EGFR/KRAS/BRAF Mutations in Primary Lung Adenocarcinomas and Corresponding Locoregional Lymph Node Metastases

Katharina Schmid, Natalie Oehl, Fritz Wrb, Robert Pirker, Christine Pirker, and Martin Filipits

Abstract

Purpose: The epidermal growth factor receptor (EGFR) and its downstream factors KRAS and BRAF are mutated with different frequencies in non–small cell lung cancer and mutations predict clinical response to EGFR inhibitors. The present study compared the mutational status of EGFR, KRAS, and BRAF in primary tumors with the one in corresponding lymph node metastases.

Experimental Design: Direct bidirectional sequencing of EGFR gene exons 18 to 21, KRAS gene codons 12/13 and 61 to 68, and BRAF exon 15 was done on 96 paired samples of primary lung adenocarcinomas and corresponding locoregional lymph node metastases. In addition, comparative genomic hybridization analyses in two pairs of corresponding primary and metastatic tumor samples with discordant EGFR mutation status were done.

Results: Mutations in EGFR, KRAS, and BRAF were observed in 7 (7%), 36 (38%), and 2 (2%) patients, respectively. Interestingly, KRAS mutations were observed in two patients with an EGFR mutation. Mutations in primary tumors and lymph node metastases were identical in 1 of 7 (14%) patients in case of EGFR and 11 of 36 (31%) patients in case of KRAS. One patient harbored different KRAS mutations in primary and corresponding metastatic tumors. Comparative genomic hybridization analysis revealed similar patterns of chromosomal changes, strongly supporting a common clonal origin of primary tumors and metastases.

Conclusions: The possibility of differences in the mutational status of EGFR, KRAS, BRAF between primary tumors and corresponding lymph node metastases should be considered whenever these mutations are used for the selection of patients for EGFR-directed tyrosine kinase inhibitor therapy.

Lung cancer is one of the leading causes of cancer-related deaths world-wide and standard therapeutic strategies such as surgery, chemotherapy, or radiotherapy have reached a plateau (1). Recently, therapies that specifically target factors involved in the development and progression of lung cancer have shown promising efficacy (2, 3). One of the most intensively investigated targeted therapies is directed against the epidermal growth factor receptor (EGFR), a 170-kDa trans-membrane glycoprotein and member of the erbB family. EGFR was shown to be frequently overexpressed in non–small cell lung cancer (NSCLC; ref. 4). In 2004, it was reported that EGFR mutations located in the tyrosine kinase domain (exon 18-21) of NSCLC are associated with an improved response to EGFR tyrosine kinase inhibitors (TKIs; refs. 5 – 8). Deletions in exon 19 and the point mutation L858R in exon 21 are the most common activating mutations predominantly found in females, never-smokers, adenocarcinomas, and Asian patients (9 –11). In addition, secondary EGFR mutations have also been proven to be involved in resistance to EGFR-directed TKI therapy (12 –15).

KRAS and BRAF function downstream of EGFR in the signaling pathway and activating mutations have previously been described to be usually mutually exclusive in EGFR-mutated tumors (9, 16, 17). KRAS mutated to an oncogenic form by introducing amino acid substitutions at codons 12, 13, and 61 are detected in 15% to 57% of patients with lung adenocarcinomas from United States and Europe (18 –24) and are associated with poor response to EGFR-directed TKI therapy (22, 23, 25 –27). BRAF, a serine/threonine kinase, is activated by somatic point mutations in exon 15 in only 1% to 2% of lung cancer patients (28, 29).

Because EGFR, KRAS, and BRAF mutations may be clinically useful for the selection of patients for EGFR-directed TKIs and other targeted therapies, the present study was done to retrospectively evaluate the relationship between the mutation status in primary lung adenocarcinomas and the one in corresponding locoregional lymph node metastases. Additionally, we did comparative genomic hybridization (CGH) in two selected pairs of samples to confirm the common origin of primary tumors and corresponding metastases.

Patients and Methods

Patients. Ninety-six patients (58 males, 38 females; median age, 62 y; range, 42-81 y) with locally advanced adenocarcinomas (all mixed subtype) of the lung were included in this study. All patients underwent thoracic surgery at the Department of Cardio-Thoracic Surgery, Medical
University of Vienna, between 1999 and 2006. None of them received preoperative systemic therapy. After surgery, tissue samples were routinely fixed in 4.5% buffered formalin and embedded in paraffin for routine diagnosis. Tumors were staged according to the pathologic tumor-node-metastasis classification of lung cancer (2002) as 45 (47%) pT1-, 39 (41%) pT2-, 4 (4%) pT3-, and 8 (8%) pT4-primary tumors; locoregional lymph node metastases corresponded to 56 (58%) pN1- and 40 (42%) pN2 cases. The International Contra Cancer stages were 31 (32%) IA, 18 (19%) IB, 38 (40%) IIA, and 9 (9%) IIB tumors. Clinical data of the patients including smoking status were obtained from our clinical database and supplemented with review of the medical record. Informed consent was obtained according to institutional guidelines.

Smoking status was defined as former if the patient had not smoked any cigarettes within 12 mo before entry. Never smokers were defined as individuals who had smoked ≤100 total cigarettes during their lifetime (5). Targeted therapies have improved and will continue to improve the outcome of NSCLC. Specific activating mutations located in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) are associated with an improved response to EGFR-directed tyrosine kinase inhibitors (TKI) in NSCLC patients. In contrast, KRAS mutations have been shown to be associated with poor response to EGFR-directed TKI therapy. In the present study, we observed a substantial discordance between EGFR, KRAS, and BRAF mutational status in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. The possibility of differences in the mutational status of primary tumors and corresponding lymph node metastases should be considered whenever these mutations are used for the selection of patients for EGFR-directed TKI therapy.

**Table 1. Primer and annealing temperatures**

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**Results**

A total of 43 (45%) patients with mutations in EGFR, KRAS, and BRAF were identified as shown in Table 2. In a second run with either re-extracted DNA or with the extracted DNA reamplified and sequenced, all results from the first run were confirmed. Tumors of seven (7%) patients (four females, three males) contained an EGFR mutation. Three (3%) patients had a classic in-frame deletion in exon 19, three (3%) patients showed the classic missense point mutation L858R (Fig. 1), and one (1%) patient harbored an in-frame insertion in exon 20. Only one (1%) patient contained the same EGFR mutation in the primary tumor and the corresponding lymph node metastasis. In the remaining six (6%) patients, EGFR mutations were identified either in the primary tumor (three patients, 3%) or in the lymph node metastasis (also three patients, 3%). Two (2%) patients with EGFR mutations (one with an EGFR mutation in the primary tumor, one with an EGFR mutation in the lymph node metastasis) had an additional KRAS mutation in the corresponding lymph node metastasis, whereas a total of 43 (45%) patients with mutations in EGFR, KRAS, and BRAF were identified as shown in Table 2. In a second run with either re-extracted DNA or with the extracted DNA reamplified and sequenced, all results from the first run were confirmed. Tumors of seven (7%) patients (four females, three males) contained an EGFR mutation. Three (3%) patients had a classic in-frame deletion in exon 19, three (3%) patients showed the classic missense point mutation L858R (Fig. 1), and one (1%) patient harbored an in-frame insertion in exon 20. Only one (1%) patient contained the same EGFR mutation in the primary tumor and the corresponding lymph node metastasis. In the remaining six (6%) patients, EGFR mutations were identified either in the primary tumor (three patients, 3%) or in the lymph node metastasis (also three patients, 3%). Two (2%) patients with EGFR mutations (one with an EGFR mutation in the primary tumor, one with an EGFR mutation in the lymph node metastasis) had an additional KRAS mutation in the corresponding lymph node metastasis, whereas a total of 43 (45%) patients with mutations in EGFR, KRAS, and BRAF were identified as shown in Table 2. In a second run with either re-extracted DNA or with the extracted DNA reamplified and sequenced, all results from the first run were confirmed. Tumors of seven (7%) patients (four females, three males) contained an EGFR mutation. Three (3%) patients had a classic in-frame deletion in exon 19, three (3%) patients showed the classic missense point mutation L858R (Fig. 1), and one (1%) patient harbored an in-frame insertion in exon 20. Only one (1%) patient contained the same EGFR mutation in the primary tumor and the corresponding lymph node metastasis. In the remaining six (6%) patients, EGFR mutations were identified either in the primary tumor (three patients, 3%) or in the lymph node metastasis (also three patients, 3%). Two (2%) patients with EGFR mutations (one with an EGFR mutation in the primary tumor, one with an EGFR mutation in the lymph node metastasis) had an additional KRAS mutation in the corresponding lymph node metastasis, whereas 

**Comparative genomic hybridization.** CGH analysis of paraffin-embedded primary tumor and lymph node metastasis samples was done as previously described (30). Briefly, after extraction, DNA was amplified and labeled by linker-adapter-PCR. Equal amounts of tumor and reference DNA were hybridized to normal human metaphase spreads. Images were captured with a Leica DMRXA fluorescence microscope (Leica Mikroskopie und System), and CGH profiles were analyzed using the Leica QCGH software.

**Translational Relevance**

Targeted therapies have improved and will continue to improve the outcome of NSCLC. Specific activating mutations located in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) are associated with an improved response to EGFR-directed tyrosine kinase inhibitors (TKI) in NSCLC patients. In contrast, KRAS mutations have been shown to be associated with poor response to EGFR-directed TKI therapy. In the present study, we observed a substantial discordance between EGFR, KRAS, and BRAF mutational status in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. The possibility of differences in the mutational status of primary tumors and corresponding lymph node metastases should be considered whenever these mutations are used for the selection of patients for EGFR-directed TKI therapy.

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in all other patients EGFR, KRAS, and BRAF mutations were mutually exclusive.

Besides the above mentioned EGFR mutations, we observed the silent EGFR polymorphism Q787Q in primary tumors as well as corresponding lymph node metastases in 80 (83%) patients. Four patients harbored the silent EGFR polymorphism R836R in lymph node metastases and in one corresponding primary tumor.

### Table 2. EGFR, KRAS, and/or BRAF mutations in primary lung adenocarcinomas and/or corresponding locoregional lymph node metastases

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NOTE: Corresponding mutations are italicized.
Abbreviations: TNM, tumor-node-metastasis; UICC, Unio Internationale Contra Cancrum; Wt, wild-type.
*New mutation.
With regard to KRAS, 36 (38%) patients had a KRAS mutation, mainly representing missense point mutations (G12A, G12C, G12D, G12F, G12R, G12V, G13C, G13D, Q61H mutation). In patient #14, two consecutive KRAS missense point mutations (g870G>T and g871G>T; G12C/F) were observed. Furthermore, in two patients (#35 and #41), a novel, thus far unpublished in-frame insertion in codon 66 to 67 was detected. Eleven (11%) patients presented with identical KRAS mutations in primary tumors and corresponding lymph node metastases. A different KRAS mutation in the primary tumor and the corresponding metastasis was seen in one (1%) patient (#19). Twenty-four (25%) patients harbored KRAS mutations exclusively in either primary (16 patients, 17%) or metastatic tumor (8 patients, 8%). Novel, thus far unpublished BRAF exon 15 mutations were observed in primary tumors of 2 (2%) patients (one in-frame deletion and one K601L missense point mutation). All lymph node metastases were wild-type for BRAF.

Next, we assessed the relationship between EGFR/KRAS/BRAF mutations and the clinicopathologic variables listed in Table 3. No significant association was observed between EGFR/KRAS/BRAF mutation status and age, sex, tumor size, lymph node status, tumor stage, and grading. As previously described, the EGFR mutation rate was significantly higher in never smoker compared with current or former smoker (23% versus 3%; $P = 0.002$), whereas KRAS mutations were more frequently observed in current or former smoker than in never smoker (43% versus 18%; $P = 0.03$; Table 4).

To investigate whether the metastases with different mutation profiles are derived from the corresponding primary tumor, CGH analyses of two cases with discordant EGFR mutation status (patients #4 and #5 with a solitary L858R mutation in the primary tumor and lymph node metastasis, respectively) were done to compare the respective genomic profiles (Fig. 2). In both cases, similar patterns of chromosomal changes were observed in the primary and corresponding metastatic tumors. Identical chromosomal changes in patient #4 were observed on chromosomes 12, 13, 14, and 18, whereas patient #5 showed identical changes of chromosome 7p and 8 (Fig. 2A and B, left). Differences between the genomic profiles of primary tumor and metastasis of patient #4 were observed on chromosomes 7 and 8. Patient #5 showed differences with regard to chromosome 1q and 5p (Fig. 2A and B, right).

**Discussion**

Mutations in genes involved in the EGFR/KRAS/BRAF pathway were recently shown to predict clinical response to
EGFR-directed TKIs in NSCLC patients. Presence of activating EGFR mutations and absence of KRAS mutations have been shown to be favorable markers for responsiveness to EGFR-directed TKI therapy (5, 6, 11). Currently, data of the EGFR/KRAS/BRAF mutation status in NSCLC are mostly based on samples obtained from a single source, either primary tumors or metastases.

In our study population, we observed EGFR, KRAS, or BRAF mutations in 45% of the patients. The KRAS and BRAF mutation rate of primary tumors (36% and 2%, respectively) are consistent with those previously described in NSCLCs (20, 21, 23, 29). The incidence of EGFR mutations (7%) is slightly lower compared with other reports (25, 31–33), which may be explained that we included patients with known low frequency of EGFR mutations such as males, smoker, patients of all ages, and Caucasians. We found three new mutations (a KRAS in-frame insertion in codon 66-67, a BRAF in-frame deletion in exon 15, and a BRAF K601L missense point mutation), which were not listed in the Catalogue of Somatic Mutations in Cancer database. Whether these new mutations have an effect on the response to EGFR-directed TKI therapy has to be investigated in further studies.

Surprisingly, 72% (31 of 43 patients with mutated tumors, in total 32% of all investigated cases) of our patients with mutations in either primary tumor, lymph node metastases, or both showed discordant results. In 6 of 7 cases with an EGFR mutation and in 25 of 36 cases with a KRAS mutation, we found a discordant mutational status in the primary tumor and the corresponding lymph node metastasis. The lack of correlation in the mutation status between primary tumors and metastasis is most likely not due to technical problems for several reasons. First, all tumor specimens analyzed were required to contain >70% tumor cells or if not were macro-dissected before analysis. Second, results were confirmed by a second run. Third, the mutation rate in the primary tumors is not different to previously published data. Finally, our results are in accordance with those from other reports (34–36).

Kalikaki et al. (34) reported discordance in EGFR and KRAS mutational status between primary tumors and corresponding metastases in 25 patients with NSCLC. Among five patients with an EGFR mutation in the primary tumor, none of the corresponding metastases had the same mutation pattern, and in five patients with a KRAS mutation, only two harbored identical mutations in the metastases. Badalian et al. (35) reported that KRAS mutations in primary NSCLCs did not predict the presence of KRAS mutations in corresponding bone metastases in 11 patients. In another report, Takahashi et al. (36) found also differences in the EGFR and KRAS mutation status between primary lung tumors and corresponding metastases. In this report, the authors investigated p53, EGFR, and KRAS in eight sets of primary and metastatic lung cancers.

### Table 3. Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;60 y</td>
<td>44 (46%)</td>
</tr>
<tr>
<td>≥60 y</td>
<td>52 (54%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>38 (40%)</td>
</tr>
<tr>
<td>Male</td>
<td>58 (60%)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>45 (47%)</td>
</tr>
<tr>
<td>pT2</td>
<td>39 (41%)</td>
</tr>
<tr>
<td>pT3</td>
<td>4 (4%)</td>
</tr>
<tr>
<td>pT4</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
</tr>
<tr>
<td>pN1</td>
<td>58 (60%)</td>
</tr>
<tr>
<td>pN2</td>
<td>38 (40%)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>31 (32%)</td>
</tr>
<tr>
<td>IIB</td>
<td>18 (19%)</td>
</tr>
<tr>
<td>IIIA</td>
<td>38 (40%)</td>
</tr>
<tr>
<td>IIIB</td>
<td>9 (9%)</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>G2</td>
<td>55 (57%)</td>
</tr>
<tr>
<td>G3</td>
<td>38 (40%)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>60 (63%)</td>
</tr>
<tr>
<td>Former</td>
<td>14 (15%)</td>
</tr>
<tr>
<td>Never</td>
<td>22 (23%)</td>
</tr>
</tbody>
</table>

NOTE: Percentages may not total 100 because of rounding.

### Table 4. Smoking status and mutations in EGFR, KRAS, and BRAF

<table>
<thead>
<tr>
<th></th>
<th>Current or former smoker</th>
<th>Never smoker</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Wild-type</td>
<td>72 (97%)</td>
<td>17 (77%)</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>2 (3%)</td>
<td>5 (23%)</td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Wild-type</td>
<td>42 (57%)</td>
<td>18 (82%)</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>32 (43%)</td>
<td>4 (18%)</td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Wild-type</td>
<td>72 (97%)</td>
<td>22 (100%)</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>2 (3%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of genomic changes of primary tumor (inner bars) and corresponding lymph node metastasis (outer bars); green bars, gains; amplifications; red bars, losses of chromosomal material. Both, patient #4 (A) and patient #5 (B), show multiple identical changes in primary tumor and metastasis on chromosome 12 to 14 and 18 (left); right, differences between primary and corresponding metastasis.
by means of a whole-genome allelic imbalance scanning and mutational analysis. They found in seven of eight investigated cases genetic alterations accumulated only in metastatic tumors. However, in one article a correlation between EGFR mutations in primary lung adenocarcinomas and corresponding brain metastases was described. Matsumoto et al. (37) detected EGFR mutations in 12 of 19 brain metastases of lung adenocarcinomas. In six cases, the corresponding primary lung tumor was also examined and in all of them the identical EGFR mutation was observed.

Previously, it was reported that EGFR and KRAS mutations in lung cancer are mutually exclusive (9, 16, 17). However, there is our group of patients, two with mutated EGFR, was observed. Interestingly, in additional KRAS was observed. Also examined and in all of them the identical KRAS mutation in the corresponding lymph node metastasis. However, in the present study, KRAS and BRAF as well as EGFR and BRAF mutations were mutually exclusive.

Our results are relevant for both the selection of patients for EGFR-directed therapies and the design of future clinical trials with EGFR-directed TKIs. Recently, the results of Iressa Pan Asia Study, a phase III randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced NSCLC were reported (39). This study showed improved progression-free survival for gefitinib compared with chemotherapy. The EGFR mutation rate in this trial was 59.7% (262 of 437). Progression-free survival was significantly longer for gefitinib than chemotherapy in patients with mutated EGFR and longer for chemotherapy than gefitinib in patients with wild-type EGFR. Thus, patients with known EGFR mutations may be candidates for first-line therapy with gefitinib, whereas patients with wild-type EGFR should be treated with first-line chemotherapy.

In conclusion, we observed a substantial discordance between EGFR, KRAS, and BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. These differences in the mutational status may be important for the selection of patients for EGFR-directed TKI therapy and should be considered the design of future clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
EGFR/KRAS/BRAF Mutations in Primary Lung Adenocarcinomas and Corresponding Locoregional Lymph Node Metastases


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