

HER Family Receptor Abnormalities in Lung Cancer Brain Metastases and Corresponding Primary Tumors

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Abstract Purpose: To compare the characteristics of deregulation of HER receptors and their ligands between primary tumor and corresponding brain metastases of non – small cell lung carcinoma (NSCLC).

Experimental Design: Fifty-five NSCLC primary tumors and corresponding brain metastases specimens were examined for the immunohistochemical expression of epidermal growth factor receptor (EGFR), phosphorylated EGFR, Her2, Her3, and phosphorylated Her3, and their ligands EGF, transforming growth factor- α , amphiregulin, epiregulin, betacellulin, heparin-binding EGFR-like growth factor, neuregulin (NRG) 1, and NRG2. Analysis of *EGFR* copy number using fluorescence *in situ* hybridization and mutation by PCR-based sequencing was also done.

Results: Metastases showed significantly higher immunohistochemical expression of EGF (membrane: brain metastases 66.0 versus primary tumors 48.5; $P = 0.027$; nucleus: brain metastases 92.2 versus 67.4; $P = 0.008$), amphiregulin (nucleus: brain metastases 53.7 versus primary tumors 33.7; $P = 0.019$), phosphorylated EGFR (membrane: brain metastases 161.5 versus primary tumors 76.0; $P < 0.0001$; cytoplasm: brain metastases 101.5 versus primary tumors 55.9; $P = 0.014$), and phosphorylated Her3 (membrane: brain metastases 25.0 versus primary tumors 3.7; $P = 0.001$) than primary tumors did. Primary tumors showed significantly higher expression of cytoplasmic transforming growth factor- α (primary tumors 149.8 versus brain metastases 111.3; $P = 0.008$) and NRG1 (primary tumors 158.5 versus brain metastases 122.8; $P = 0.006$). In adenocarcinomas, a similar high frequency of *EGFR* copy number gain (high polysomy and amplification) was detected in primary (65%) and brain metastasis (63%) sites. However, adenocarcinoma metastases (30%) showed higher frequency of *EGFR* amplification than corresponding primary tumors (10%). Patients whose primary tumors showed *EGFR* amplification tended to develop brain metastases at an earlier time point.

Conclusions: Our findings suggest that NSCLC brain metastases have some significant differences in HER family receptor – related abnormalities from primary lung tumors.

Lung cancer is the leading cause of cancer-related deaths in the United States (1). Lung cancer includes several histologic types, the most frequently occurring of which (~80%) are two types of non – small cell lung carcinoma (NSCLC): adenocarcinoma

and squamous cell carcinoma (1). The brain is one of the main sites of metastasis in patients with lung cancer: brain metastasis has an incidence of up to 60% in patients with lung adenocarcinoma (2–5). The median survival for lung cancer patients with brain metastasis is usually 3 to 6 months (5, 6). The use of systemic chemotherapy and cranial irradiation is unsuccessful in the treatment of NSCLC brain metastasis (2, 7), and this in turn has motivated the search for new therapeutic strategies for this disease.

During the past few years, significant advances have been made in the development of new molecularly targeted agents for lung cancer (8). One example of such targets is the epidermal growth factor receptor (EGFR) that belongs to the HER family tyrosine kinase (TK) receptors composed of four homologous cell membrane receptors, including Her2 and Her3 (9). These three receptors are activated by nine known ligands, including EGF, transforming growth factor- α (TGF- α), amphiregulin, epiregulin, betacellulin, heparin-binding EGFR-like growth factor (HB-EGF), neuregulin (NRG) 1, and NRG2 (10–12). Deregulation of HER receptors, especially EGFR, seems to play an important role in the pathogenesis and

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Translational Relevance

Brain metastasis occurs in up to 60% of non-small cell lung carcinomas, and there is little information on the molecular differences between primary tumor and metastases. Our findings indicate that non-small cell lung carcinoma brain metastases have some significant differences in HER family receptor-related abnormalities from primary lung tumors. These differences could be related to tumor progression and may cause diverse responses to epidermal growth factor receptor and other HER receptor-targeted therapy of primary and metastatic tumor sites.

progression of NSCLC (13). In lung cancer cells, the constitutive activation of EGFR is achieved by several mechanisms, including increased production of ligands, increased levels of the receptor, and mutation of *EGFR* (13–15).

Small-molecule inhibitors that target the TK domain of the *EGFR* produce tumor responses in ~10% of patients with advanced NSCLC that has progressed despite prior chemotherapy (13, 16). However, the brain is still a frequent site of disease recurrence in NSCLC patients after an initial response to treatment with EGFR TK inhibitors (TKI), regardless of disease control in the lungs (3). Activating mutations in the *EGFR* TK domain, an increased *EGFR* copy number, and increased EGFR protein expression have been associated with a favorable response to treatment with EGFR TKIs (13, 16). Previous reports showed that metastatic NSCLC brain tumors respond to EGFR TKIs (17, 18). However, it is still unclear whether the abnormalities of EGFR and its related receptors differ in metastases compared with primary NSCLC tumors.

The identification of differences in the deregulation of HER ligands and receptors in NSCLC primary tumors and corresponding metastases may explain differences in the response to HER receptor-targeted therapy in this tumor type, and these differences need to be considered in the analysis of predictive biomarker used in clinical trials. In the present study, we investigated the immunohistochemical expression of three HER receptors (EGFR, Her2, and Her3) implicated in the pathogenesis of NSCLC cancer and eight of their nine known ligands (EGF, TGF- α , amphiregulin, epiregulin, betacellulin, HB-EGF, NRG1, and NRG2) in 55 paired primary and brain metastasis NSCLCs. In addition, we compared *EGFR* copy number and mutation abnormalities using fluorescence *in situ* hybridization (FISH) and PCR-based sequencing analyses, respectively.

Materials and Methods

NSCLC tissue specimens. We obtained archived formalin-fixed paraffin-embedded material from surgically resected specimens from 55 NSCLC patients with primary lung cancers and corresponding brain metastases containing tumor tissues and collected between 1988 and 2002. These cases were selected based on the availability of enough archival tissue for the immunohistochemistry and FISH analyses. All specimens were from the lung cancer tissue bank at The University of Texas M. D. Anderson Cancer Center, which is approved by the M. D. Anderson institutional review board. After histologic examination,

tissue microarrays were constructed using three 1-mm-diameter cores per tumor.

Detailed clinical and pathologic information, including demographic, pathologic tumor-node-metastasis staging, overall survival, and time of brain metastasis occurrence, was obtained for all patients (Table 1). Pathologic tumor-node-metastasis stage had been determined for lung cancers according to the revised International System for Staging Lung Cancer (19) at time of primary tumor surgery with curative intent. In all cases, the NSCLC brain metastases were solitary, and 11 patients also developed metastases at other brain sites over a median period of 12 mo (range, <1-27 mo). Forty-four (80%) of 55 patients developed clinically detectable brain metastases after primary lung cancer surgical resection (metachronous tumors; median, 13 mo; range, <1-94 mo); in 11 (20%) patients, the brain metastases were detected at the same time as the lung tumors (synchronous tumors), and they were surgically removed before (median, <1 mo range, <1-11 mo) the primary lung cancer surgery.

Immunohistochemical staining and evaluation. For our analysis, antibodies against the following proteins were purchased and used: EGF (dilution, 1:50; EMD Biosciences), amphiregulin (dilution, 1:150; Lab Vision), TGF- α (dilution, 1:150; EMD Biosciences), epiregulin (dilution, 1:10; R&D Systems), betacellulin (dilution, 1:10; R&D Systems), HB-EGF (dilution, 1:10; R&D Systems), NRG1 (dilution, 1:10; R&D Systems), NRG2 (dilution, 1:50; Abcam, Inc.), EGFR (clone 31G7; dilution, 1:100; Zymed), p-EGFR Tyr¹⁰⁸⁶ (dilution, 1:100; Invitrogen), Her2 (dilution, 1:100; Dako), Her3 (dilution, 1:50; GenTex), and phosphorylated Her3 (p-Her3; dilution, 1:100; Cell Signaling). Immunohistochemical staining was done using 5- μ m-thick tissue microarray histologic sections as previously described (20). The immunohistochemical protein expression was quantified, using white light microscopy with $\times 20$ magnification, by two experienced thoracic pathologists (M.S. and I.I.W.) blinded to clinical and other molecular variables. All markers were examined for membrane, cytoplasm, and nucleus localization in tumor cells. As previously described (21–23), immunohistochemical expression was quantified using a three-value intensity score (0, 1+, 2+, and 3+) for all markers, except for membrane EGFR and phosphorylated EGFR (p-EGFR), for which a four-value intensity score (0, 1+, 2+, 3+, and 4+) and the percentage (0-100%) of the extent of reactivity were used. Next, expression scores

Table 1. Summary of clinicopathologic features of 55 NSCLC patients with primary tumors and corresponding brain metastases

Characteristic	Number	%
Tumor histology		
Adenocarcinoma	40	73
Squamous cell carcinoma	13	23
Large cell carcinoma	1	2
Adenosquamous carcinoma	1	2
Age (y)		
≤ 60	30	55
> 60	25	45
Gender		
Female	19	35
Male	36	65
Pathologic stage*		
I	13	24
II	10	18
III	18	33
IV [†]	14	25

* The staging was done at time of surgical resection of the primary lung tumor.

[†]In 11 cases, the brain metastases were surgically removed before the primary tumor.

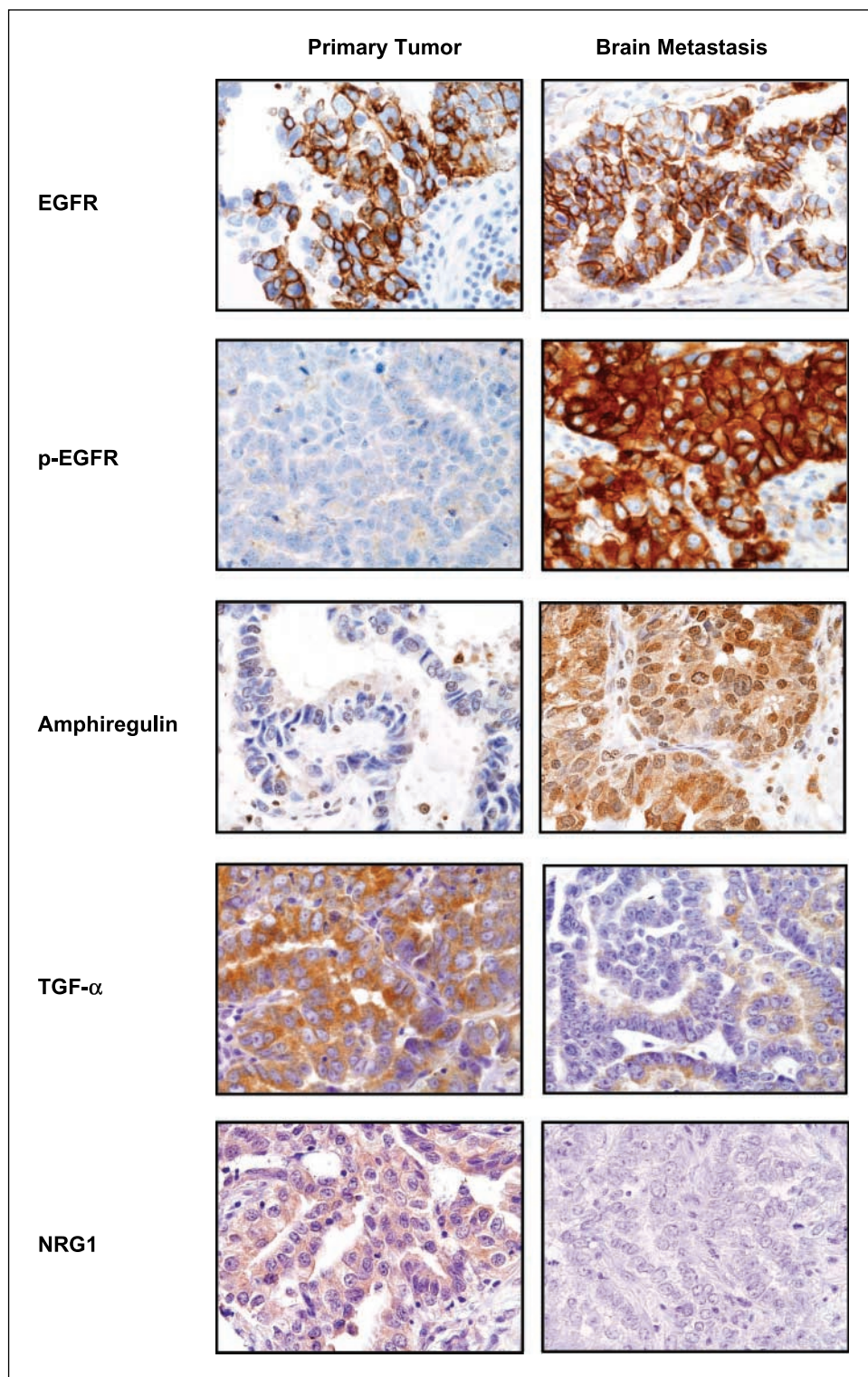


Fig. 1. Representative microphotographs of immunohistochemical expression of EGFR and p-EGFR and the ligands amphiregulin, TGF- α , and NRG1 in primary tumors and corresponding brain metastases. Magnification, $\times 400$. All markers showed protein expression (*brown staining*) in tumor cells from primary and/or metastasis sites at the membrane and cytoplasm levels. Amphiregulin showed also nuclear expression in malignant cells.

were obtained by multiplying the intensity and reactivity extension values (range, 0-300 for all markers, except for membrane EGFR and p-EGFR with a range of 0-400). For each case of primary tumor and metastasis, the immunohistochemical expression of the markers was averaged using the cores available per tumor site.

EGFR FISH analysis. We analyzed the gene copy number per cell using the LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen Probe

(Abbott Molecular), as previously described (24, 25). Tumor specimens were classified into six FISH strata according to the frequency of cells with each *EGFR* gene copy number and referred to the chromosome 7 centromere, as follows: (a) disomy (3 or 4 copies in <10% of cells), (b) low trisomy (3 copies in 10% to <40% of cells and 4 copies in <10% of cells), (c) high trisomy (3 copies in $\geq 40\%$ of cells and 4 copies in <10% of cells), (d) low polysomy (4 copies in 10% to <40% of cells),

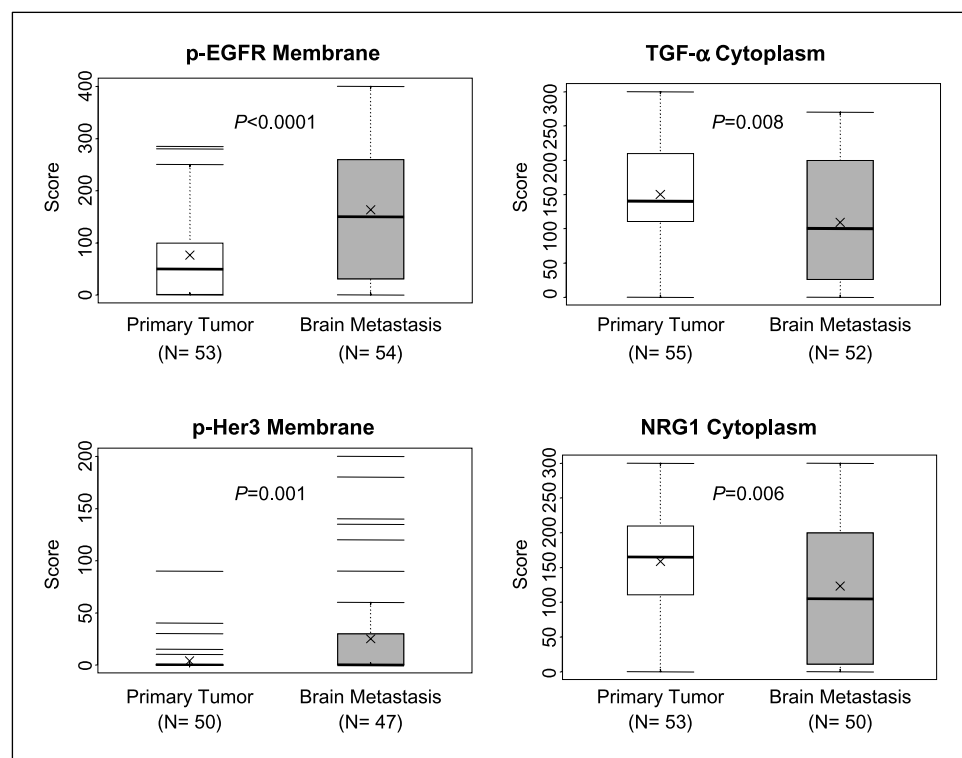


Fig. 2. Box plots showing scores of immunohistochemical expression of p-EGFR, TGF- α , p-Her3, and NRG1 markers comparing primary tumors with corresponding brain metastases. *P* values comparing primary tumors and brain metastasis are shown for all comparisons. Bars, median; x, mean score.

(e) high polysomy (≥ 4 copies in $\geq 40\%$ of cells), and (f) gene amplification (presence of loose or tight *EGFR* gene clusters with ≥ 4 copies, *EGFR* gene to CEP 7 ratio ≥ 2 , or 15 copies of *EGFR* per cell in $\geq 10\%$ of cells). The high polysomy and gene amplification categories were considered to indicate high *EGFR* copy number (*EGFR* FISH positive), and the other categories were considered to indicate no significant increase in the *EGFR* copy number (*EGFR* FISH negative), as previously described (24, 25). For each case of primary tumor and metastasis, the FISH *EGFR* copy number was quantified by counting cells representing each core available per tumor site.

***EGFR* mutation analysis.** Exons 18 to 21 of *EGFR* were PCR amplified using intron-based primers as previously described (26). Approximately 200 microdissected formalin-fixed paraffin-embedded cells were used for each PCR amplification. All PCR products were directly sequenced using the Applied Biosystems Prism dye terminator cycle sequencing method. All sequence variants were confirmed by independent PCR amplifications from at least two independent microdissections and DNA extraction, and the variants were sequenced in both directions, as previously reported (26).

Statistical analysis. Data were summarized using standard descriptive statistics and frequency tabulations. Associations between the marker expression and patients' clinical demographic variables, including age, sex, histology type, and pathologic stage, were assessed using appropriate methods, including the χ^2 test or Fisher's exact test for categorical variables and the Wilcoxon rank sum test or the Kruskal-Wallis test for continuous variables. The Wilcoxon signed rank test was used to test the differences in biomarker expression between primary lung tumors and brain metastases. Cox proportional hazard models were used for univariate analysis of time to metastasis according to biomarker expression. Hazard ratios (HR) with 95% confidence intervals (95% CI) and *P* values are reported. All tests were two sided. *P* values < 0.05 were considered to indicate statistical significance.

Results

Immunohistochemical expression of HER receptors and ligands in NSCLC primary tumors and corresponding brain metastases.

Most markers, including the ligands EGF, amphiregulin, TGF- α , epiregulin, betacellulin, NRG1, and NRG2 and the receptors EGFR, p-EGFR, Her2, Her3, and p-Her3, showed protein expression in tumor cells from primary and metastasis sites at the membrane and cytoplasm levels (Fig. 1; Supplementary Table S1). Of those, EGF, amphiregulin, epiregulin, NRG1, NRG2, p-EGFR, and p-Her3 showed also nuclear expression in malignant cells (Fig. 1; Supplementary Fig. S1). The ligand HB-EGFR expressed only in the cytoplasm of cancer cells. Although showing overlapping, brain metastases had significantly higher immunohistochemical expression scores of EGF (membrane: metastasis 66.0 versus primary 48.5; *P* = 0.027; nucleus: metastasis 92.2 versus primary 67.4; *P* = 0.008), amphiregulin (nucleus: metastasis 55.4 versus primary 33.7; *P* = 0.019), p-EGFR (membrane: metastasis 161.5 versus primary 76.0; *P* < 0.0001; cytoplasm: metastasis 101.5 versus primary 55.9; *P* = 0.014), and p-Her3 (membrane: metastasis 25.0 versus primary 3.7; *P* = 0.001) than did corresponding primary tumors (Fig. 2; Supplementary Table S1). Only the protein expression score of TGF- α (primary 149.8 versus metastasis 111.3; *P* = 0.008) and NRG1 (primary 158.5 versus metastasis 122.8; *P* = 0.006) at the cytoplasmic level was significantly higher in malignant cells from primary tumors than in brain metastasis cells (Fig. 2; Supplementary Table S1).

***EGFR* copy number analysis by FISH in NSCLC primary tumors and corresponding brain metastases.** Overall, the presence of high frequency of gain in *EGFR* copy number (FISH positive: high polysomy and amplification; Fig. 3) was similar in NSCLC primary (34 of 55, 62%) and brain metastasis (35 of 55, 64%) sites (Table 2). Although a relatively lower frequency of high polysomy was detected in metastases than in primary tumors (33% versus 47%), brain metastases showed a nonsignificant higher frequency of *EGFR* amplification than

corresponding primary tumors did (31% versus 15%; $P = 0.53$). In adenocarcinomas ($n = 40$ cases), a similar frequency of gain in *EGFR* copy number was detected in primary tumors (65%) and corresponding metastases (63%). However, brain metastases of lung adenocarcinoma showed a nonsignificant higher frequency of *EGFR* amplification than primary lung tumors (30% versus 10%; $P = 0.53$). Although a higher frequency in *EGFR* copy number gain was detected in brain metastases (62%) than primary tumors (46%) among squamous cell carcinomas (Table 2), the data were difficult to interpret because of the small number of cases available for analysis. The concordance of *EGFR* copy number abnormalities between both tumor sites was higher for the cases with primary and

metastasis tumors clinically detected as synchronous lesions (11 of 11, 100%) than those diagnosed as metachronous tumors (34 of 44, 77%).

A relatively high level of concordance (46 of 55, 84%) for gain in *EGFR* copy number gain was found between primary tumors and metastases (Supplementary Table S2). Sixteen (29%) paired primary/metastasis cases were *EGFR* FISH negative in both sites, whereas 30 (55%) paired cases showed gain in *EGFR* copy number at both tumor sites. Discordance in *EGFR* copy number status was detected in nine cases (16%); in six of these, brain metastasis sites had a gain in copy number, whereas primary tumors were FISH negative. The levels of concordance for high polysomy (15 of 30, 50%) and

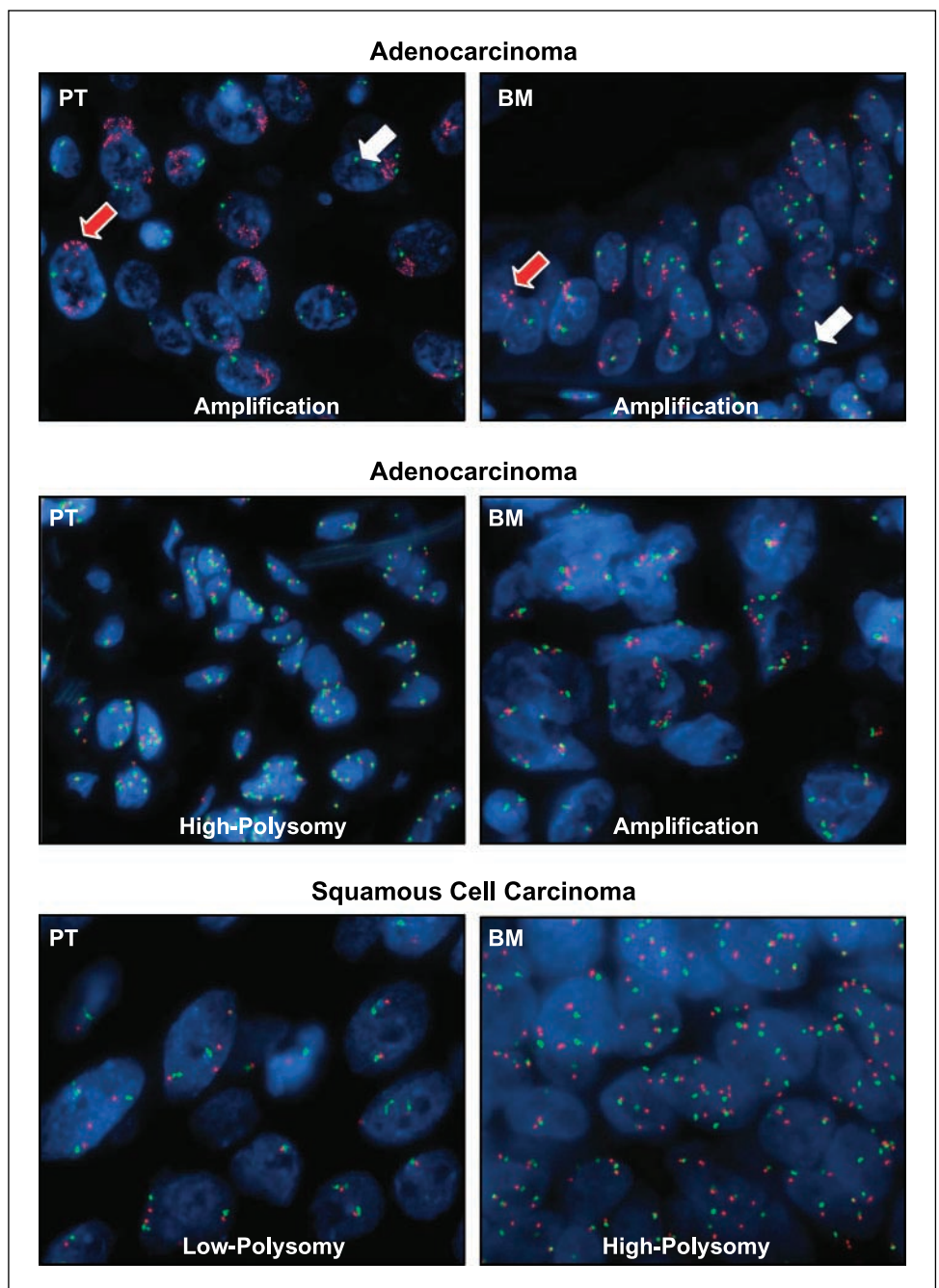


Fig. 3. Representative microphotographs of FISH showing *EGFR* copy number in primary tumors (PT) and corresponding brain metastases (BM). Magnification, $\times 1,000$. Red signals (red arrows) represent *EGFR* gene copies and green signals (white arrows) represent the chromosome 7 centromere probe. Cell nuclei stained blue with 4',6'-diamidino-2-phenylindole. High polysomy is defined by ≥ 4 copies in $\geq 40\%$ of cells, and gene amplification by the presence of loose or tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of 2 or 15 copies of *EGFR* per cell in 10% of the analyzed cells.

Table 2. *EGFR* copy number by FISH in 55 NSCLC primary and corresponding brain metastases by tumor histology

Copy number categories	Adenocarcinoma (n = 40)		Squamous cell carcinoma (n = 13)		Total (N = 55)*	
	Primary	Metastasis	Primary	Metastasis	Primary	Metastasis
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
FISH negative	14 (35)	15 (37)	7 (54)	5 (38)	21 (40)	20 (36)
Disomy	1 (3)	2 (5)	0 (0)	0 (0)	1 (2)	2 (4)
Trisomy	2 (5)	2 (5)	1 (8)	0 (0)	3 (6)	2 (4)
Low polysomy	11 (28)	11 (27)	6 (46)	5 (39)	17 (31)	16 (29)
FISH positive	26 (65)	25 (63)	6 (46)	8 (62)	34 (62)	35 (64)
High polysomy	22 (55)	13 (33)	2 (15)	5 (39)	26 (47)	18 (33)
Amplification	4 (10)	12 (30)	4 (31)	3 (23)	8 (15)	17 (31)

*One adenosquamous carcinoma and one large cell carcinoma showed *EGFR* high polysomy in the primary tumors and amplification in the brain metastasis specimen.

amplification (6 of 18, 33%) were low when primary tumors and corresponding brain metastases were compared. To assess heterogeneity in *EGFR* copy number abnormalities in the different cores examined per tumor sample, we examined in paired primary tumors and metastases from 14 cases the level of concordance between the level of the most advanced gene copy number abnormality detected in all cores examined per tumor site. In primary tumors, the concordance was 100% (22 comparisons), and in the brain metastases was 90% (27 of 30 comparisons).

Two consecutive brain metastasis samples were available for analysis from each of five adenocarcinoma cases. The length of time between the consecutive brain metastasis was 1, 6, 10, 16, and 31 months. In all comparisons, paired consecutive brain metastasis specimens showed identical *EGFR* copy number status. One pair was FISH negative and the other four were FISH positive. FISH-positive specimens included two pairs showing *EGFR* high polysomy and two showing gene amplification.

We correlated *EGFR* copy number status with the immunohistochemical expression of the markers at both tumor sites. The only associations detected were that *EGFR* FISH-positive primary tumors and brain metastases showed significantly higher protein expression scores of nuclear p-EGFR ($P = 0.018$) and cytoplasmic Her2 ($P = 0.015$) than FISH-negative tumors. In contrast, FISH-positive tumors showed lower expression of betacellulin ($P = 0.0293$) than FISH-negative tumors.

EGFR mutations in NSCLC primary tumors and corresponding brain metastases. We successfully amplified and sequenced DNA obtained from primary tumors and metastases samples from 42 cases, including 30 adenocarcinomas, for exons 19 and 21 of *EGFR*, which harbor >90% of TK activating mutations of the gene (27). We detected only one case with *EGFR* mutation (exon 19, point mutation TTA2239-2240CCA, Leu747Pro), which was present in both sites, primary and metastasis, in adenocarcinoma obtained from a patient female and never smoker.

Correlation between immunohistochemical expression of markers and EGFR copy number and time to brain metastasis. We investigated the correlation between the immunohistochemical expression of the markers examined and *EGFR* copy number abnormalities in primary lung tumors and the time to brain metastasis development. In this analysis, we included only the 44 patients whose brain metastases were

diagnosed after surgical resection of the primary tumor. Overall, the median time to brain metastasis for all 44 patients was 1.23 years (95% CI, 0.89-1.62 years). The median time to brain metastasis development for patients with adenocarcinoma was 1.43 years (95% CI, 0.96-2.04 years) and that for patients with squamous cell carcinoma was 0.89 years (95% CI, 0.63 to not available). Using the Cox proportional hazard regression models, we identified that adenocarcinoma, compared with squamous cell carcinoma, was significantly correlated with a longer time to brain metastasis occurrence ($P = 0.009$; HR, 0.347; 95% CI, 0.157-0.769), whereas *EGFR* membrane protein expression scores ($P = 0.025$; HR, 1.003; 95% CI, 1.000-1.006) and *EGFR* amplification (versus no amplification) ($P = 0.0039$; HR, 3.492; 95% CI, 1.494-8.162) were significantly correlated with a shorter time to brain metastasis development. None of these markers was shown in the multivariate analysis to be statistically significant predictors of metastasis development. However, in the multivariate analysis, when we analyzed time to brain metastasis development in the subset of 39 patients who developed a single brain metastasis (excluding the 5 patients who developed two consecutive metastases in the brain) after primary tumor diagnosis, we found that adenocarcinoma, compared with squamous cell carcinoma, was significantly correlated with a longer time to brain metastasis occurrence ($P = 0.031$; HR, 0.373; 95% CI, 0.152-0.917). In contrast, *EGFR* amplification (versus no amplification) was significantly correlated ($P = 0.0033$; HR, 4.452; 95% CI, 1.645-12.053) with a shorter time to brain metastasis development.

Discussion

In NSCLC, overexpression and activation of *EGFR*, Her2, and Her3 are well-known phenomena (9, 13). However, to the best of our knowledge, the overexpression of those TK receptors has not been previously reported in NSCLC brain metastasis. In this study, we have described for the first time higher levels of immunohistochemical expression of *EGFR*, p-*EGFR*, Her2, Her3, and p-Her3 in a series of NSCLC brain metastases using tissue microarray specimens. Interestingly, we found that the expression of phosphorylated forms of *EGFR* and Her3 proteins at the cytoplasmic and membrane level of malignant cells was significantly increased in brain metastasis compared with expression in corresponding primary lung tumors. Although

these data need to be validated in a larger set of specimens, these findings are consistent with the notion that activation of the EGFR and Her3 pathways is important in the progression and metastasis of lung cancer (13, 16). Similarly to brain metastasis, we recently showed that in *EGFR* mutant lung adenocarcinomas, p-EGFR immunohistochemical expression was significantly increased in nine lymph node metastases compared with expression in corresponding primary tumors (20).

It is known that the receptors of the HER family are activated after binding to ligands or peptide growth factors (10, 12). Ligand binding induces clustering of HER family receptors and produces subsequent autophosphorylation of cytoplasmic tyrosine residues (10, 12). There are 11 HER ligands identified, and they can be divided into four groups based on the receptor binding specificity: (a) exclusive EGFR binding: EGF, amphiregulin, TGF- α , and epigen; (b) EGFR and Her4 binding: betacellulin, HB-EGF, and epiregulin; (c) Her3 and Her4 binding: NRG1 and NRG2; and (d) exclusive Her4 binding: NRG3 and NRG4 (10, 12). No ligand binding Her2 has been identified (10, 12). In our study, we examined the protein expression of eight of nine ligands that bind to EGFR and Her3 receptors (10, 12). Of these, TGF- α (28, 29), epiregulin (30), and amphiregulin (31) are frequently expressed in primary NSCLC tumors, and EGF, betacellulin, HB-EGF, and NRG1 have been shown to be expressed in NSCLC cell lines (32–34). However, none of them has been characterized in primary lung tumors and corresponding brain metastasis. In addition, to the best of our knowledge, there is no report of the expression of NRG2 in lung cancer. We found that, compared with the corresponding primary tumors, NSCLC brain metastases had significantly higher immunohistochemical expression of membrane and nuclear EGF and of nuclear amphiregulin—ligands associated with activation of EGFR dimmers (10). These findings are consistent with the concomitant high level of overexpression of p-EGFR in the NSCLC brain metastasis that we studied and indicate the presence of an autocrine secretion mechanism of these ligands. In contrast to EGF and amphiregulin, the cytoplasmic expression of TGF- α and NRG1, which bind to EGFR and Her3 receptors, respectively (10, 12), was significantly higher in malignant cells from primary tumors than in cells from brain metastases. Overexpression of TGF- α has been associated with the metastatic potential of NSCLC (32) and colon cancer (35) cell lines in favoring modifications of the tumor microenvironment conducive to metastasis, such as increasing angiogenesis.

In our study, we have identified that six of ligands, EGF, amphiregulin, epiregulin, NRG1, and NRG2, and two receptors, p-EGFR and p-Her3, had nuclear expression in malignant NSCLC cells. There is evidence that TK receptors, as well as their ligands, translocate into the nucleus via receptor-mediated endocytosis for degradation or to be recycled back to the cell surface (36–40). However, it now seems clear that these complexes reach into the cell nucleus where they participate directly in the control of cell proliferation, cell differentiation, and cell survival (40).

The current concept of metastasis development states that metastases are the result of tumor cells interacting with a specific organ microenvironment, also called the “seed and soil” hypothesis (41). Thus, the microenvironments of different organs, including the brain, are biologically unique and can explain the expression of HER receptors and ligands in the brain metastasis tissue specimens differing from expression in

the corresponding primary lung tumors. In addition, these observations have important implications for the development of molecularly targeted therapy in lung cancer patients. The fact that potential therapeutic targets (EGF, amphiregulin, TGF- α , NRG1, EGFR, and Her3) are expressed differently in metastases from corresponding primary tumors suggests that different molecular properties among tumor sites may influence differing responses to treatment and affect the levels of biomarkers that may be predictive of the response to treatment. Although immunohistochemical testing of EGFR has been shown not to be an optimal method for identifying patients who may respond to treatment with anti-EGFR drugs (16), there are preliminary data suggesting that the expression in tumor tissue of Her3 (15), amphiregulin (42), and TGF- α (31) correlates with sensitivity and resistance to EGFR TKI therapy. The immunohistochemical overexpression of Her3 in NSCLC tissue specimens has been correlated with EGFR TKI sensitivity (15). In contrast, increased expression of amphiregulin and TGF- α has been correlated with resistance to such therapy (31). In breast cancer, the transmembrane expression of neuroregulin has been correlated with improved survival in patients treated with Her2 inhibitor (43).

An increase in *EGFR* gene copy number, including high polysomy and gene amplification (as shown by FISH), has been detected in 22% of patients with surgically resected (stages I-IIIa) NSCLC (21). Higher frequencies (40-50%) of *EGFR* high copy number have been reported in patients with more-advanced metastatic NSCLC (stage IV; refs. 44–48). In the present study, we have identified even a higher frequency (62%) of gain in *EGFR* copy number in surgically resected primary NSCLC specimens from patients who developed brain metastases. Recently, we reported that a gain in *EGFR* gene copy number was detected in 74% of primary NSCLC tumors from patients who developed brain metastasis (25). Altogether, these data suggest a stepwise increase in the frequency of gain in *EGFR* copy number in primary tumors with increasing tumor stage and, more important, with the development of brain metastasis. Interestingly, in our cases, the presence of *EGFR* amplification, along with membrane EGFR protein overexpression, was significantly correlated with shorter time to brain metastasis development in the univariate analysis, further suggesting the important role of this genetic abnormality in the progression and metastasis of NSCLC.

Recently, we (20) and others (49) have shown that *EGFR* copy number gain, and specifically gene amplification, is a late phenomenon in the development of lung adenocarcinoma, appearing at invasive tumor stages and progressing in lymph node metastases, and that it is preceded by gene mutation. In the present study, we have expanded some of these observations to NSCLC brain metastasis. Although it was not statistically significant, we found that brain metastases of lung adenocarcinomas had a higher frequency of *EGFR* amplification than the corresponding primary tumors (30% versus 10%). Although a relatively high level (84%) of concordance for gain in *EGFR* copy number (when high polysomy and amplification were analyzed together) was detected when primary tumors and metastases were compared, there were nine discordant cases (16%), including six brain metastases that had increased copy numbers, whereas primary tumors did not. In contrast, we found that *EGFR* gene amplification had a low level of concordance (33%) when primary and metastatic

tumors were compared, indicating a high level of heterogeneity for this phenomenon. The distinct rate of *EGFR* gene amplification between primary tumors and corresponding brain metastases may support the influence of this phenomenon on differing responses to treatment and may affect the assessment of this specific biomarker for anti-EGFR therapy.

The low frequency of *EGFR* mutations in exons 19 and 21 detected in our series of 42 primary NSCLC and corresponding metastases examined, including 30 adenocarcinomas, did not allow us to compare differences between both tumor sites. The single case having *EGFR* mutation (exon 19, point mutation) in the primary tumor showed identical mutation in the metastasis. The concordance on *EGFR* mutation between primary tumors and brain metastases has been previously reported in NSCLC in Japanese patients (50).

In summary, our findings indicate that NSCLC brain metastases exhibit important differences in abnormalities related

to the HER family receptors from primary lung tumors. These differences may cause different responses to EGFR and other HER receptor-targeted therapy of primary and metastatic tumor sites and suggest that the site of origin (primary versus metastasis) of the tumor specimen should be factored into the biomarker analyses in the clinical trials testing the efficacy of HER receptor inhibitor in patients with metastatic NSCLC. Although our series of cases is relatively small and restricted to one metastatic site per patient, the data strongly suggest that the analysis of both primary and metastasis tumor sites may be critical for the identification of novel therapeutic targets and corresponding predictive biomarkers in lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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