Abstract
Recent evidence indicates that high epidermal growth factor receptor (EGFR) gene copy number evaluated by fluorescence in situ hybridization is an excellent predictive biomarker for response and survival benefit in patients with non–small cell lung cancer who receive epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors. Data on EGFR protein expression by immunohistochemistry as a selection marker are conflicting, although several studies showed that the treatment benefit was confined to EGFR-positive patients. Our studies and others showed that fluorescence in situ hybridization and immunohistochemistry were associated with the best predictive value. Expeditious validation of this information in prospective clinical trials with patient selection to first-line treatment is currently being done or planned by several cancer research groups worldwide.

Therapeutic strategies of blocking the epidermal growth factor receptor (EGFR)–mediated signal transduction in non–small cell lung cancer (NSCLC) include small-molecule tyrosine kinase inhibitors (TKI; e.g., gefitinib and erlotinib) and monoclonal antibodies (e.g., cetuximab). Several new compounds targeting the EGFR pathway are in development, many of which interfere with multiple receptors of the EGFR family or other target tyrosine kinases (1). Current data indicate that patients with advanced NSCLC whose tumors are characterized by high EGFR gene copy numbers, as assessed by fluorescence in situ hybridization (FISH), derive clear response and survival benefit from treatment with EGFR TKIs (2–4). The role of EGFR immunohistochemistry for predicting sensitivity to EGFR TKIs might be less clear. There is now compelling evidence to use FISH to select patients for EGFR-based targeted therapy of NSCLC. This article focuses on clinical trials that provided support for the predictive role of immunohistochemistry and FISH EGFR analysis in NSCLC, provides discussion on the combination of immunohistochemistry and FISH data with other predictive factors, and highlights possibilities for future clinical studies exploiting immunohistochemistry and FISH as biomarkers of treatment benefit. There are other articles that focus on the prognostic and predictive value of mutations in the EGFR gene, and the reader is referred to them for more details.

Clinical Markers for Treatment Benefit

Several patient characteristics associate with increased responsiveness to EGFR TKIs, as shown in clinical studies with gefitinib and erlotinib: “never smoking” history, Asian ethnicity, female gender, and adenocarcinoma histology (5–7). The response rates of these patient categories to single-agent EGFR TKI therapy in large clinical trials in refractory advanced NSCLC vary between 13% and 25%. However, all above characteristics, except Asian ethnicity, are linked to favorable clinical prognosis regardless of the use of EGFR TKIs (8). In a subgroup analysis of the BR.21 placebo-controlled trial, which evaluated erlotinib in patients who failed first-line or second-line chemotherapy and led to the registration of erlotinib, survival benefit was observed regardless of gender [hazard ratio (HR) of death, 0.8 in both females and males], cancer histology (HR, 0.7 in patients with adenocarcinoma versus 0.8 in other histologic types), and ethnicity [HR, 0.6 in Asians versus 0.8 in non-Asians (5)]. Negative smoking history was the only factor that predicted greater survival benefit in this trial (HR, 0.4 in never smokers versus 0.9 in ever smokers). The overall result of Iressa Survival Evaluation in Lung Cancer (ISEL) Study, a clinical trial comparing gefitinib to placebo in chemotherapy-pretreated NSCLC, did not show significant survival benefit with gefitinib (HR, 0.89). However, subsets of patients who never smoked and Asians derived significant benefit (9). In view of the above data, some investigators are testing the efficacy of EGFR TKIs in patient populations selected by clinical characteristics (e.g., never smokers), although this represents relatively small proportion of total population of patients.

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EGFR Immunohistochemistry

High EGFR protein expression determined by immunohistochemistry is observed in the majority of squamous cell carcinomas and a smaller majority of large cell and adenocarcinomas and is also often seen in bronchial preneoplastic lesions, implicating its significance in lung carcinogenesis (10, 11). Systematic review of several investigations on the prognostic significance of EGFR expression revealed no association with survival, which was in accordance with our findings (10, 12). However, other studies have reported high EGFR expression to be an adverse prognostic factor in NSCLC (13, 14).

Early studies evaluating the predictive role of immunohistochemistry for response and survival benefit to EGFR TKIs showed no value of EGFR immunohistochemistry. The biomarker analysis of two phase II gefitinib Iressa Dose Evaluation in Lung Cancer (IDEAL) studies was done with the DAKO Cytomation EGFR PharmDx (DAKO Corp., Glostrup, Denmark) assay and reported as quantitative assessment, taking into account both percentage of positive cells and staining intensity (15). No association was found between staining intensity and response or symptom improvement, but survival data were not reported. EGFR immunohistochemistry analysis from 516 patients participating in two phase III trials evaluating addition of gefitinib to first-line chemotherapy (INTACT 1 and 2, Iressa NSCLC Trials Assessing Combination Treatment) also did not reveal any predictive value of EGFR immunostaining for treatment benefit (16). In contrast to the above, Cappuzzo et al. reported on 102 gefitinib-treated patients evaluated for EGFR immunostaining by Zymed antibody (Zymed Laboratories, San Francisco, CA) using the “Colorado scoring system,” based on the fraction of positive cells times staining intensity (Table 1; ref. 3). The cutoff level for positive immunostaining was 200 on a scale of 0 to 400, indicating that immunohistochemistry-positive tumors show strong EGFR expression, whereas immunohistochemistry-negative tumors have low or no expression. Positive EGFR immunostaining, observed in 59% of patients, was associated with a significantly increased response rate (21% versus 5%), median progression-free survival (5.2 versus 2.3 months), and overall survival (11.5 versus 5.0 months). In multivariate analysis, EGFR protein expression remained associated with overall survival (HR, 0.60; 95% confidence interval [95% CI], 0.36-1.01). An evaluation of a subgroup of 325 patients participating in the BR.21 study confirmed the value of EGFR immunohistochemistry to predict treatment outcome (2). Positive EGFR immunostaining with DAKO antibody could not well discriminate responding from nonresponding patients (11% response rate in EGFR-positive versus 4% in EGFR-negative patients) but associated with better survival (HR, 0.68 versus 0.93, respectively). Discordant results among the reported studies may be due to different methodologies, different study populations, and different efficacies of gefitinib versus erlotinib. The cutoff value for EGFR-positive immunostaining in the Colorado studies is set on a continuous scale to discriminate between strong or moderate versus weak or no staining using Zymed antibody (3, 10), whereas many other studies with DAKO antibody select different cut points (2, 15, 16). The optimal cut point remains to be established and validated in larger series of patients participating in prospective studies.

Low EGFR protein expression may also be considered as valuable information on whom we should not treat with EGFR TKIs. In two large clinical monotherapy studies with erlotinib and gefitinib, patients with negative EGFR immunohistochemistry did not derive survival benefit from EGFR TKIs (HR, 0.93; 95% CI, 0.63-1.36 for erlotinib versus placebo in a subset of EGFR immunohistochemistry-negative patients (2); similar results were presented for gefitinib-treated patients in the ISEL trial; ref. 17). Both studies used DAKO EGFR PharmDx and classified tumors as positive when >10% of cells showed membranous staining of any intensity.

EGFR, HER-2, and HER-3 Gene Copy Numbers by FISH

High EGFR gene copy number or gene amplification is noted by FISH in 22% to 45% of NSCLC patients (2–4, 10, 17). A positive correlation between EGFR protein expression and high EGFR gene copy number was observed in a study of 183 surgically treated patients (10). Both abnormalities carried no significant prognostic information in this report, but a tendency of shorter survival was noted for patients with high EGFR copy number. The study of Cappuzzo et al. (3) was the first to show that high EGFR copy number (high polysomy and gene amplification evaluated by FISH) correlated significantly with improved survival in univariate and multivariate analysis of patients treated with gefitinib (Table 1). FISH results were classified according to six categories with ascending number of gene copies per cell: disomy, low trisomy, high trisomy, low polysomy, high polysomy, and gene amplification. The two latter categories were considered as FISH positive, whereas all the other categories were classified as FISH negative.

The results of three other studies confirmed that FISH-positive patients have prolonged survival after treatment with EGFR TKIs. Hirsch et al. analyzed 81 samples from 137 eligible participants in Southwest Oncology Group S0126, a phase II study evaluating the effectiveness of gefitinib in bronchoalveolar carcinoma and adenocarcinoma with bronchoalveolar carcinoma features (4). Objective response and disease control rates were 26% and 63% in the EGFR FISH-positive group compared with 11% and 39%, respectively, in the FISH-negative group. The median survival was significantly better in EGFR FISH-positive group compared with negative patients with a HR of 0.50 (95% CI, 0.25-0.97). HER-2 copy numbers were not associated with response or survival in this study. In the BR.21 phase III clinical trial, data on the subgroup of 125 patients, in whom FISH analysis was successful, showed a survival difference favoring erlotinib confined to the EGFR FISH-positive patients (HR, 0.44; 95% CI, 0.23-0.82) as contrasted to EGFR FISH-negative patients (HR, 0.85; 95% CI, 0.48-1.51; ref. 2). In a subset analysis of the ISEL trial, FISH-positive gefitinib-treated patients had median survival of 8.3 months compared with 4.3 months for patients receiving placebo (HR, 0.61; 95%CI, 0.36-1.04; ref. 17).

High gene copy number of HER-2, as assessed by FISH, might be important for sensitivity to gefitinib in NSCLC. In a report by Cappuzzo et al., high HER-2 copy number, present in 22% of gefitinib-treated patients, was associated with
significantly better response rate, time to progression, and a trend for improved survival (Table 1; ref. 18). This study showed that increased HER-2 copy number correlates with increased EGFR copy number, and patients with both abnormalities have the highest response rates and the longest survival.

In vitro studies suggest that high expression of HER-3 also affects gefitinib sensitivity (19). Cappuzzo et al. evaluated the predictive value of HER-3 gene copy number by FISH in a subset of 82 patients previously characterized for other abnormalities of EGFR pathway (Table 1; ref. 20). HER-3 FISH-positive patients (27%) had significantly higher response rate (36% versus 10%), time-to-progression (7.7 versus 2.7 months), but not overall survival (10 versus 11 months).

**EGFR Gene Copy Number by Quantitative Real-time PCR**

Bell et al. reported recently on the predictive value of EGFR copy number, evaluated by quantitative real-time PCR (qPCR), and EGFR mutations in a series of NSCLC patients treated with single-agent gefitinib in the phase II IDEAL trials and gefitinib in combination with chemotherapy in the INTACT studies (Table 1; ref. 21). EGFR amplification (defined as more than four copies of EGFR relative to the housekeeping gene) was found in 7 of 90 (8%) patients in the IDEAL and 33 of 453 (7%) patients in the INTACT studies. In the latter trials, subset of patients with amplified EGFR did not benefit from the addition of gefitinib to chemotherapy (HR, 2.03; 95% CI, 0.67-6.13). Takano et al. reported that high EGFR gene copy number evaluated by qPCR correlated with EGFR mutations and predicted for time to progression but not overall survival benefit in a group of 66 gefitinib-treated patients, whereas EGFR mutations were associated with improved survival (22). In our study of 82 gefitinib-treated NSCLC patients, EGFR gene copy number evaluated by qPCR did not correlate with EGFR FISH data and did not predict for response, progression-free nor overall survival (23).

It should be noted that FISH and qPCR methods for EGFR gene copy number assessment differ substantially. The former

![Table 1. Predictive value of EGFR immunohistochemistry, EGFR, HER-2, and HER-3 gene copy numbers for response and survival in advanced NSCLC treated with EGFR TKIs](https://www.aacrjournals.org)
method allows for direct determination of gene copy number in single tumor cells and is available in many clinical laboratories. Determination of gene copy number by qPCR (referred also as gene dosage) is relatively fast and inexpensive. However, the result is relative to a housekeeping gene, making interstudy comparisons and cutoff determinations difficult. It also remains unknown if reference gene copy number is disomic and not amplified or deleted in tumor cells. Quantification of gene copy number in tumor cells may be affected by the proportion of disomic inflammatory and tumor stroma cells if tumor microdissection is not done.

Combination of Immunohistochemistry, FISH, and Other Predictive Factors

Data on the predictive value of combined EGFR immunohistochemistry and FISH assessment are relatively sparse, due to the limited number of patients analyzed for these abnormalities in currently available studies. Combined analysis of an Italian cohort of gefitinib-treated patients and the Southwest Oncology Group S0126 study was presented by Hirsch et al. at the 11th World Conference on Lung Cancer (24). Patients with both tests positive had excellent response rate and prolonged survival, whereas patients with both tests negative had dismal outcome. This study suggests that patients with negative EGFR immunohistochemistry and FISH results (who represented ~30% of study population) do not derive any clinical benefit from EGFR TKIs. Multivariate analysis of the BR.21 study is unfortunately inconclusive due to the limited number of evaluable patients with available biomarker results (2).

A detailed summary of predictive information carried by EGFR mutations is not within the scope of this report. It should be noted, however, that EGFR mutations have been linked to increased response rate and prolonged survival of patients treated with EGFR TKIs in several studies, with survival benefit mainly in Asian populations. EGFR mutations associate with EGFR copy number both in vitro and in clinical data sets (3, 22, 25), and long-lasting responses had been reported in EGFR-mutant patients (26, 27). In the study of Cappuzzo et al., patients harboring EGFR mutations had prolonged survival (21 versus 8 months); the difference was, however, not statistically significant (3). A recent study indicated that patients with exon 19 deletions have longer survival compared with patients with exon 21 point mutations (28). Table 2 shows the available data on predictive significance of EGFR protein expression, EGFR copy number by FISH, and EGFR mutations in large randomized placebo-controlled trials with a brief explanation of the trial design. The value of EGFR mutations as a marker for patient selection to treatment with EGFR TKIs remains unclear. As contrasted to the data indicating significant and clinically meaningful improvement in survival for FISH-positive patients, no randomized trial has shown the predictive value of EGFR mutations for survival benefit in patients treated with EGFR TKIs. There might be several explanations why EGFR mutations did not predict survival benefit from EGFR TKIs compared with placebo in these studies. The prevalence of EGFR

<table>
<thead>
<tr>
<th>Author (reference)</th>
<th>Study description</th>
<th>EGFR protein, percentage positive; HRs in positive vs. negative subsets</th>
<th>EGFR copy number by FISH, percentage positive; HRs in positive vs. negative subsets</th>
<th>EGFR mutations, percentage positive; HRs in positive vs. negative subsets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsao (2)</td>
<td>BR.21, phase III trial comparing erlotinib to placebo in second or third-line treatment; trial result positive</td>
<td>57%; 0.68 vs. 0.93</td>
<td>45%; 0.44 vs. 0.85</td>
<td>22.6%; 0.77 vs. 0.73</td>
</tr>
<tr>
<td>Hirsch (17) and Holloway (31)</td>
<td>ISEL, phase III trial comparing gefitinib to placebo in second or third-line treatment; trial result negative</td>
<td>68%; NR</td>
<td>31%; 0.61 vs. 1.14</td>
<td>12%; NR*</td>
</tr>
<tr>
<td>Bell (21) and Bailey (16)</td>
<td>INTACT1 and 2, phase III trials comparing addition of gefitinib or placebo to chemotherapy in first-line treatment; trials results negative</td>
<td>NS</td>
<td>ND</td>
<td>10%; 1.77 vs. 0.91</td>
</tr>
<tr>
<td>Eberhard (30) and Herbst (35)</td>
<td>TRIBUTE, phase III trial comparing addition of erlotinib or placebo to chemotherapy in first-line treatment; trial result negative</td>
<td>NR; 1.00 vs. 1.02</td>
<td>ND</td>
<td>13%; NS</td>
</tr>
</tbody>
</table>

NOTE: HR comparing EGFR TKI versus placebo. Abbreviations: ND, not done; NS, not significant (exact value(s) not reported). *Not reported due to too few events.
mutations are included in a larger FISH-positive subset (31). Not available from the above studies, but the molecular analysis into those with mutations and those with wild-type EGFR are treated with chemotherapy alone; refs. 21, 30).

The exact data breaking down EGFR FISH-positive patients into those with mutations and those with wild-type EGFR are not available from the above studies, but the molecular analysis of the ISEL study indicates that most patients with EGFR mutations are included in a larger FISH-positive subset (31).

The predictive value of EGFR mutations may be different in East Asia, where EGFR mutations are more prevalent, response rates seem higher, and improved survival was consistently reported in retrospective studies (22, 32–34). Unfortunately, there are no data from placebo-controlled trials in these populations to derive any meaningful conclusion on the clinical value of mutations and on combination of these biomarkers.

Current and Future Clinical Trials Exploiting Biomarkers to Select Patients for Treatment with EGFR TKIs

Consistent findings from independent institutions and from prospective placebo-controlled studies indicate improved survival from EGFR TKIs in NSCLC patients with tumors characterized by high EGFR copy number detected by FISH, supporting further validation of this marker in trials with enriched populations. The strategy of selecting patients for EGFR TKI therapy based on a positive FISH result is very similar to the successful strategy selecting breast cancer patients for treatment with trastuzumab, a monoclonal antibody against HER-2. In the United States, there is an ongoing randomized phase II study comparing erlotinib alone to chemotherapy alternating with erlotinib in untreated advanced NSCLC patients who are FISH positive or immunohistochemistry positive. A randomized trial of erlotinib or placebo after surgery and adjuvant chemotherapy in patients selected by FISH and immunohistochemistry is planned. The evidence to select patients based solely on EGFR immunohistochemistry is less clear, although combination of EGFR gene copy number by FISH and immunohistochemistry seems promising. A phase II trial of gefitinib in EGFR mutation-positive patients has been conducted in Japan, and other studies may be contemplated, although there is no evidence from placebo-controlled trials to support the selection of patients based on EGFR mutations.

Open Discussion

Dr. Thomas Lynch: We have seen different data from different groups, and some of the differences may be related to methodologic issues. When we talk about immunohistochemistry, is everyone using the same techniques for determining what are considered to be IHC-positive cells?

Dr. Bunn: The answer is that there are completely different methodologic approaches. First, there are different antibodies, but I don’t think the antibody matters. I think the definition does matter. These cells can be scored in two ways once they are stained. One way is to score the intensity as 0, 1+, 2+, 3+. Another way is to score the fraction of cells that are positive, 0 to 100. A third approach, which is what we have done, is to multiply those two together, intensity times the percent positive. There, you get a numeric score of 0 to 400. Using a cutoff of 200, our pathologists found 61% of the patients were positive. I would submit that, at the moment, nobody knows what is the best test and the best cutoff.

Dr. Lynch: In the study that Daphne Bell is publishing (J Clin Oncol, 2005), she did not find a relationship between amplification and IHC positivity. That was defined as the fraction of cells that was positive, correct?

Dr. Daniel Haber: Pathologists typically use the percentage of cells positive, but if there were a reliable way of quantifying the amount of expression per cell, that would make more sense in correlating with gene amplification. However, quantifying the degree of staining or extent of staining by IHC is very difficult because you need standards for each assay.

Dr. Lynch: Dr. Bunn, did you find a relationship between the degree of amplification and the intensity of immunohistochemical staining in your samples?

Dr. Bunn: There is a significant correlation between amplification by FISH and protein by IHC, but in a multivariable analysis, they are independent. In our experience, 61% of patients are EGFR protein positive, but only a third are FISH positive. FISH is different from quantitative PCR. There are positive patients in one and not the other. It still remains to be determined which is a better test. For whatever reason, in our hands, FISH is a better test with correlating with the clinical outcome than quantitative PCR.

Dr. Haber: When you define a FISH-positive tumor, what fraction of cells are FISH positive for specific EGFR amplification versus those that may be aneuploid? How do you deal with tumor heterogeneity?

Dr. Bunn: The good news is that different groups have gotten the same result in six different cohorts now. For high polysomy, you have to have four copies in more than 40% of the cells. So, even in high polysomy, there may be plenty of diploid cells.

Dr. Matthew Meyerson: I’m curious about the reproducibility of the distinction between high polysomy and low polysomy. If you take different sections and have it measured by different observers, what is the precision of that distinction?

Dr. Bunn: We haven’t done a formal study of that, and it needs to be done. When Dr. Cappuzzo was at our institution, all of the specimens were read by Dr. Varella-Garcia and Dr. Cappuzzo. When they disagreed, they got together and discussed it. The rate of agreement was very high, it was over 90%. But we have not done that scientifically so that I can give you an accurate number.

Dr. Rogerio Lilenbaum: Assuming that IHC and FISH will have the importance that we hope in terms of selecting patients and therapies, how does the situation compare to breast cancer and how they establish HER2 status? They have been dealing with this for over a decade. From a clinician’s perspective, is the
EGFR inhibitors?

Dr. Bunn: No, they are identical in technology, differing only in the specific process. I think the results are also similar for their ability to select patients. I think the biology is very similar, and clinically, I believe that is what we are all going to be doing 2 or 3 years from now. Every patient going on a trial in our institution has these tests done, but we don’t test routinely at the moment. Until we have prospective trials, I’m not sure that we should be doing this testing clinically.

Dr. Meyerson: Are any large-scale trials being planned to validate different diagnostic approaches and to see if any of them can determine who would be the best patients to receive EGFR inhibitors?

Dr. Lynch: Yes, actually, those trials are either about to all from retrospective studies involving a fraction of the patients on trial. As Dr. Haber pointed out this morning, we have to consider what might be the bias in terms of which subset of patients you have tissue for. So it really is critical to do this prospectively.

Dr. Panos Fidias: So on the one hand we have to do trials, but at the same time we have to treat people. For me, the difficulty has been to say, what do these studies add to day-to-day decision-making? The clear thing that has emerged is the importance of smoking status. Smoking status is much more important than female sex or adenocarcinoma or BAC histology in predicting response to an EGFR TKI. In my mind, the main importance in decision-making for knowing EGFR mutation or amplification would be for those patients who do not have clinical characteristics. So, for those patients who were double positive, do you know their smoking status?

Dr. Bunn: The question in my mind isn’t who to treat, it is who not to treat. If the patient has the mutation or is a never smoker, I have no doubt that patient should be treated with an EGFR inhibitor. But those are 10% of the patients. There are many more patients than that who are going to benefit from these drugs, and there are many more patients than that who are going to have better survival with these pills than they are going to get with triple-agent chemotherapy. So the clinical question is, which patients should not get a pill first. I would argue that it is the double-negative patients for protein by IHC and copy number by FISH.

Dr. Fidias: Even if they are nonsmokers?

Dr. Bunn: Even if they are nonsmokers. There is a statistical correlation between never smoking and FISH positivity. There is a statistical correlation between never smoking and amplification. But in a multivariable analysis, because there are so many more FISH-positive patients, FISH comes up as an independent prognostic variable even when smoking status is in the equation.

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