 \) (the threshold value identified by receiver-operating characteristic analysis) had a 43% probability of response (6 of 14 patients), whereas patients with EGFR mRNA expression \( \leq 5.01 \) had an 8% response probability (4 of 48 patients, \( P = 0.006 \)). Disease control probabilities in these subsets were 57% and 38%, respectively (\( P = 0.19 \)). In the analyzed population, response probabilities for EGFR FISH-positive and FISH-negative patients were 41% and 2%, and for

<table>
<thead>
<tr>
<th>Table 1. Clinical patient characteristics according to median relative EGFR mRNA expression and median EGFR gene dosage</th>
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<tr>
<td>Clinical variable</td>
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</tr>
<tr>
<td>Gender</td>
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<td>Female</td>
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<tr>
<td>Smoking history</td>
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<td>Never-smokers</td>
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<td>Performance status</td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>Age</td>
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</tbody>
</table>

NOTE: Values in table expressed as n(%).
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Mutant and wild-type EGFR patients were 50% and 6%, respectively. In the univariate analysis, high EGFR mRNA expression (>5.01) was associated with longer median progression-free survival as compared with low EGFR mRNA expression (5.3 versus 2.8 months, respectively; log-rank, \(P = 0.028\); Fig. 1A). This difference was not significant when mRNA expression was analyzed as a continuous variable (dichotomized (median, 13.8 versus 10.9 months for patients with high and low expression, respectively; log-rank, \(P = 0.87\)) or as a continuous variable (\(P = 0.66\)). Objective response probability, progression-free survival, and overall survival data according to EGFR mRNA expression are summarized in Fig. 1A and B. In the multivariate progression-free survival model which included EGFR mRNA expression, clinical variables (age, gender, histology, performance status, and stage), and other biomarkers (\(EGFR\) copy number by FISH, \(EGFR\) mutations, and \(EGFR\) protein expression), high relative mRNA expression was not significant after adjusting for other covariates. This model is, however, difficult to interpret due to the relatively small number of patients with correlated biomarker data.

Median relative mRNA expression was significantly different between FISH-positive (\(n = 22\)) and FISH-negative patients (\(n = 42\); 5.02; range, 0.2-28.3, and 1.44; range, 0.2-28.3, respectively, \(P = 0.001\)).

In the group of 64 patients with known EGFR mRNA expression, there were 50 patients with wild-type \(EGFR\), and 14 patients with tumors having \(EGFR\) mutations; 7 patients with exon 19 deletions, and 7 patients with exon 21 point mutations. Median relative EGFR mRNA expression tended to be higher in patients with exon 19 deletions (4.0; range, 0.6-21.6) as compared with wild-type \(EGFR\) (1.9; range, 0.2-28.3) or exon 21 mutations (0.8; 0.3-8.5; \(P = 0.19\); Table 2). We subsequently analyzed the outcome of 14 \(EGFR\)-mutant patients according to relative EGFR mRNA expression and the response to gefitinib. In seven nonresponding patients, median relative EGFR mRNA expression was 0.8 (range, 0.3-5.2) as compared with 5.3 (range, 0.7-21.6) in seven patients who responded to gefitinib (\(P = 0.025\)). Patients with \(EGFR\) mutations (\(n = 5\) and high mRNA expression had a median progression-free survival of 19.7 months (95% confidence interval, 0-44.1 months), whereas patients with \(EGFR\) mutations and low mRNA expression (\(n = 9\)) had a median progression-free survival of 4.0 months (95% confidence interval, 0.9-7.9 months), similar to 48 patients with wild-type \(EGFR\) (2.9 months; 95% confidence interval, 2.0-3.8 months; \(P = 0.016\) for comparison of patients with mutant \(EGFR\)/high EGFR mRNA expression versus mutant \(EGFR\)/low EGFR mRNA expression and wild-type \(EGFR\); Fig. 2). There was no difference in overall survival of patients with mutant \(EGFR\)/high relative EGFR mRNA expression, mutant \(EGFR\)/low EGFR mRNA expression, and wild-type \(EGFR\) (\(P = 0.55\); results not shown).

Tumors with positive EGFR immunostaining (≥200, \(n = 42\)) had higher mRNA expressions compared to tumors with low or negative immunostaining (<200, \(n = 22\); median, 3.3 and 1.4, respectively; \(P = 0.012\); results not shown). The correlation between \(EGFR\) protein and mRNA expression was also significant when both variables were considered to be continuous (Spearman’s \(r = 0.46; P < 0.001\); Table 2).

\(EGFR\) gene dosage assessed by qPCR. \(EGFR\) gene dosage was analyzed by qPCR in 82 patients. Median \(EGFR\) gene dosage was 0.36 (range, 0-4.51). Patient characteristics according to median \(EGFR\) gene dosage are shown in Table 1. There was no association of \(EGFR\) gene dosage with the analyzed clinical variables except for age. Patients with \(EGFR\) gene dosage >0.36 were younger as compared to patients with \(EGFR\) gene dosage ≤0.36 (median age, 58 versus 66 years, respectively; \(P = 0.031\)).

In a group of 82 patients, there were 80 patients evaluable for response. \(EGFR\) gene dosage did not differ between patients who responded to gefitinib and nonresponders (median, 0.36 and 0.34, respectively; \(P = 0.54\); results not shown). There was no difference in \(EGFR\) gene dosage between patients who achieved disease control as compared with patients with progressive disease (median, 0.36 and 0.34, respectively; \(P = 0.95\); results not shown). We used median relative \(EGFR\) gene dosage (≤0.36 versus >0.36) for comparisons in the response and survival analysis. Response rates in evaluable patients with high (\(n = 41\)) versus low \(EGFR\) dosage (\(n = 39\)) were 12% versus 10%,
respectively \((P = 0.78)\). Progression-free survival was the same in patients with low and high \(EGFR\) dosage (median of 2.8 months in each group, \(P = 0.73\); Fig. 3A). There was no difference in overall survival in patients with low and high \(EGFR\) gene dosage (median, 9.4 and 11.3 months, respectively; \(P = 0.89\); Fig. 3B). There was no association between \(EGFR\) gene dosage, progression-free survival, and overall survival when gene dosage was entered as a continuous variable in the univariate Cox model (\(P = 0.072\) and \(P = 0.52\), respectively).

The median \(EGFR\) gene dosage was 0.40 (range, 0-4.51) in the FISH-positive group \((n = 24)\) as compared with 0.36 (range, 0-1.58, \(P = 0.15\)) in the FISH-negative group of patients \((n = 58, \text{Table } 2)\).

\(EGFR\) mutations were present in 13 of 82 patients (16\%), including seven deletions in exon 19 and six point mutations in exon 21. Median relative \(EGFR\) gene dosage was nonsignificantly higher in patients with exon 19 deletions (0.54; range, 0-4.51) as compared with wild-type \(EGFR\) (0.35; range, 0-3.13) or exon 21 mutations (0.29; range, 0.17-2.05; \(P = 0.39\); Table 2). In the subset of \(EGFR\)-mutant, nonresponding patients \((n = 7)\), median \(EGFR\) gene dosage was 0.37 (range, 0.17-0.69) as compared with 1.01 (0-4.51) in six patients who responded to gefitinib \((P = 0.22)\). Five of six patients who responded to gefitinib were FISH-positive, whereas only two of seven nonresponders were scored as FISH-positive \((P = 0.10)\).

There was no statistically significant correlation between \(EGFR\) gene dosage and protein expression, when the latter was analyzed as categorical \((P = 0.23); \text{results not shown}) or continuous variables (Spearman’s \(r = 0.11, P = 0.35\); Table 2). There was no significant correlation between \(EGFR\) gene dosage evaluated by qPCR and relative \(EGFR\) mRNA expression (Spearman’s \(r = 0.14, P = 0.27\); Table 2).

**Table 2. Correlations among analyzed biomarkers**

<table>
<thead>
<tr>
<th>(EGFR) gene dosage (qPCR)</th>
<th>(EGFR) gene copy number (FISH)</th>
<th>Relative (EGFR) mRNA expression (qRT-PCR)</th>
<th>(EGFR) mutation status (sequencing)</th>
<th>(EGFR) protein expression (immunohistochemistry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>(P)</td>
<td>(n)</td>
<td>(P)</td>
<td>(r)</td>
</tr>
<tr>
<td>(EGRF) genedosage (qPCR)</td>
<td>—</td>
<td>82</td>
<td>0.15*</td>
<td>61</td>
</tr>
<tr>
<td>(EGRF) genecopynumber (FISH)</td>
<td>—</td>
<td>64</td>
<td>0.001*</td>
<td>82</td>
</tr>
</tbody>
</table>

\*Mann-Whitney \(U\) test.

\*Spearman’s correlation (Spearman’s correlation coefficient \(r\) is provided).

\*Kruskal-Wallis test.

\*\(\chi^2\) test.

Discussion

In two randomized phase III trials comparing placebo to erlotinib \((7)\) or to gefitinib \((8)\), various biological markers were evaluated to compare clinical outcomes. In both studies, the \(EGFR\) copy number by FISH was the best predictor of survival. Several large phase II or retrospective studies came to similar conclusions \((5, 6)\). The FISH positivity seems to be purely predictive and not prognostic because FISH-positive and FISH-negative patients receiving placebo have similar rates of survival (in the ISEL study, median survival in FISH-positive patients was 4.5 months and 6.2 months for FISH-negative patients; ref. 8) and because another study showed a tendency for shorter survival in FISH-positive patients diagnosed with stage I to III NSCLC \((24)\).

There are fewer data on the relationship between \(EGFR\) TKI treatment outcome, \(EGFR\) mRNA expression, and gene dosage determined by qPCR assessment. In the current study, we showed that \(EGFR\) mRNA expression evaluated by qRT-PCR from paraffin-embedded tumor tissue is a predictive marker for response to gefitinib and for progression-free survival. The
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EGFR was significantly longer than expression than nonresponders. Moreover, progression-free survival of patients who respond to gefitinib have significantly higher EGFR mRNA expression (43% and 10%, respectively) were comparable with the information obtained by EGFR FISH (41% and 2%, respectively) and EGFR mutation analysis (50% and 6%, respectively). Multivariate analysis shows that the predictive value of EGFR mRNA expression is lost when other biomarkers are included (EGFR immunohistochemistry, FISH, and mutational analysis). However, this model is difficult to interpret due to the high level of EGFR mRNA expression in tumors is dependent on several molecular events. We have shown that high EGFR mRNA expression correlated with increased EGFR gene copy number, as evaluated by FISH. A similar correlation was reported by Bhargava et al. in breast cancer (25). Both mRNA expression analysis and FISH could be successfully applied in paraffin-embedded tissue. FISH analysis is available in many diagnostic laboratories; mRNA expression evaluation often requires tissue microdissection and validation of “positive” versus “negative” cutoff points, but is less costly and could potentially be done as a high-throughput method. Moreover, HER2 gene dosage by qPCR and HER2 mRNA expression by qRT-PCR have been proposed as alternative methods of HER2 testing with potential clinical applications in breast cancer (26, 27). At present, routine use of gene dosage analysis by qPCR is limited by the lack of standardization regarding the choice, measurement of the reference genes, and the potential to amplify nontumor DNA.

Evaluation of EGFR gene dosage as a predictive marker for response to EGFR TKIs was addressed by Takano et al. (14) in a group of 66 NSCLC patients treated with gefitinib for relapse after surgery. EGFR dosage was evaluated after tumor microdissection by real-time duplex PCR using RNaseP as the endogenous reference gene. In the latter study, increased EGFR gene dosage was associated with higher response rate to gefitinib and longer time-to-progression in univariate and multivariate analysis. Bell and colleagues successfully analyzed gene amplification in the study of Bell and colleagues does not allow for a direct comparison of their results with our findings. In the INTACT studies, increased EGFR gene dosage was associated with higher response rate to gefitinib and longer time-to-progression in univariate and multivariate analysis. Bell and colleagues successfully analyzed EGFR dosage by qPCR in 90 patients who participated in two IDEAL phase II studies and in 453 patients who participated in two INTACT phase III studies that compared chemotherapy and gefitinib to chemotherapy and placebo (16). The frequency of gene amplification by qPCR was 8% in IDEAL and 7% in INTACT studies, a result markedly different from ~30% of FISH-positive patients observed in the University of Colorado Cancer Center studies (5, 6, 8). Differences in the definition of gene amplification in the study of Bell and colleagues does not allow for a direct comparison of their results with our findings. In the INTACT studies, EGFR amplification was not predictive of treatment benefit, i.e., was not associated with better progression-free or overall survival in patients receiving chemotherapy and gefitinib as compared with chemotherapy and placebo. Patients with EGFR-amplified tumors had longer overall and progression-free survival irrespective of gefitinib treatment, suggesting that gene amplification analyzed by qPCR may be a positive prognostic factor in NSCLC.

Contrary to the findings of Takano et al. (14), we could not establish any association between EGFR gene dosage evaluated by qPCR and gefitinib treatment outcome in our series of patients. The reason of this discrepancy is unclear. However, it is likely that the proportion of patients with high EGFR gene

response rates in patients with high and low EGFR mRNA expression (43% and 10%, respectively) were comparable with the information obtained by EGFR FISH (41% and 2%, respectively) and EGFR mutation analysis (50% and 6%, respectively). Multivariate analysis shows that the predictive value of EGFR mRNA expression is lost when other biomarkers are included (EGFR immunohistochemistry, FISH, and mutational analysis). However, this model is difficult to interpret due to the relatively small group of patients with highly correlated covariates. We have also shown that EGFR-mutant patients who respond to gefitinib have significantly higher EGFR mRNA expression than nonresponders. Moreover, progression-free survival of EGFR-mutant patients with high mRNA expression was significantly longer than EGFR-mutant patients with low EGFR mRNA expression and patients with wild-type EGFR, implicating that effective transcription is needed for effective treatment in EGFR-mutant patients, and mRNA expression may add valuable information to EGFR mutation status if the latter is considered for patient selection, as may be the case in Asian populations. To our knowledge, these associations have not been reported previously.

The predictive value of EGFR mRNA expression has been addressed, according to our best knowledge, only sporadically. In a recent report by Taron et al., the analysis of EGFR mRNA expression was done in a subset of 28 patients from a cohort of 68 patients who were analyzed for EGFR mutations (15). Similarly to our report, this study showed a tendency towards higher EGFR mRNA expression in EGFR-mutant as compared with wild-type patients, but the response and survival data according to mRNA expression were not reported.

Fig. 3. Progression-free survival (A) and overall survival (B) according to relative EGFR gene dosage.
dosage is larger in Japanese versus Western NSCLC patients (14, 16), and thus progression-free advantage could more easily be detected in patients with high EGFR gene dosage from Far East. In the study of Bell and colleagues (16), high EGFR gene dosage was not predictive of gefitinib treatment benefit, but indicated better prognosis of patients regardless of treatment received. Much larger sample size would be needed in our study to detect a survival difference of this magnitude.

We did not find a significant correlation between FISH positivity according to University of Colorado Cancer Center scoring criteria and EGFR gene dosage by qPCR. The two measurements are related to the same process—EGFR gene quantification, but their results yield different information and cannot be interchangeably used. Potential reasons for this discrepancy include the direct measurement of gene copy numbers in single tumor cells by FISH, which cannot be achieved by qPCR even when microdissection is done, and relative measurement of EGFR gene dosage to the reference gene, with no guarantee that the reference gene is always diploid and not amplified or deleted.

It can be concluded from the current study that high EGFR mRNA expression analyzed by qRT-PCR is associated with increased response to gefitinib and prolonged progression-free survival. Our study supports further evaluation of EGFR mRNA expression as a biomarker of sensitivity for EGFR TKIs in larger data sets. In contrast to EGFR gene copy number assessed by FISH, we could not show any predictive value of EGFR gene dosage by qPCR for response or survival.

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Epidermal Growth Factor Receptor Messenger RNA Expression, Gene Dosage, and Gefitinib Sensitivity in Non–Small Cell Lung Cancer

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