Epidermal growth factor receptor activating mutations in Spanish gefitinib-treated non-small-cell lung cancer patients


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Background: North American and Japanese non-small-cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR) activation via tyrosine kinase (TK) mutations respond dramatically to gefitinib treatment. To date, however, the frequency and effect of EGFR TK mutations have not been examined in European patients.

 Patients and methods: Eighty-three Spanish advanced NSCLC patients who had progressed after chemotherapy, were treated with compassionate use of gefitinib. Patients were selected on the basis of available tumor tissue. Tumor genomic DNA was retrieved from paraffin-embedded tissue obtained by laser capture microdissection. EGFR mutations in exons 19 and 21 were examined by direct sequencing.

Results: EGFR mutations were found in 10 of 83 (12%) of patients. All mutations were found in adenocarcinomas, more frequently in females ($P = 0.007$) and non-smokers ($P = 0.01$). Response was observed in 60% of patients with mutations and 8.8% of patients with wild-type EGFR ($P = 0.001$). Time to progression for patients with mutations was 12.3 months, compared with 3.6 months for patients with wild-type EGFR ($P = 0.002$). Median survival was 13 months for patients with mutations and 4.9 months for those with wild-type EGFR ($P = 0.02$).

Conclusions: EGFR TK mutational analysis is a novel predictive test for selecting lung adenocarcinoma patients for targeted therapy with EGFR TK inhibitors.

Key words: EGFR, gefitinib, mutations, NSCLC, predictive markers

Introduction

Although the majority of lung cancers are linked to environmental carcinogens such as tobacco smoke and environmental pollutants [1], it is lung cancers in non-smokers, especially adenocarcinomas, which are the most likely to respond to tyrosine kinase (TK) inhibitors targeting the epidermal growth factor receptor (EGFR) gene. These drugs achieve unpredictable but often spectacular ‘Lazarus responses’, regardless of the metastatic site or number of prior chemotherapy regimens [2–4]. Increased EGFR expression is common in lung cancers, but neither EGFR expression levels nor phosphorylation state correlates with response to gefitinib [2].

TKs are central regulators of signaling pathways that control differentiation, transcription, cell cycle progression, apoptosis, motility and invasion. Mutations in several genes have been identified in key regions of the TK domain in several cancers [5], paving the way for the identification of biomarkers for gefitinib sensitivity. The hypothesis that mutations in the EGFR TK domain may play a role in non-small-cell lung cancer (NSCLC) is supported by several lines of evidence. In three studies in NSCLC [6–8], mutations were identified in the EGFR TK domain, with the majority clustering within exons 19 and 21. Mutations were either in-frame deletions or amino acid substitutions clustered around the ATP binding pocket. Missense mutations changing leucine 858 to arginine (L858R) and leucine 861 to glutamine (L861Q), as
well as multiple deletions clustered in the region spanning codons 746 to 759, were found [6–8]. In a very small number of patients, other mutations have been found in exons 18 and 20, some of which were identified in patients who did not respond to gefitinib. Duplication mutations in exon 20 have been described in Taiwanese patients [9]. Another recent study [10] examined 38 patients, of whom 21 were treated with gefitinib; a point mutation was found in exon 18 in only one case, while 19 cases had a deletion in exon 19, and 18 cases had a point mutation in exon 21. No mutations were found in exon 20. EGFR TK mutations represent bona fide somatic mutations in NSCLC and have not been identified in other primary tumors such as breast, colon, kidney, pancreas and brain, or in 108 cancer cell lines [6]. Mutations were found more frequently in women, adenocarcinomas and Japanese patients [7]. However, EGFR mutations were found in 11 of 96 (12%) primary NSCLCs resected from untreated patients, none of whom were East Asians [8]. Fourteen of 182 (8%) primary lung cancers in the United States harbored EGFR mutations [6–8]. The NSCLC H3255 cell line, harboring the L858R mutation, was 50-fold more sensitive to gefitinib than other adenocarcinoma cell lines containing wild-type EGFR TK [7]. Accumulated data of the three studies [6–8] show that 25 of 31 (81%) tumors from patients having partial responses or marked clinical improvement while taking gefitinib or erlotinib contained mutations in the EGFR TK domain. In contrast, none of 29 specimens from patients refractory to gefitinib or erlotinib had such mutations. The frequency of EGFR mutations across different populations and the robustness of the correlation between the mutations and clinical benefit [11] prompted us to examine the presence of EGFR TK mutations in exons 19 and 21 and their association with clinical outcome in chemoresistant advanced NSCLC patients who received gefitinib in a compassionate-use program in Spain.

Patients and methods

Patients

From October 2001 to June 2004, 220 patients with previously treated NSCLC received gefitinib, based on the attending oncologist’s decision at the time of chemotherapy failure, at a daily dose of 250 mg administered until disease progression, as part of a compassionate-use program. Eighty-three patients were selected for the present study based on the availability of tumor tissue. Acquisition of tissue specimens and examination of clinical records were approved by the ethical committees of participating institutions.

Laboratory methods

Pure tumor genomic DNA was derived from paraffin-embedded tissue obtained by laser capture microdissection (Palm, Oberlensheim, Germany). For isolation of DNA from deparaffinized, microdissected tissue, the material was incubated with proteinase K, and DNA was extracted with phenol-chloroform and ethanol precipitation. Primers and cycling conditions for PCR amplification and direct sequencing for exons 19 and 21 of EGFR (Gen Bank accession number: X00558) are shown in Table 1. Sequencing was performed using forward and reverse primers with the ABI Prism 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). Electropherograms were analyzed for the presence of mutations using Seqscape v2.1.1 software in combination with Factura to mark heterozygous positions. The NSCLC cell line (PC9) derived from an adenocarcinomas [12] was also examined using the same methods.

Evaluation criteria and statistical analysis

Patients were divided into smokers and non-smokers. Non-smokers were defined as those who had smoked less than 100 cigarettes in their lifetime [13]. Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST) [14]. Time to progression was calculated from the start of gefitinib treatment until disease progression. Survival was calculated from the start of gefitinib treatment until death or last follow-up.

Age differences were analyzed using the Mann–Whitney U-test. Normality of the distribution of continuous variables was assessed with the Kolmogorov–Smirnov test. To identify relevant parameters of influence, a multivariable logistic regression model was used, and the fit of the models was evaluated with the Hosmer–Lemeshow likelihood ratio test. The Wald test was used to test the statistical significance of each variable in the model. Survival and time to progression curves were drawn with the Kaplan–Meier product limit method. All reported P values are two-sided; P < 0.05 was considered statistically significant. SPSS software version 11.5 (SPSS Inc, Chicago, IL) was used for all analyses.

Results

Patient characteristics are shown in Table 2. EGFR mutations were identified in 10/83 (12%) patients (Table 2). Mutations were more frequent in females (P = 0.007) and in non-smokers (P = 0.01). All 10 mutations were observed in adenocarcinomas (Table 2, Figure 1). Eight tumors had in-frame nucleotide deletions in exon 19, adjacent to K745;

<table>
<thead>
<tr>
<th>Exon 19</th>
<th>Forward 1st PCR</th>
<th>Reverse 1st PCR</th>
<th>Forward nested PCR</th>
<th>Reverse nested PCR</th>
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<tr>
<td>CATGAAAGTTGAACATTTAGGATGTG</td>
<td>3’</td>
<td>CATACATACCCCTTAGGCCCCTCC</td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>GTGCATCGCTGTAACTCC</td>
<td>3’</td>
<td>TGTGGAGATGAGCAGGGTCT</td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>Exon 21</td>
<td>Forward 1st PCR</td>
<td>Reverse 1st PCR</td>
<td>Forward nested PCR</td>
<td>Reverse nested PCR</td>
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<tr>
<td>CTACGGTGTGCCCCAGCATAAGTCC</td>
<td>3’</td>
<td>GCTGCGAGCTACCGAATGCTGG</td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>CATCCTCCCCCTGCTATGTG</td>
<td>3’</td>
<td>GCTGACGCTCCTGGATGAAA</td>
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</tr>
</tbody>
</table>
patients with wild-type EGFR (four partial responses), in contrast with six (8.8%) of the 73 attained objective radiographic response (one complete and mutation in exon 21 (Figure 1).

Six of the 10 patients (60%) carrying EGFR mutations attained objective radiographic response (one complete and four partial responses), in contrast with six (8.8%) of the 73 patients with wild-type EGFR (P = 0.001) (Table 2). Four of the six responders with wild-type EGFR were male; three were squamous cell carcinomas, two large cell carcinomas and one adenocarcinoma. Five of these six patients have died. The remaining four patients with EGFR mutations had stable disease. Three of these four patients were female; all were adenocarcinomas. Three had in-frame deletions in exon 19 (two delE746-A750), one of which contained an amino acid insertion (delL747-P753insS); the fourth patient with stable disease had a missense mutation in exon 21 (L858R). All four patients died. Overall median time to progression was 3.9 months (95% CI 3.5–4.3). Median time to progression for patients with EGFR mutations was 12.3 months (95% CI 6.4–18.3), while for those with wild-type EGFR, it was 3.6 months (95% CI 2.6–4.7; P = 0.002) (Figure 2). Patients with wild-type EGFR had 3.1 times greater probability of progressive disease than those with EGFR mutations [hazard ratio (HR), 3.7; 95% CI 1.5–9.3; P = 0.005]. Neither gender, number of prior chemotherapy lines, nor histology modified this risk. Overall median survival from the start of gefitinib treatment was 5.7 months (95% CI 3.7–7.6). Median survival for patients carrying EGFR mutations was 13 months (95% CI 6.5–19.4), in contrast to 4.9 months (95% CI 3.8–5.9) for those patients carrying wild-type EGFR (P = 0.02) (Figure 3). Patients with wild-type EGFR had 3.1 times greater probability of death than those with EGFR mutations (HR, 3.1; 95% CI 1.1–8.5; P = 0.03). Neither gender, number of prior chemotherapy lines, nor histology modified this risk.

### Discussion

This study confirms and expands on findings of previous studies [6–8] that EGFR TK mutations are present in a small sub-group of NSCLC tumors and can predict clinical outcome in gefitinib-treated chemoresistant stage IV NSCLC patients. We observed EGFR TK mutations in 12% of the Spanish NSCLC patients, which is in line with the frequency reported in North American NSCLC patients [6–8]. As in previous studies [6–8], mutations were observed more frequently in adenocarcinomas, women and non-smokers. The EGFR TK mutations found in our study, mainly in-frame deletions delE746-A750 and delL747-P753insS and the missense mutation L858R, are identical to those previously described [6–8]. Interestingly, in human lung adenocarcinoma cell lines, these same mutations were associated with selective activation of Akt and of signal transducer and activator of transcription 5 (STAT 5). It is well recognized that Akt is constitutively active in NSCLC cells and linked to chemoresistance [15]. Lung cancer cell lines harboring EGFR TK mutations exhibited increased sensitivity to disruption of Akt and markedly increased resistance to several chemotherapeutic agents such as cisplatin [16]. Moreover, these cell lines were 100-fold more sensitive to gefitinib than those with wild-type EGFR [16].

The 60% objective response rate observed in our study among NSCLC tumors harboring EGFR mutations is somewhat lower than that previously described [6–8]. This disparity may be due to the retrospective nature of our study, where patients were included based on the availability of tumor tissue. In addition, the limitations imposed by the use of computed tomography to measure tumor size, especially in non-spherical lesions for which one-dimensional measurements are subject to inaccuracy [17], may have led to discrepancies. Nevertheless, a highly significant difference in response rate (P = 0.001) and survival (P = 0.02) was observed between patients whose tumors contained EGFR TK mutations and those harboring wild-type EGFR.

### Table 2. Patient characteristics according to presence of EGFR mutations

<table>
<thead>
<tr>
<th></th>
<th>Mutation n (%)</th>
<th>Wild-type n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>10</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>59 (28–75)</td>
<td>64 (37–84)</td>
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</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>0.007</td>
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<tr>
<td>Male</td>
<td>3 (30)</td>
<td>55 (75.3)</td>
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</tr>
<tr>
<td>Female</td>
<td>7 (70)</td>
<td>18 (24.7)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>10 (100)</td>
<td>32 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>–</td>
<td>7 (9.7)</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>–</td>
<td>31 (43.1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>–</td>
<td>2 (2.8)</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Smokers</td>
<td>4 (40)</td>
<td>59 (80.8)</td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>6 (60)</td>
<td>14 (19.2)</td>
<td></td>
</tr>
<tr>
<td>No. prior regimens (range)</td>
<td>2 (0–3)</td>
<td>2 (0–5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Response to gefitinib</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Complete and partial response</td>
<td>6 (60)</td>
<td>6 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>4 (40)</td>
<td>34 (46.5)</td>
<td></td>
</tr>
<tr>
<td>Progressive disease</td>
<td>–</td>
<td>26 (38.3)</td>
<td></td>
</tr>
<tr>
<td>Not evaluable</td>
<td>–</td>
<td>5 (6.9)</td>
<td></td>
</tr>
<tr>
<td>Duration of gefitinib, months (range)</td>
<td>8.5 (3.7–22.5)</td>
<td>3.1 (0.4–20.5)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Response to previous chemotherapy</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Complete and partial response</td>
<td>3 (37.5)</td>
<td>30 (44.1)</td>
<td></td>
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<tr>
<td>Stable disease</td>
<td>4 (50)</td>
<td>27 (39.7)</td>
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<tr>
<td>Progressive disease</td>
<td>1 (12.5)</td>
<td>11 (16.2)</td>
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<tr>
<td>Not evaluable</td>
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<td>5</td>
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</tr>
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</table>
There is a need for markers that can predict the efficacy of chemotherapy, and genetic changes in metastases in different organs may be a contributing factor in second-line chemotherapy failure. Objective responses after second-line treatment with docetaxel do not necessarily translate into longer time to progression [18]; median time to progression with either docetaxel or pemetrexed was only 2.9 months in previously-treated NSCLC patients [19]. Even in first-line chemotherapy, median time to progression is rather short: 4.2 months in a randomized phase III trial comparing four different platinum-based doublets [20]. In the present study, the first to examine the relation between EGFR TK mutational status and time to progression in gefitinib-treated stage IV NSCLC chemotherapy failures, patients with EGFR mutations had a remarkable 12.3-month time to progression.

The presence of EGFR TK mutations clearly identifies a subset of NSCLC patients with oncogene addiction [3, 16] who will respond dramatically to EGFR TK inhibitors such as gefitinib or erlotinib [6–8]. The frequency of EGFR TK mutations in our Spanish NSCLC cohort is clinically meaningful, particularly in adenocarcinomas, and represents

Figure 1. Electropherograms of nucleotide sequences of the EGFR TK domain. Patients 1–8 showed nucleotide sequence deletions in their tumor specimens (horizontal arrows). Patients 9–10 harbored L858R missense mutations (vertical arrows).
a new paragon in lung cancer management. EGFR TK mutational analysis constitutes a novel predictive test for selecting NSCLC patients for upfront treatment with EGFR TK inhibitors in preference to chemotherapy [21]. Based on accumulated evidence and the results of our study, the Spanish Lung Cancer Group is initiating a new trial of first-line gefitinib in stage IV NSCLC patients carrying EGFR TK mutations.

Acknowledgements

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References