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

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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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Lung cancer is the most common cause of cancer death in both men and women throughout the world. Disease stage is usually advanced at presentation, median survival time is stage dependent, and globally, the overall survival remains poor. Lung cancer extrathoracic spread occurs more commonly to the bone, liver, adrenal gland, and brain. Approximately 40% of all patients with lung cancer suffer from brain metastases in the course of their disease,[1] although the frequency of metastasis to the adrenal gland found at autopsy ranges from 18 to 42%.[2] Although patients with brain metastases are generally treated with corticosteroids and whole-brain radiation therapy, their prognosis is still disappointing. Whole-brain radiation therapy extends survival by only 14 to 21 weeks, even when it achieves palliative improvement for neurologic symptoms.[3,4] Moreover, refractory brain metastases cause death in 25 to 50% of these patients.[4] In the case of adrenal metastases a palliative chemotherapy should be considered, even though most recent studies suggest the adrenalectomy as a therapeutic option for patients with a single metachronous or synchronous adrenal metastases with a encouraging 5-year survival rate of 25%.[5]

During the last few years, new molecularly targeted agents aimed to inhibit specific pathways and key molecules in tumor growth and progression have been developed. One example of such a target is the epidermal growth factor receptor (EGFR), a tyrosine kinase (TK) receptor, overexpressed in many human epithelial malignancies, including non-small cell lung cancer (NSCLC)[6,7] in which its expression is associated with poor survival.[8] Ligand binding to EGFR leads to receptor TK activation and to a series of downstream signaling activation that mediate proliferation, migration, invasion, and suppression of apoptosis.[9] The EGFR signaling can be blocked by small-molecule EGFR TK inhibitors, such as gefitinib and erlotinib. These inhibitors produce objective response rates of 12 to 27% in previously treated or untreated advanced NSCLC.[10–13] Interestingly, some recent reports have demonstrated that gefitinib and erlotinib are capable of reducing brain metastases from NSCLC, sometimes with a dramatic improvement,[14–17] and a complete responsiveness was reported in a single case of a Taiwanese female patient with an adrenal metastasis from lung adenocarcinoma.[18] Acquired mutations of *EGFR* gene in exons 18 to 21 and *EGFR* gene copy number, which is

assessed by fluorescence in situ hybridization (FISH), could be used to predict a patient's responsiveness.[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 *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 *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 *EGFR* mutations was evaluated in the series of brain metastases. We show that the *EGFR* gene gains, as detected by FISH, are more frequent in brain (but not in adrenal) metastases than in the corresponding primary tumors.

PATIENTS AND METHODS

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 (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.

EGFR FISH Analysis

FISH analysis was performed on the 38 cerebral/adrenal metastasis and on the correspondent primary tumors. Probes for *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 increasing ethanol (70%, 85%, 100%), and air dried. Specimens were covered with 10 μL of probe (LSI *EGFR*/CEP7 Dual color probe, Vysis Inc., Downers Grove, IL) and a glass coverslip sealed with rubber cement, codenatured in Hybrite System (Vysis Inc., Downers Grove, IL) for 10 minutes at 80°C, and overnight hybridized at 37°C. Finally, slides were washed with posthybridization buffer at 73°C and counterstained with 4′, 6′-diamidino-2-phenylindole. Tumor sections were first scanned at low power with a 4′, 6′-diamidino-2-phenylindole filter to identify areas of optimal tissue digestion and nonoverlapping nuclei. Patients were classified into two strata: (i) FISH-negative, with no or low genomic gain ($<$four copies of the gene in $>$40% of cells) or (ii) FISH-positive, with either a high level of polysomy ($\geq$four copies of the gene in $>$40% of cells) or with gene amplification. Gene amplification was defined by the presence of tight gene clusters, and a gene/chromosome per cell ratio $\geq$two, or $\geq$15 copies of the gene per cell in $\geq$10% of analyzed cells.[20,21] For the evaluation of the FISH results in primary tumor specimens obtained from FNA and from bronchial biopsies we considered as positive cutoff the presence of at least 10 neoplastic cells showing gene gain, as previously stated.[22]

DNA Extraction and Polymerase Chain Reaction

Mutational analysis was performed in the 28 brain metastases. Genomic DNA was extracted from four 10-μm 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 (BioPhotomer Eppendorf AG, Hamburg, Germany). The quality of DNA extracted from FFPE was tested by performing amplification of a 300-bp fragment of the human major histocompatibility complex class II DRβ gene with the following primers: DRBF 5′-CCG GTC GAC TGT CCC CCC AGC ACG TTT C-3′ and DRBR 5′-GAA TTC TCG CCG CTG CAC TGT GAA GC-3′. Polymerase chain reaction (PCR) amplification of *EGFR* (exons 19 and 21) was performed using the following primers: *EGFR*19F 5′-CAA TAT CAG CCT TAG GTG CGG CTC-3′; *EGFR*19R 5′-CAT AGA AAG TGA ACA TTT AGG ATG TG-3′; *EGFR*21F 5′-CTA ACG TTC GCC AGC CAT AAG TCC-3′; *EGFR*21R 5′-GCT GCG AGC TCA CCC AGA ATG TCT GG-3′. PCR was performed in a total volume of 50 μL, containing 1X PCR buffer (Tris-HCl 20 mM, KCl 50 mM), $MgCl_2$ 1.5 mM, 0.2 mM dNTPs, 0.4 μM each primer, 0.2 U *Taq*DNA 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.

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).

Statistical Analysis

Pearson's correlation test confirmed by Spearman's correlation test was used to compare the 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 ($n = 38$)

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

[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

	EGFR FISH in Brain Mts		EGFR FISH in Adrenal Mts		
	FISH −	FISH +	FISH −	FISH +	Total
EGFR FISH in primary tumor					
FISH −	14	6	4	1	25
FISH +	1	4	0	5	10
Total	15	10	4	6	35
	$p < 0.05$		$p < 0.05$		

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization.

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.

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. Three samples were inadequate because of a large amount of autofluorescent hemosiderin or necrotic background. EGFR data 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%) brain metastases 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).

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

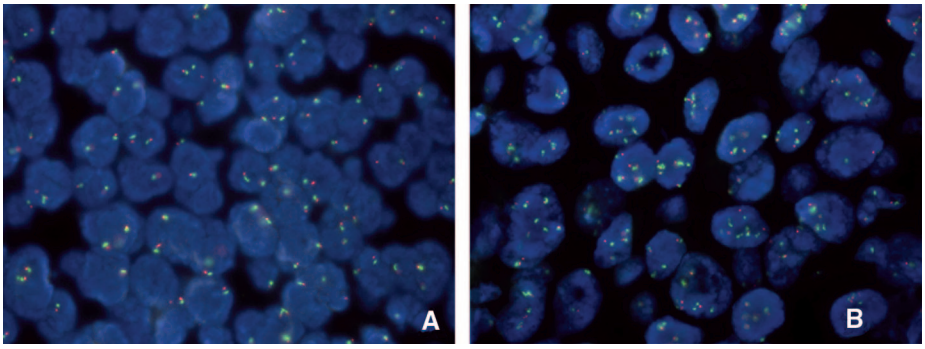

FIGURE 1. Dual-color FISH assays using EGFR (red) and chromosome-7 centromere (CEP7, green) probes: balanced disomy in the primary lung tumor (*A*) and high level of polysomy 7 in the corresponding brain metastasis (*B*).

for response.[22] However, the issue of EGFR expression consistency throughout the metastatic process for NSCLC is not yet clear. In fact, the EGFR receptor status has been determined in primary tumors, whereas data referring to the distant metastases are almost nonexistent. A limited number of studies[23,24] reported that the correlation between EGFR status in primary NSCLC cancer and in the corresponding metastatic sites is relatively poor, suggesting a change during metastatic progression. This discordance has been observed also by Petersen et al.,[25] who identified chromosomal imbalances at the 7p11 region, to which the *EGFR* gene has been mapped, by comparative genomic hybridization in a series of paired primary tumor and metastases of NSCLC. A recent study by the group of Kalikaki et al.[26] found a substantial discordance in *EGFR* and *K-RAS* mutational status between the primary tumors and corresponding metastases in patients with NSCLC. To the best of our knowledge, only one study has performed *EGFR* FISH analysis on a selected series of 30 patients with NSCLC and the corresponding metastasis and found that the *EGFR* gene copy number was discordant in a significant proportion of cases.[24] In our study, we aimed to ascertain the EGFR status in metastatic brain or adrenal lesions in comparison with the corresponding primary tumors. Our results revealed a discordance in 23% of cases, comparing primary tumors versus metastases. Brain metastases acquired a specific *EGFR* gene gain (driven by a high polysomy of chromosome 7) in six cases that were FISH negative in the corresponding primary tumor, with one case positive at FISH analysis in the primary tumor but negative in the metastatic site. In adrenal metastases, only one case acquired a specific *EGFR* 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,[27] 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%,[28] 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.[29] 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.[30] 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.[31] 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.[32,33] 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.

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