Epidermal Growth Factor Receptor Gene in Primary Tumor and Metastatic Sites from Non-small Cell Lung Cancer

Lorenzo Daniele, MD, PhD,* Paola Cassoni, MD, PhD,* Elisa Bacillo, BSc,† Susanna Cappia, BSc,‡ Luisella Righi, MD, PhD,† Marco Volante, MD, PhD,† Fabrizio Tondat, BSc,§ Giorgio Inghirami, MD,‡ Anna Sapino, MD,* Giorgio V. Scagliotti, MD,§ Mauro Papotti, MD,† and Silvia Novello, MD, PhD§

Introduction: The majority of patients with non-small cell lung cancer (NSCLC) develop distant metastases. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are capable of reducing brain and adrenal metastases. However, the EGFR status may be discordant between primary NSCLC and the corresponding metastases.

Methods: Using fluorescence in situ hybridization (FISH) analysis, the EGFR gene status was evaluated in a series of 38 cerebral or adrenal metastases collected from two institutions and in the corresponding primary tumors. Also, EGFR mutational analysis was performed using direct sequencing on the cerebral metastases.

Results: EGFR FISH was positive in 28% of the primary tumors and in 45% of the metastases (p < 0.05). Among the seven cases FISH-positive at the metastatic site but negative in the primary tumor, six were brain metastases, and one was an adrenal metastasis; all were polysomic for chromosome 7, none were amplified. No EGFR mutations have been found in the cerebral metastases.

Conclusion: Because the molecular asset of EGFR may change during the metastatic progression of NSCLC to brain (but not to adrenal), the selection of patients with brain metastasis for specific targeted therapies by EGFR FISH analysis should be performed on metastatic lesions rather than on their corresponding primary tumors.

Key Words: FISH, EGFR polysomy, Brain metastases, Lung cancer.

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assessed by fluorescence in situ hybridization (FISH), could be used to predict a patient’s responsiveness.\textsuperscript{19,20} However, an association of such molecular features with brain and adrenal metastases is not well referenced, and the possibility should be considered that the actual status of the \textit{EGFR} gene in the metastases could differ from that of the primary tumor.

The aim of the current study is to investigate possible changes in the \textit{EGFR} gene copy number between primary lung cancer and the corresponding brain or adrenal metastases. These two metastatic sites were selected because of the availability of a relatively large number of single secondary lesions from two surgical series. In addition, the presence of specific \textit{EGFR} mutations was evaluated in the series of brain metastases. We show that the \textit{EGFR} gene gains, as detected by FISH, are more frequent in brain (but not in adrenal) metastases than in the corresponding primary tumors.

\section*{PATIENTS AND METHODS}

\subsection*{Patients and Tissue Samples}

Between January 2004 and December 2006 tumor specimens from 80 consecutive patients with surgically excised cerebral metastases from lung cancer were analyzed in the Pathology Department of the San Giovanni Hospital (Turin, Italy). Thirteen other patients affected by lung cancer underwent adrenal metastasis resection at the San Luigi Gonzaga Hospital (Orbassano, Turin, Italy). In 38 of these 93 cases, representative paraffin blocks of either cerebral metastasis or adrenal metastasis and of the corresponding primary tumor were available. Primary tumor specimens were constituted by alcohol-fixed and paraffin-embedded transthoracic fine-needle aspirate (FNA) in 12 cases, by formalin-fixed and paraffin-embedded transthoracic fine-needle aspirate (FFPE) bronchial biopsies in three cases, and by FFPE surgical specimens obtained from radical surgery in 23 cases, respectively. This study was approved by the institutional ethical review board.

\subsection*{EGFR FISH Analysis}

FISH analysis was performed on the 38 cerebral/adrenal metastasis and on the correspondent primary tumors. Probes for \textit{EGFR} (Vysis Inc., Downers Grove, IL) were used for FISH according to the manufacturer’s instructions. Briefly, sections were baked overnight at 56°C, deparaffinized in xylene, dehydrated in 100% ethanol, and air dried; then they were pretreated in sodium thiocyanate for 20 minutes at 80°C, and then with proteases for 15 minutes at 37°C; finally, they were washed in 2X SSC, dehydrated using ethanol (70%, 85%, 100%), and air dried. Specimens were covered with 10\textmu m thick sections of FFPE blocks. After deparaffinizing with xylene-ethanol, specimens were incubated overnight at 55°C in lysis buffer containing proteinase K (20 mg/ml) followed by DNA isolation after phenol-isopropanol extraction. DNA concentration was measured with a spectrophotometer (BioPhotometer Eppendorf AG, Hamburg, Germany). The quality of DNA extracted from FFPE was tested by performing amplification of 300-bp fragment of the human major histocompatibility complex class II DRB gene with the following primers: DRBF 5’-CGG GTG CAC TGG CCG CCC AGC ACG TTC TTT C-3’ and DRBR 5’-GAA TTC TCG CCG CTG CAC TGT GAA GC-C-3’. Polymerase chain reaction (PCR) amplification of \textit{EGFR} (exons 19 and 21) was performed using the following primers: \textit{EGFR}F19R 5’-CAA TAC GCC GCC GCT-3’; \textit{EGFR}R19R 5’-CAT AGA AAG TGA ACA TTT AGG ATG TG-3’; \textit{EGFR}F21R 5’-CCT CGG AGC TCC ACC TTC GGG TAC-3’; \textit{EGFR}R21R 5’-GCT CGG AGC TCA CCC AGA ATG TCT CTG-3’. PCR was performed in a total volume of 50 \mu l, containing 1X PCR buffer (Tris-HCl 20 mM, KCl 50 mM), MgCl\textsubscript{2} 1.5 mM, 0.2 mM dNTPs, 0.4 \mu M each primer, 0.2 U TaqDNA polymerase (Invitrogen, Carlsbad, CA), and 500 ng of genomic DNA. Thermal cycling conditions were 5 minutes at 94°C, followed by 40 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, with a final extension step of 72°C for 7 minutes.

\subsection*{DNA Sequencing}

PCR products were separated on a 2% agarose gel, purified using the PCR clean-up gel extraction kit (Macherey-Nagel, Dueren, Germany), and sequenced in both directions by dye-terminator sequencing with the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing fragments were detected by capillary electrophoresis on an ABI Prism 310 DNA analyzer (Applied Biosystems).

\subsection*{Statistical Analysis}

Pearson’s correlation test confirmed by Spearman’s correlation test was used to compare the \textit{EGFR} status between primary tumors and related metastatic sites and statistical significance was defined as \(p < 0.05\). Statistical analyses were performed using the “R 1.7.1” statistical software package.
TABLE 1. Patients Characteristics (n = 38)

<table>
<thead>
<tr>
<th></th>
<th>No. Patients</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>66 (45–82)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>87</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Histology</td>
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<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
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<td>47</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>7</td>
<td>18</td>
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<tr>
<td>Large cells carcinoma</td>
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<td>8</td>
</tr>
<tr>
<td>Small cells carcinoma</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>NSCLC NOS</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>Stage at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>39</td>
</tr>
<tr>
<td>Metastatic sites analyzed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>28</td>
<td>73</td>
</tr>
<tr>
<td>Adrenal</td>
<td>10</td>
<td>27</td>
</tr>
</tbody>
</table>

a Stage at the time of primary tissue sampling.

NSCLC, non-small cell lung cancer; NOS, not otherwise specified.

TABLE 2. Correlation Between EGFR FISH Status in Primary Lung Cancer and Corresponding Metastatic (Brain or Adrenal) Site

<table>
<thead>
<tr>
<th></th>
<th>EGFR FISH in Brain Mts</th>
<th>EGFR FISH in Adrenal Mts</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR FISH in primary tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FISH −</td>
<td>14</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>FISH +</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

p < 0.05

EGFR FISH Analysis

EGFR FISH was assessable on 36 of the 38 archival histologic and cytologic sections from the primary lung cancer and on 37 of the 38 histologic sections from the corresponding metastatic (brain or adrenal) sites. The results of FISH analysis obtained by FISH analysis in the 35 primary NSCLC and corresponding brain or adrenal metastases are shown in Table 2. EGFR FISH was positive in 28% (10 of 35) of the primary tumors and in 45% (16 of 35) of the metastatic sites (p < 0.05). In particular, FISH was positive in 10 of 25 (40%) primary tumors and in six of 10 (60%) adrenal metastases. Among the 10 cases that were EGFR FISH positive in the primary tumor, only two were amplified, whereas the others were polysomic for Chromosome 7. The two cases with EGFR amplification in the primary tumor confirmed their status in the corresponding metastatic site. Among the seven cases that were EGFR FISH positive in the metastatic site but negative in the primary tumor, six were brain metastases, and only one was an adrenal metastasis; all were polysomic for Chromosome 7, and none were amplified (Figure 1). Nine of the 35 cases (26%) were EGFR FISH positive for both the primary tumor and the metastasis and 18 (51%) were negative for both the primary tumor and the metastasis. Eight of the 35 cases (23%) showed primary tumor versus metastasis discordance; in seven cases, EGFR FISH was positive in the metastatic site but negative in the primary tumor, and one sample was EGFR positive in the primary tumor but not in the metastasis. Interestingly, the tissue source (and relative availability of neoplastic cells) was not a major cause of discrepancy, because among the eight discrepant cases, only one consisted of cytologic FNA from the primary tumor, whereas all other cases were represented by histologic specimens (bronchial biopsies and surgical resections).

EGFR Mutation Status

Among the 28 brain metastases from lung cancer, no specific EGFR mutation was detected in the analyzed exons (19 and 21).

RESULTS

Clinical and Pathologic Features

The characteristics of the 38 lung cancer patients are reported in Table 1. The median age of patients at diagnosis of metastasis was 66 years (with a range of 45–82 years). The thirty-eight analyzed metastases were synchronous in 19 cases (50%) and metachronous in 19 cases (50%). There were 33 men and five women. Eighteen patients (47%) had adenocarcinoma, seven (18%) squamous cell carcinoma, three (8%) large cells carcinoma, three (8%) small cell carcinoma, and seven (18%) had NSCLC not otherwise specified. In particular, of the 28 patients with brain metastases, 10 had adenocarcinoma (36%), seven squamous cell carcinoma (25%), three large cells carcinoma (10%), three small cell carcinoma (10%), and five (18%) had NSCLC not otherwise specified. Of the 10 patients with adrenal metastases, eight (80%) had adenocarcinoma and two (20%) had NSCLC not otherwise specified.

None of the patients received prior EGFR-targeted therapy.

DISCUSSION

The majority of patients with NSCLC develop distant metastases either at the time of the initial diagnosis or during the disease progression. Several reports have shown that EGFR specific tyrosine kinase inhibitors such as gefitinib and erlotinib are capable of reducing brain and adrenal metastases in NSCLC, sometimes with a highly dramatic response.14–17 Both mutations and amplifications or gene gains of EGFR have been reported in association with clinical responses to such drugs.19,20 A previous study demonstrated that EGFR FISH analysis may be used as the first-choice laboratory test, as an alternative to gene mutation analysis, for endoscopic biopsies or cytologic specimens of NSCLC to select patients.
has performed with NSCLC. To the best of our knowledge, only one study the primary tumors and corresponding metastases in patients the primary lesions.

Other five cases were consistent with the EGFR status of other studies, and this surprising lack of This frequency is lower than the frequencies reported by

one case acquired a specific gene gain, whereas the other five cases were consistent with the EGFR status of the primary lesions.

In our study, no specific EGFR mutations were found. This frequency is lower than the frequencies reported by other studies, and this surprising lack of EGFR mutation was observed in a consistent series of 28 brain metastases from lung cancer. However, in Italy, the expected occurrence of EGFR mutations for nonselected NSCLCs is not higher than 4.5%, i.e., 1/28 cases, at best. It might be speculated that lung cancers that metastasize to the brain contain particular EGFR gene alterations characterized by polysomy of chromosome 7 rather than by known EGFR mutations. This observation seems to conform to the available data in the literature, because recent studies suggest that EGFR mutations, when present, are an early event in the development of lung adenocarcinoma. Yoshida et al. showed that EGFR mutations were present in 3% of atypical adenomatous hyperplasias, which is considered to be a precursor lesion of lung adenocarcinoma. Tang et al. reported that nine of 21 patients carrying lung adenocarcinoma EGFR mutations also had identical mutations in the histologically normal respiratory epithelium. Thus, EGFR mutations are likely to be an early genetic alteration in the multistage carcinogenic processes of lung adenocarcinoma, but different genetic alterations responsible for metastases could be required. A recent study from the group of Yatabe et al. reported that a specific EGFR amplification may be acquired in association with tumor progression, even if this group suggests that the selection of the metastatic clone could be defined by factors other than amplification. Our study demonstrated that the development of brain metastasis could be correlated with a trend to acquire a specific EGFR gene gain, whereas the progression to adrenal metastasis could be related to other molecular mechanisms. However, it should be considered as an alternative explanation of our findings that tumor heterogeneity might be responsible of the differences between primary and metastatic tumors observed in our study. In fact, it has been recently reported that even in a context of a high polysomy there may be plenty of diploid cells, and—with special reference to cytologic FNA specimens with paucicellular material—the risk of underestimating FISH results has to be taken into account. However, it should be underlined that in our series only one of eight discrepant cases corresponded to FNA sample of the primary tumor.

Our findings must be taken into account when considering the recent evidence of the efficacy of gefitinib and erlotinib in the treatment of brain metastases from lung cancer. Because the molecular asset of EGFR may change during the metastatic progression, our data suggest that the selection of patients for specific targeted therapies by EGFR FISH analysis should, whenever possible, be performed on metastatic lesions rather than on their corresponding primary tumors.

FIGURE 1. Dual-color FISH assays using EGFR (red) and chromosome-7 centromere (CEP7, green) probes: balanced disomy in the primary lung tumor (A) and high level of polysomy 7 in the corresponding brain metastasis (B).

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