Epidermal growth factor receptor gene copy number and protein level are not associated with outcome of non-small-cell lung cancer patients treated with chemotherapy

R. Dziadziozsko¹,², B. Holm³, B. G. Skov⁴, K. Osterlind⁵, M. V. Sellers⁶, W. A. Franklin¹, P. A. Bunn Jr¹, M. Varella-Garcia¹ & F. R. Hirsch¹*

¹University of Colorado Health Sciences Center, Aurora, CO, USA; ²Medical University of Gdańsk, Gdańsk, Poland; ³Herlev University Hospital, Herlev; ⁴Gentofte University Hospital, Copenhagen; ⁵Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark; ⁶AstraZeneca, Macclesfield, UK

Received 5 July 2006; revised 21 September 2006; accepted 25 September 2006

Background: Survival benefit of non-small-cell lung cancer (NSCLC) patients treated with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors is predicted by high EGFR gene copy number and by strong EGFR protein expression. Clinical relevance of these features in patients treated with chemotherapy has not been reported.

Patients and methods: This study included 82 NSCLC patients treated with chemotherapy. There were 45% of females, 6% of never smokers and 45% of patients diagnosed with adenocarcinoma. EGFR gene copy number was evaluated by fluorescence in situ hybridization and EGFR protein level by immunohistochemistry.

Results: High EGFR gene copy number and protein level were found in 33% and 71% of patients, respectively. Both markers were significantly associated (P = 0.01). For objective response and disease control, there was no difference between patients defined as negative or positive for both EGFR gene copy number (P = 0.39 and P = 1.00, respectively) and for EGFR protein (P = 1.00 and P = 0.80, respectively). There were no differences in progression-free and overall survival according to EGFR gene copy number (P = 0.76 and P = 0.92, respectively) and protein level (P = 0.67 and P = 0.62, respectively).

Conclusion: In chemotherapy-treated NSCLC patients, EGFR gene copy number was positively associated with protein level but none of the features were predictive for either treatment response or survival.

Key words: epidermal growth factor receptor, fluorescence in situ hybridization, gene copy number, immunohistochemistry, lung cancer, protein

Introduction

Novel emerging compounds in the treatment of non-small-cell lung cancer (NSCLC) include epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs, gefitinib and erlotinib) and monoclonal antibodies against EGFR (cetuximab) [1]. Many other agents targeting EGFR and other signaling pathways are in preclinical or clinical development in lung cancer [2]. In the pivotal phase III BR.21 study testing erlotinib versus placebo in the second- or third-line treatment of advanced NSCLC, median survival in erlotinib-treated patients was significantly longer than in patients receiving placebo [3]. Treatment benefit was particularly pronounced in never smokers and in patients of Asian origin. In the Iressa Survival Evaluation in Lung Cancer (ISEL) clinical trial exploring the efficacy of gefitinib compared with placebo in the second- or third-line treatment of advanced NSCLC, the overall reduction in the risk of death was not significant, although subsets of never-smoking and Asian patients had a significant survival benefit [4]. Although clinical predictive markers of prolonged survival may be used to identify the patients most likely to benefit from EGFR TKIs, they only identify a fraction of these patients. Reduction in the risk of death was also observed in the subsets of patients with very low response rates to erlotinib—e.g. smoking men with squamous cell histology [5].

Biomarkers that could be used to select patients most likely to benefit from EGFR-targeted therapy include activating EGFR mutations, high EGFR gene copy number, high EGFR and phospho-Akt protein levels and absence of Kirsten sarcoma virus (K-ras) mutations. EGFR mutations are associated with high response rates to EGFR TKIs irrespective of the EGFR TKI and ethnicity, and are prognostic for a favorable outcome irrespective of therapy. EGFR mutations are more frequent in individuals from East Asia and have been associated with increased survival in these patients. Survival prediction, however, was not apparent in the phase II studies of North American and European patients [6, 7] and in the randomized BR.21 study [8]. The low prevalence of EGFR mutations in Western NSCLC populations and its positive prognostic value...
regardless of treatment with EGFR TKIs are limiting its practical use as a selection factor, and further studies are needed to define the role of particular types of mutations for survival benefit from EGFR TKIs [9].

High EGFR gene copy number, evaluated by fluorescence in situ hybridization (FISH), was associated with increased responsiveness and prolonged survival to EGFR TKIs in both phase II single-arm studies [6, 10] and both randomized phase III trials comparing placebo with erlotinib or gefitinib in NSCLC patients [8, 11]. The proportion of patients who are EGFR FISH positive (high polysomy or gene amplification) ranged from 32% to 45% [8, 10, 11]. The relative reduction in the risk of death of FISH-positive patients treated with erlotinib or gefitinib compared with placebo was 56% and 39% in two phase III trials, respectively [8, 11]. The prognostic relevance of high EGFR gene copy number determined by FISH is less well studied. We reported that early-stage NSCLC patients with high EGFR gene copy numbers treated by surgery had a slightly worse survival than patients with low EGFR gene copy numbers [12]. There are no reports of the prognostic or predictive relevance of EGFR gene copy number in advanced NSCLC patients treated with chemotherapy. EGFR gene copy number by FISH, either alone or in combination with other biomarkers, is now explored as a selection marker in prospective clinical trials. In the present report, we evaluated the predictive/prognostic significance of EGFR gene copy number by FISH and EGFR protein expression by immunohistochemistry (IHC) in NSCLC patients treated with chemotherapy, using methodology that was demonstrated to be clinically useful to predict survival benefit from EGFR TKIs.

The presence of K-ras mutations in tumors is associated with decreased sensitivity to EGFR TKIs in the retrospective [13] and prospective [14] clinical studies with EGFR TKIs. High levels of activated Akt, measured with antibodies against the phosphorylated form of this protein, are indicative of signaling through antiapoptotic pathways [15] and have been demonstrated to associate with gefitinib sensitivity in one study [16] but not confirmed by other data [11].

patients and methods

Records and paraffin-embedded tumor samples of 82 consecutive NSCLC patients treated by combination chemotherapy at Herlev University Hospital (Herlev, Denmark) were identified and retrieved for the purpose of this study. Inclusion criteria included no history of other malignancies, no treatment with EGFR TKIs and adequate amount of tumor cells as evaluated by the pathologist before commencement of the study. The study was approved by the Danish ethical committee on research involving human subjects.

The primary objectives of this study were to determine whether EGFR gene copy number evaluated by FISH and EGFR protein expression evaluated by IHC in samples of consecutive NSCLC patients treated with chemotherapy associated with progression-free survival (PFS) or overall survival (OS). All tumor samples were collected for routine histopathological diagnosis before commencement of chemotherapy.

EGFR gene copy number assessment by FISH

EGFR gene copy number was determined according to our previous reports [6, 10]. Commercially available LSI EGFR SpectrumOrange/centromere DNA probe (CEP) 7 SpectrumGreen probes were used (Vysis/Abbott Molecular, Downers Grove IL). Specimens containing high polysomy (more than four copies of the gene in 240% of the cells) or gene amplification (EGFR gene clusters, ratio of EGFR/CEP 7 ≥ 2, or >15 EGFR signals in ≥100% of cells) were scored as FISH positive, whereas all other specimens were scored as FISH negative.

EGFR protein expression assessment by IHC

Evaluation of EGFR protein expression was carried out as described previously using Dako EGFR PharmDx kit (DAKO, Glostrup, Denmark). Consistent with the molecular analysis of the phase III clinical trial BR.21 with erlotinib [8] and the ISEL trial with gefitinib [11], a cut off point of >10% of stained cells was used to define IHC positivity.

statistical analysis

Response evaluation was carried out according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria [17]. Computed tomography examinations were carried out every three cycles of chemotherapy in all patients and every 3 months during follow-up according to the guidelines for evaluation and treatment of advanced NSCLC in the Danish hospitals participating in this study. Disease control was defined as stable disease (less than a 30% reduction and less than a 20% increase in the sum of the longest diameter of target lesions and the appearance of no new lesions) during at least two subsequent evaluations after the start of chemotherapy, without clinical progression on physical examinations. Categorical variables were compared using the chi-square or Fisher’s exact test. Continuous variables were compared using the Mann–Whitney U test. Survival analysis was carried out by the Kaplan–Meier method and survival curves were compared using a log-rank test. Median follow-up time was calculated according to the method proposed by Schepner and Smith [18]. PFS was measured from day 1 of the first-line chemotherapy to the diagnosis of progressive disease or to death without progression. OS was measured from the first day of chemotherapy to the date of death of any cause, or the date of last follow-up. Univariate Cox proportional hazards model was used to calculate univariate hazard ratios and their 95% confidence intervals (95% Cls). Additionally, other known prognostic factors (sex, performance status (PS) 0–1 versus 2 and stage III versus other) were included in multivariate model to calculate if adjusted hazard ratios would change these results. Significance level of 5% was used for hypothesis testing. Calculations were carried out using statistical software SPSS 13.0 (SPSS, Chicago, IL).

results

patient characteristics

The study population consisted of 45 males (55%) and 37 females (45%), 29 patients had World Health Organization PS0 (35%), 43 patients PS1 (52%) and 10 patients PS2 (12%). Median age was 61 years (range: 40–79 years) and five patients were never smokers (6%). Histological types were adenocarcinoma (37 patients, 45%), squamous cell or large cell carcinoma (39 patients, 48%), mixed histologies (two patients, 2%) and NSCLC not otherwise specified (four patients, 5%). There were six patients in clinical stage I–IIA (7%), 35 patients in stage IIIB (43%) and 37 patients in stage IV (45%); the stage was unknown for four patients (5%). First-line chemotherapy regimens were gemcitabine/carboplatin (81 patients, 99%) and docetaxel/carboplatin (one patient, 1%). Seventy-five patients (91%) were treated with palliative intent (most stage III patients were not candidates for combined modality treatment due to extent of disease, significant comorbid diseases or malignant pleural effusion). Three patients (4%) were given chemotherapy after nonradical surgery and four patients (5%) received chemotherapy as part of the induction protocol before definite radiotherapy or surgery. The treatment was completed
according to protocol in 40 patients (49%). In the remaining patients, chemotherapy was terminated due to clinical or radiological signs of progression (22 patients, 27%), toxicity (10 patients, 12%) or deterioration of PS/early death (10 patients, 12%). Fourteen patients (17%) received second-line chemotherapy (most frequently docetaxel) and two patients (2%) received docetaxel as third-line treatment. At the time of analysis, 61 patients have died (74%) and the median follow-up was 19 months. Median survival in the analyzed group was 8.2 months (95% CI 5.4–11.0 months). Known prognostic factors, PS and stage, were significantly associated with survival (log-rank test, \( P < 0.001 \) for both features).

**EGFR gene copy number and EGFR protein expression**

EGFR gene copy number was successfully analyzed by FISH in 76 samples (93%), EGFR amplification was observed in nine patients (12%) and high polysomy in 18 patients (24%), accounting for 27 patients classed as FISH positive (36%). There were no differences in FISH positivity according to smoking history, gender, PS, age, histology and stage (Table 1). EGFR protein level was successfully analyzed in 78 patients (95%) and 58 patients (71%) were positive. Positive immunostaining was more common in nonadenocarcinomas (89%) as compared with adenocarcinomas (65%, \( P = 0.04 \)). No associations with other clinical characteristics were observed (Table 1). There was a significant association between EGFR FISH and IHC positivity (Fisher’s exact test \( P = 0.01 \)). FISH+/IHC− combination was found in 21%, FISH−/IHC+ in 43%, FISH+/IHC− in 3% and FISH+/IHC+ in 33% of patients.

**EGFR gene copy number, EGFR protein level and treatment outcome**

There were no differences in objective response or disease control rates according to EGFR gene copy number or protein level (Table 2).

There were no differences in PFS or OS according to EGFR gene copy number (log-rank test, \( P = 0.76 \) and \( P = 0.82 \), respectively, Table 2 and Figure 1). Univariate hazard ratio estimates for progression and death in FISH-positive versus FISH-negative patients were 0.92 (95% CI 0.55–1.54) and 0.94 (95% CI 0.54–1.62), respectively.

Protein EGFR levels were not associated with PFS or OS (log-rank test, \( P = 0.67 \) and \( P = 0.62 \), respectively, Figure 2). Univariate hazard ratio estimates in EGFR IHC-positive versus -negative patients were 1.13 (95% CI 0.64–1.99) and 1.16 (95% CI 0.64–2.13), respectively.

There were no significant differences in PFS or OS for patients with both positive FISH and IHC, patients with one positive test or patients with both negative tests (log-rank test, \( P = 0.67 \) and \( P = 0.94 \), respectively; data not shown). In multivariate Cox regression models adjusted for other known prognostic factors in NSCLC (gender, PS 0–1 versus 2 and stage IV versus other), hazard ratio estimates for EGFR gene copy number and protein expression remained insignificant in both OS and PFS analyses (data not shown).

**discussion**

The optimal method of patient selection for treatment with EGFR TKIs remains a subject of intensive discussion.

### Table 1. Patient characteristics according to EGFR gene copy number and EGFR protein levels

<table>
<thead>
<tr>
<th>Smoking history</th>
<th>( P )</th>
<th>EGFR protein level</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or past smoker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Performance status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer.

*Chi-square or Fisher’s exact test.

**Mann–Whitney \( U \) test.
EGFR gene copy number by FISH, protein expression by IHC and EGFR tyrosine kinase mutations are all potential markers to be used as selection criteria in clinical practice. Two large randomized placebo-controlled studies comparing EGFR TKIs with placebo confirmed the results of single-arm studies showing the predictive value of EGFR FISH and IHC for survival benefit in advanced NSCLC [8, 11]. There are no previous studies exploring the relationship between EGFR gene copy

Table 2. Response rates, disease control rates, univariate PFS and OS analysis according to EGFR gene copy number and EGFR protein level

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>E</th>
<th>Response rate (%)</th>
<th>P*</th>
<th>Disease control rate (%)</th>
<th>P*</th>
<th>OS median (95% CI) (months)</th>
<th>P**</th>
<th>PFS median (95% CI) (months)</th>
<th>P**</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR FISH negative</td>
<td>49</td>
<td>36/41</td>
<td>27</td>
<td>0.39</td>
<td>49</td>
<td>1.00</td>
<td>8.2 (5.8–10.6)</td>
<td>0.82</td>
<td>5.2 (4.1–6.3)</td>
<td>0.76</td>
</tr>
<tr>
<td>EGFR FISH positive</td>
<td>27*</td>
<td>21/23</td>
<td>15</td>
<td>0.60</td>
<td>50</td>
<td>1.00</td>
<td>12.4 (6.2–18.7)</td>
<td>0.82</td>
<td>7.5 (2.4–12.5)</td>
<td>0.67</td>
</tr>
<tr>
<td>EGFR protein negative</td>
<td>20</td>
<td>15/16</td>
<td>20</td>
<td>1.00</td>
<td>45</td>
<td>0.80</td>
<td>7.3 (2.2–12.4)</td>
<td>0.62</td>
<td>5.2 (3.6–6.9)</td>
<td>0.67</td>
</tr>
<tr>
<td>EGFR protein positive</td>
<td>58b</td>
<td>44/50</td>
<td>21</td>
<td>1.00</td>
<td>49</td>
<td>0.80</td>
<td>9.1 (6.6–11.7)</td>
<td>0.62</td>
<td>5.3 (2.2–8.4)</td>
<td>0.67</td>
</tr>
</tbody>
</table>

PFS, progression-free survival; OS, overall survival; EGFR, epidermal growth factor receptor; CI, confidence interval; FISH, fluorescence in situ hybridization; N, number of patients; E, number of events for OS/PFS.

* Twenty-six patients were assessable for response.

** Fifty-seven patients were assessable for response.

**Fisher’s exact test.

** Log-rank test.

Figure 1. Progression-free (A) and overall survival (B) according to EGFR gene copy number by fluorescence in situ hybridization (FISH).

Figure 2. Progression-free (A) and overall survival (B) according to EGFR protein level by immunohistochemistry (IHC).
number, protein expression and outcome of chemotherapy in advanced NSCLC using the methodology of biomarker evaluation employed in clinical trials with EGFR TKIs. Our report indicates that these biomarkers do not associate with response, PFS or OS of NSCLC patients treated with chemotherapy.

Unlike the lack of prognostic significance of high EGFR gene copy number, EGFR mutations are associated with better prognosis for advanced NSCLC patients [14, 19]. The predictive value of EGFR mutations for survival benefit from EGFR inhibitor therapy is debated. Prospective single-arm clinical studies with selection of NSCLC patients to gefitinib or erlotinib based on EGFR mutation status indicated impressive response rates and survival in the first-line setting [20, 21]. Molecular analysis of the BR.21, phase III clinical trial comparing erlotinib with placebo in chemotherapy-pretreated population of patients, failed to establish the differential effect of erlotinib versus placebo on survival in EGFR mutant and wild-type patients [8]. Update of this analysis taking into account ‘classical mutations’ (i.e. exon 19 deletions and exon 21 point mutations) did not change this conclusion [22]. In the ISEL study, there were too few mutations to allow for meaningful subset comparisons with regard to survival [11]. Thus, we currently do not know how much the superior survival of patients harboring EGFR mutations is attributed to the treatment with EGFR inhibitor or to the biology of the disease.

There is some evidence for the clinical use of EGFR IHC as a predictive marker for survival benefit in NSCLC patients treated with EGFR TKIs [8, 11], although the benefit in IHC-positive patients seems smaller compared with that observed in EGFR FISH-positive patients. Because this study demonstrated that EGFR protein level has no predictive significance in advanced NSCLC patients treated with chemotherapy, this marker may also be regarded as purely predictive for survival benefit from EGFR TKIs. Although the present study includes relatively small number of patients, hazard ratios are very close to one suggesting that survival differences according to FISH or IHC status are unlikely.

Data presented in this report are consistent with the molecular analysis of the ISEL trial [11]. In this placebo-controlled study, EGFR gene copy number was assessed by FISH according to the same method and scoring system as in the present report. Patients receiving placebo who were FISH positive had a nonsignificantly shorter survival (median of 4.5 months) as compared with survival of placebo-pretreated patients who were FISH negative (median of 6.2 months). EGFR gene copy number was analyzed by quantitative polymerase chain reaction (qPCR) in tumor samples from gefitinib-treated NSCLC patients who participated in the phase II IDEAL studies (testing the efficacy of two different doses of gefitinib) and the phase III INTACT studies (testing the addition of gefitinib to chemotherapy in advanced NSCLC) [19]. In survival analysis of the INTACT studies, patients with EGFR amplification had better prognosis regardless of treatment with gefitinib or placebo in addition to chemotherapy. The results from this trial, however, cannot be directly compared with the results from the current study due to different assessment methods of EGFR gene copy number. A direct comparison of EGFR copy number evaluation by FISH and by qPCR was carried out in 82 gefitinib-treated NSCLC patients and failed to establish a significant association of these two measurements [23].

The prognostic significance of EGFR protein expression has been addressed in numerous studies. Almost all of them have focused on surgically treated NSCLC patients. Two meta-analyses of these studies showed that EGFR is more frequently expressed in squamous cell carcinomas. One of the meta-analyses concluded that high EGFR protein level had significant impact on prognosis, whereas the other did not confirm this finding [24, 25]. No prognostic impact of EGFR IHC was observed in a previous study from our institution [13], while others have reported worse [26–28] or better prognosis for NSCLC patients with tumors demonstrating high EGFR levels by IHC [29, 30]. Both meta-analyses have also demonstrated significant heterogeneity among the studies, most likely due to the different antibodies used, staining protocols and the definitions of high EGFR expression.

In conclusion, we found no prognostic or predictive association to clinical outcome of EGFR gene copy number by FISH and EGFR protein expression by IHC in a cohort of NSCLC patients who were treated with chemotherapy. Our data support the previously published observation [8, 11] that the two markers are specifically predictors of treatment benefit if the treatment is EGFR inhibitor.

Acknowledgements

This work was supported by a research grant from AstraZeneca. RD is supported by a fellowship from the International Association for the Study of Lung Cancer.

References

7. Hirsch FR, McCoy J, Capuzzo F et al. FISH and immunohistochemistry can be used to select NSCLC patients, who will not benefit from gefitinib treatment. Lung Cancer 2005; 49 (Suppl 2): S38.