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EGFR and HER-2 status of non-small cell lung cancer brain metastasis and corresponding primary tumor

J. S. KOO, S. H. KIM*

Department of Pathology, Yonsei University College of Medicine, Severance Hospital, Seoul, South Korea, e-mail: paxco@yuhs.ac

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We investigated EGFR and HER-2 status in brain metastatic non-small cell lung cancer (NSCLC) and compared them to EGFR and HER-2 status of primary NSCLC. Evaluated were 66 cases of brain metastatic NSCLC, including 20 cases of corresponding primary NSCLC. HER-2 status was investigated by immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH), and EGFR status was evaluated by IHC. HER-2 overexpression and/or amplification was/were observed in three cases (4.5 %) of 66 cases of brain metastatic NSCLC, and 23 cases (34.8%) demonstrated EGFR overexpression. Among 20 cases of primary and corresponding metastatic NSCLC, one case showed HER-2 overexpression and amplification in both primary and metastatic tumor. On the other hand, EGFR overexpression was noted in four cases of primary NSCLC and nine cases of metastatic NSCLC. Five cases showed EGFR gain in metastatic NSCLC. Brain metastatic NSCLC demonstrated different expression patterns of the abovementioned biomarkers, particularly EGFR when compared to primary NSCLC. Therefore, HER-2 and EGFR status are suggested to be evaluated in brain metastatic NSCLC for targeted monotherapy.

Key words: brain metastasis, EGFR, lung neoplasm, HER-2, immunohistochemistry

Although the development of new chemotherapeutic agents and surgical skills, lung cancer is still one of the malignant tumors with high mortality [1]. Particularly, brain metastasis is one of important factors that determine morbidity and mortality of lung cancer patients [2, 3]. Therefore, the effective treatment of brain metastasis of lung cancer is essential for improving clinical symptoms and prolonging survival. However, the effective treatment modality for brain metastasis has not been developed [4, 5]. In this circumstance, it is thought that targeted therapy which is directed to a specific biomarker of lung cancer could be very effective. It is reported that selective overexpression of EGFR and/or HER-2 was observed in non-small cell lung cancer (NSCLC). Overexpression of EGFR is observed in 40-80% of NSCLC [6-8] and overexpression of HER-2 in 16-30% of NSCLC [9-11]. Overexpression of EGFR and/or HER-2 is reported to be a prognostic and predictive factor of NSCLC [10-16]. Synchronous expression of both EGFR and HER-2 is associated with a high recurrence rate and lower overall survival in NSCLC [17]. In contrast, with higher sensitivity to EGFR tyrosine kinase inhibitor (TKI) such as gefitinib, EGFR TKI is reported to improve response rate, disease control rate, time to progression and overall survival in NSCLC with strong HER-2 and EGFR expression confirmed by FISH or IHC [13, 18]. Although the status of EGFR and HER-2 is important to prognosis and prediction of NSCLC, the status for EGFR and HER-2 in brain metastasis has not been thoroughly investigated [19-21].

The purpose of this study is to investigate EGFR and HER-2 status in paired primary and brain metastatic NSCLC by immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH) and to study clinicopathologic implication of EGFR and HER-2 status in paired primary and brain metastatic NSCLC.

Patients and methods

Patient selection and analysis of clinicopathologic parameters. From the files of the Department of Pathology in Severance Hospital, tissue samples from patients with metastatic NSCLC in the brain were retrieved. Tissue samples of corresponding primary NSCLC were included if available. The study was approved by the Institutional Review Board of Severance Hospital. All patients were diagnosed as metastatic carcinoma by pathologists. All tissues were fixed in 10% buffered formalin and embedded in paraffin. All archival hematoxylin and eosin (H&E)–stained slides for each case

were reviewed by two pathologists. Histologic parameters were evaluated from the H&E–stained slides. Clinicopathologic parameters evaluated in each tumor included patient age, sex, tumor recurrence, metastasis-free interval, relapse-free interval, survival and the overall length of survival.

Immunohistochemistry and interpretation. Immunohistochemical stain was performed with formalin-fixed, and paraffin-embedded tissue sections. We obtained 5μm-thick sections with a microtome, transferred into adhesive slides, and dried at 62°C for 30 min. After incubation with primary antibodies against HER-2 (1:1500; DAKO, Glostrup, Denmark) and EGFR (1:50; Novocastra, UK), immunodetection was performed with biotinylated antimouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as substrate. Slides were counterstained with Harris hematoxylin. HER-2 staining was scored according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0, no immunostaining; 1+, weak incomplete membranous staining in any proportion of tumor cells; 2+, complete membranous staining, either nonuniform or weak staining in at least 10% of tumor cells; and 3+, uniform intense membranous staining in > 30% of tumor cells. Cases showing HER-2 0 or 1+ were considered negative. Cases showing HER-2 2+ were considered equivocal and cases showing HER-2 3+ were considered positive for overexpression. In addition, FISH study for the amplification of HER-2 was performed with cases showing HER-2 2+ or 3+. EGFR staining was scored as below; 0, no membrane staining; 1+, faint, partial membrane staining; 2+, weak, complete membrane staining in >10% of tumor cells; 3+, intense complete membrane staining in >10% of tumor cells. Tumors with a score of 2+ or 3+ were interpreted as positive for overexpression.

FISH. FISH analysis (Vysis pathvision c-erbB2 probe + DAKO FISH histology accessory kit) was performed manually. In brief, sections from formalin-fixed, paraffin-embedded tissue were mounted on Superfrost Plus slides, deparaffinized in xylene, and subsequently rehydrated in ethanol. Afterward, they were boiled for 10 min in pre-treatment solution, incubated with pepsin solution for 10 min, dehydrated in ethanol for 6 min, and finally air-dried. For hybridization, the buffered probe (Her-2/neu and centromere 17) was brought onto the slide and protected by a coverslip that was sealed with rubber cement. For denaturation, slides were heated to 82°C and incubated overnight at 45°C in a dark humidified chamber. The rubber cement and coverslip were then removed, and the slides were transferred to stringent wash buffer for 10 min at 65°C. Then, they were dehydrated in ethanol for 6 min and air-dried. Finally, they were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Signals were evaluated using an epifluorescence microscope (Olympus, Japan) equipped with a fluorescein, Cy3, and DAPI filter set and 100 W mercury lamp. Signals were counted out according to the Vysis manual (the Her-2/neu gene appears as orange and centromere 17 as green). We counted signals in at least 20 tumor nuclei in two separate regions of the tissue section according to the ASCO/CAP guideline. As proposed by the ASCO/CAP guideline, an absolute HER-2 gene copy number lower than four or HER-2 gene/chromosome 17 copy number ratio (HER-2/Chr17 ratio) of less than 1.8 was considered HER-2 negative; an absolute HER-2 copy number between four and six or HER-2/Chr17 ratio between 1.8 and 2.2 was considered HER-2 equivocal; and an absolute HER2 copy number greater than 6 or HER-2/Chr17 ratio higher than 2.2 was considered HER-2 positive.

Statistical analysis. Data were processed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL). For determination of the significance of various parameters between cases with overexpression and/or amplification of HER-2 and cases without overexpression and/or amplification of HER-2. Student's *t* test was used for continuous variables, and Fisher's exact test for categorical variables. Significance was assumed when $p < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Clinicopathologic characteristics and HER-2/EGFR status of 66 cases of brain metastatic NSCLC. Table 1 demonstrates the clinicopathologic characteristics and HER-2/EGFR status of 66 cases of metastatic NSCLC in the brain. Three patients (4.5%) showed HER-2 overexpression or amplification, and 23 patients (34.8%) demonstrated EGFR overexpression. Out of three patients with HER-2 overexpression or amplification, two patients also demonstrated EGFR overexpression. Two patients showed 3+ HER-2 overexpression in IHC and HER-2 amplification in FISH study, and one patient displayed 2+ HER-2 expression and HER-2 amplification in FISH study. The mean age of patients showing HER-2 overexpression or amplification was higher than that of patients showing no HER-2 overexpression or amplification without significant difference ($p = 0.439$). As such, patients with EGFR overexpression tended to be older than patients without EGFR overexpression ($p = 0.064$). All three patients with HER-2 overexpression or amplification were male, and patients with EGFR overexpression also showed male dominance (65.2%). There was no significant difference between metastatic NSCLC with HER-2 overexpression or amplification and metastatic NSCLC without HER-2 overexpression or amplification in clinicopathologic parameters such as histologic subtype ($p = 0.964$), T stage ($p = 0.467$), N stage ($p = 0.630$), M stage ($p = 0.628$), and treatment modality ($p = 0.800$). Like HER-2 status, EGFR status did not give rise to significant difference in clinicopathologic parameters such as histologic subtype ($p = 0.131$), T stage ($p = 0.732$), N stage ($p = 0.305$), M stage ($p = 0.536$), and treatment modality ($p = 0.624$). Normal brain tissue did not show EGFR and HER-2 expression.

Table 1. Clinicopathologic characteristics and HER-2/EGFR status of 65 cases of brain metastatic NSCLC.

Parameters	Total N = 66 (%)	HER-2 status			EGFR status		
		Overexpressed or amplified n = 3 (%)	Not Overexpressed or not amplified n = 63 (%)	p value	Overexpressed n = 23 (%)	Not Overexpressed n = 43 (%)	p value
Age (years, mean ± SD)	56.8 ± 10.9	61.6 ± 4.6	56.6 ± 11.1	0.439	60.2 ± 10.0	55.0 ± 11.0	0.064
Sex				0.117			0.273
Male	37 (56.1)	3 (100)	34 (54.0)		15 (65.2)	22 (51.1)	
Female	29 (43.9)		29 (46.0)		8 (34.8)	21 (48.9)	
Histologic subtype				0.964			0.131
AD	46 (69.7)	2 (66.7)	44 (69.8)		13 (56.5)	33 (76.7)	
SC	19 (28.8)	1 (33.3)	18 (28.6)		10 (15.8)	9 (20.9)	
LC	1 (1.5)		1 (1.6)			1 (2.3)	
Time to metastasis (months, mean ± SD)	11.3 ± 15.3	10.0 ± 17.3	11.3 ± 15.3	0.882	11.0 ± 12.7	11.4 ± 16.7	0.908
Follow-up duration (months, mean ± SD)	28.7 ± 21.7	15.6 ± 17.6	29.4 ± 21.8	0.289	28.0 ± 17.7	29.0 ± 23.7	0.866
T stage[a]				0.467			0.737
1	4 (6.1)		4 (6.3)		2 (8.7)	2 (4.7)	
2	21 (31.8)	1 (33.3)	20 (31.7)		8 (34.8)	13 (30.2)	
3	6 (9.1)	1 (33.3)	5 (7.9)		3 (13.0)	3 (7.0)	
4	22 (33.3)		22 (34.9)		7 (30.4)	15 (23.8)	
X	13 (19.7)	1 (33.3)	12 (19.0)		3 (13.0)	10 (15.9)	
N stage[a]				0.630			0.305
0	14 (21.2)	1 (33.3)	13 (20.6)		8 (34.8)	6 (9.5)	
1	6 (9.1)		6 (9.5)		1 (4.3)	5 (7.9)	
2	23 (34.8)		23 (36.5)		8 (34.8)	15 (23.8)	
3	10 (15.2)	1 (33.3)	9 (14.3)		3 (13.0)	7 (16.3)	
X	13 (19.7)	1 (33.3)	12 (19.0)		3 (13.0)	10 (23.3)	
M stage[a,b]				0.628			0.536
0	31 (47.0)	1 (33.3)	30 (47.6)		12 (19.0)	19 (44.2)	
1	35 (53.0)	2 (66.7)	33 (52.4)		11 (17.5)	24 (55.8)	
Treatment modality[c]				0.800			0.624
CTx	24 (36.4)	2 (66.7)	22 (34.9)		6 (26.1)	18 (41.9)	
CTx + RTx	4 (6.1)		4 (6.3)		1 (4.3)	3 (7.0)	
CTx + RTx + Surgery	4 (6.1)		4 (6.3)		2 (8.6)	2 (4.7)	
CTx + Surgery	11 (16.7)		11 (17.5)		6 (26.1)	5 (11.6)	
Surgery	13 (20.0)	1 (33.3)	13 (20.6)		5 (21.7)	9 (20.9)	

[a] Clinical stage for patients without surgery and pathologic stage for patients with surgery.
[b] M stage of the first diagnosis.
[c] Treatment modality for primary lung cancer.
AD, adenocarcinoma; SC, squamous cell carcinoma; LC, large cell carcinoma; CTx, chemotherapy; RTx, radiotherapy.

HER-2/EGFR status of 20 cases of primary NSCLC and corresponding brain metastatic NSCLC. Table 2 shows EGFR status of 20 cases of primary NSCLC and corresponding metastatic NSCLC in the brain. HER-2 status between primary tumor and metastatic tumor showed concordant results (p = 1.000). Only one (5.0%) case demonstrated overexpression and amplification of HER-2 in both primary and metastatic tumor (Fig. 1). EGFR status between primary tumor and metastatic tumor showed significant difference (p = 0.025). Four (20.0%) patients showed overexpression of EGFR in primary

Table 2. HER-2/EGFR status of 20 cases of primary NSCLC and corresponding brain metastatic NSCLC.

Tumor	EGFR status	
	Number of Overexpressed NSCLC (%)	Number of non-overexpressed NSCLC (%)
Primary NSCLC	4 (20.0)	16 (80.0)
Metastatic NSCLC	9 (45.0)	11 (55.0)

NSCLC, non-small cell lung cancer.

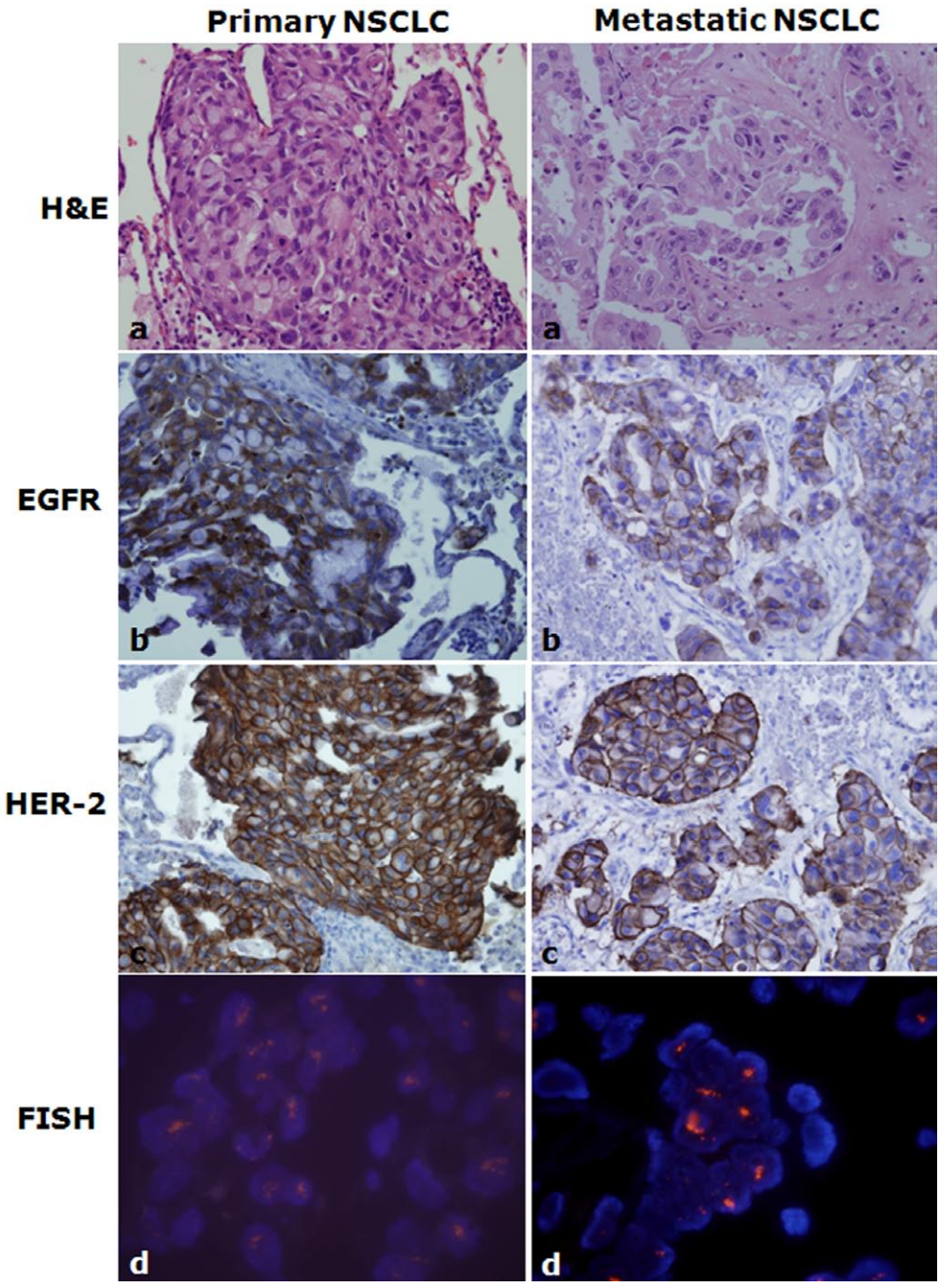

Figure 1. Concordant HER-2 and EGFR status in both primary and corresponding brain metastatic NSCLC. Primary and metastatic tumors show the histology of low differentiated adenocarcinoma (A, X200, H&E). Primary, metastatic NSCLC demonstrate EGFR overexpression (B, X200, EGFR), and HER-2 overexpression (C, X200, HER-2) in IHC. FISH study for HER-2 status shows HER-2 amplification in primary and corresponding metastatic NSCLC (D, X1000, FISH).

tumor, and this overexpression of EGFR was also observed in metastatic tumor (Fig. 1). Five patients (25.0%) did not display overexpression of EGFR in primary tumor, but showed overexpression of EGFR in metastatic tumor (Fig. 2). Among five patients who showed EGFR gain in metastatic tumor, histologic type of four patients was squamous cell carcinoma, and that of the remaining one was adenocarcinoma. *Normal lung tissue did not show EGFR and HER-2 expression.*

Effects of clinicopathologic parameters and HER-2/EGFR status of brain metastatic NSCLC on time to tumor

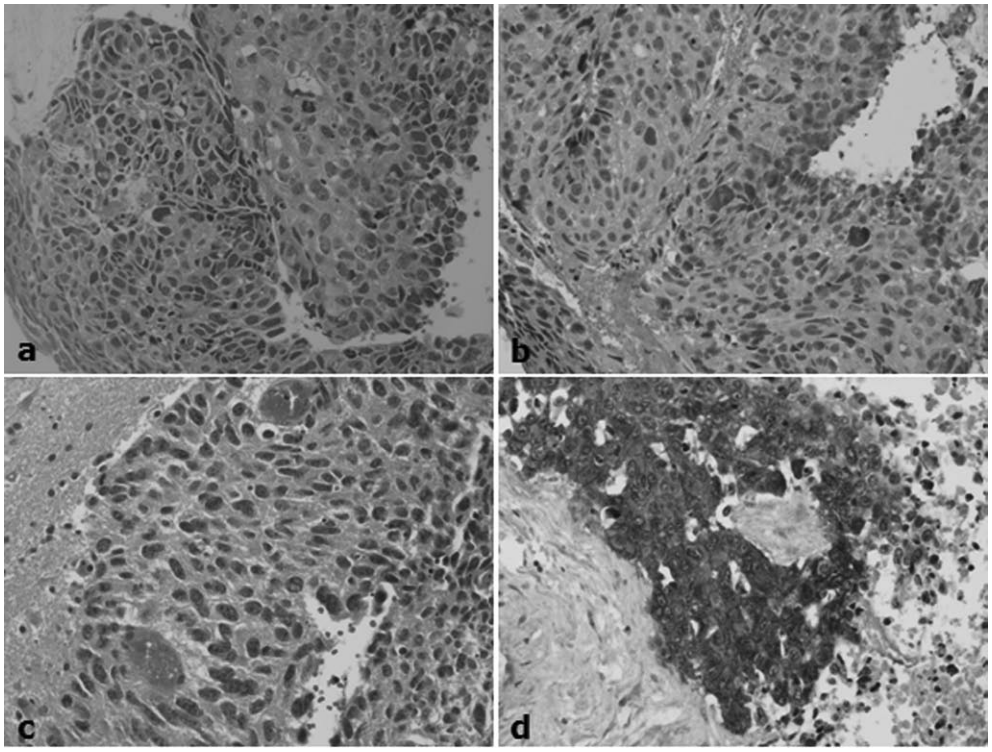

Figure 2. Discordance of EGFR status between primary and corresponding brain metastatic NSCLC. Primary tumor shows histology of squamous cell carcinoma (A, X200, H&E) and no EGFR expression (B, X200, EGFR) in immunohistochemistry. However, brain metastatic squamous cell carcinoma (C, X200, H&E) demonstrates EGFR overexpression (D, X200, EGFR) in immunohistochemistry.

metastasis and time to overall survival. Table 3 shows univariate analyses of clinicopathologic factors and HER-2/EGFR status which were identified in metastatic NSCLC of 66 patients on time to metastasis-free survival and overall survival. The results of univariate analyses of clinicopathologic factors and HER-2/EGFR status on time to brain metastasis revealed significance in only N stage ($p = 0.003$). Namely, patients with lower N stage showed longer metastasis free survival time than patients with higher N stage. Multivariate Cox regression analysis displayed no significance. The results of univariate analyses of clinicopathologic factors and HER-2/EGFR status on time to overall survival revealed significance in M stage ($p = 0.000$). Patients without metastasis in initial stage work-up demonstrated longer overall survival time than patients with metastasis in initial stage work-up. In multivariate Cox regression analysis, there was no significance in any variable.

Discussion

This study investigated HER-2 and EGFR status of brain metastatic NSCLC and evaluated the difference of HER-2 and EGFR status between primary NSCLC and corresponding brain metastatic NSCLC. In 66 patients with brain metastatic NSCLC, three patients (4.5%) showed HER-2 overexpression or/and amplification, and 23 of them (34.8%) demonstrated EGFR overexpression. The previous studies reported 16-30% of HER-2 overexpression [9-11] and 40-80% of EGFR overexpression [6-8] in NSCLC. This study showed that the rate of EGFR overexpression was higher than the rate of HER-2 overexpression in brain metastatic NSCLC, which is consistent with previous reports. However, the rate of HER-2 overexpression in this study was lower than that of HER-2 overexpression in other studies. HER-2 expression criteria of the previous studies were more than 2+ expression, but this study used 3+ expression as HER-2 overexpression. In patients with 2+ HER-2 expression, HER-2 amplification was evaluated by FISH test. The rate of NSCLC with 3+ HER-2 expression in IHC or HER-2 amplification in FISH was 4-8% in previous studies [9-11], which was compatible with the results of this study. HER-2 overexpression in NSCLC was reported to result from chromosome 17 polysomy [9], but this study showed no polysomy in cases with HER-2 overexpression, but HER-2 amplification in all cases with 3+ HER-2 overexpression. Generally, HER-2 expression in NSCLC was most frequently noted in adenocarcinoma, and was associated with shorter survival [9, 10]. However, to our knowledge, the study on HER-2 status in brain metastatic NSCLC has been hardly performed. Because HER-2 overexpression or/and amplification status in brain metastatic NSCLC revealed no association with metastasis free survival and overall survival, HER-2 status in brain metastatic NSCLC

Table 3. Univariate analysis of various clinopathologic and HER-2/EGFR status in metastatic NSCLC of 66 patients on time to metastasis-free survival and overall survival by log-rank test.

Parameters	No. of patients (n = 66) (%)		Metastasis-free survival		Overall survival	
	No. of cases	Patient death	Mean survival (95% CI) months	p value	Mean survival (95% CI) months	p value
Sex				0.157		0.422
Male	37 (56.1)	24 (64.9)	10 (6-13)		52 (38-67)	
Female	29 (43.9)	20 (68.9)	13 (7-20)		60 (45-75)	
Histologic subtype				0.510		0.661
AD	46 (69.7)	30 (65.2)	12 (7-17)		58 (46-69)	
SC	19 (28.8)	14 (73.7)	9 (4-14)		29 (22-36)	
LC	1 (1.5)	0 (0.0)	12 (12-12)		36 (36-36)	
T stage[a]				0.160		0.217
1	4 (6.1)	1 (25.0)	18 (0-46)		28 (0-61)	
2	21 (31.8)	18 (85.7)	16 (9-23)		65 (54-75)	
3	6 (9.1)	4 (66.7)	4 (0-10)		40 (19-60)	
4	22 (33.3)	15 (68.2)	7 (2-12)		39 (27-52)	
X	13 (19.7)	6 (46.2)	13 (4-22)		53 (30-76)	
N stage[a]				0.003		0.582
0	14 (21.2)	10 (71.4)	20 (9-31)		57 (35-78)	
1	6 (9.1)	5 (83.3)	20 (14-26)		54 (42-66)	
2	23 (34.8)	15 (65.2)	8 (3-13)		36 (26-47)	
3	10 (15.2)	8 (80.0)	0 (0-0)		12 (8-16)	
X	13 (19.7)	6 (46.2)	13 (4-22)		53 (30-76)	
M stage[a],[b]				0.000		0.000
0	31 (47.0)	23 (74.2)	24 (19-29)		70 (60-80)	
1	35 (53.0)	21 (60.0)	0 (0-0)		22 (16-29)	
Treatment modality[c]				0.086		0.081
CTx	24 (36.4)	16 (66.7)	5 (0-11)		24 (13-34)	
CTx + RTx	4 (6.1)	3 (75.0)	7 (0-20)		30 (7-53)	
CTx + RTx + Surgery	4 (6.1)	4 (100)	26 (2-50)		42 (19-64)	
CTx + Surgery	11 (16.7)	6 (54.5)	19 (13-24)		45 (32-59)	
Surgery	13 (20.0)	11 (84.6)	18 (9-26)		38 (26-51)	
HER-2 status				0.965		0.173
Overexpressed or amplified	3 (4.5)	1 (33.3)	10 (0-30)		16 (0-32)	
Non-overexpressed or amplified	63 (95.5)	43 (68.3)	11 (8-15)		57 (47-67)	
EGFR status				0.696		0.750
Overexpressed	23 (34.8)	15 (65.2)	11 (6-16)		49 (27-70)	
Non- overexpressed	43 (65.2)	29 (67.4)	11 (6-16)		57 (44-69)	

[a] Clinical stage for patients without surgery and pathologic stage for patients with surgery.
[b] M stage of the first diagnosis.
[c] Treatment modality for primary lung cancer.
AD, adenocarcinoma; SC, squamous cell carcinoma; LC, large cell carcinoma; CTx, chemotherapy; RTx, radiotherapy.

was not an important prognostic factor. Like HER-2 status, EGFR status had no impact on prognosis in this study.

In 20 patients with primary and corresponding metastatic NSCLC, one patient showed HER-2 overexpression and amplification in both primary and corresponding metastatic NSCLC. On the other hand, EGFR overexpression was noted in patients with primary NSCLC and patients with metastatic NSCLC, revealing five patients with EGFR gain in brain metastatic NSCLC. The discordance rate of EGFR status between primary and corresponding metastatic NSCLC was reported to be 32.5-33.0% [19, 20], which was compatible with the discordance rate of this study (25%). While one reported that metastatic NSCLC showed EGFR downregulation compared to primary NSCLC [19], the other study demonstrated that EGFR gain was more frequently noted in metastatic NSCLC than primary NSCLC [21]. Among five cases with EGFR gain in metastatic NSCLC, fourwere squamous cell carcinoma, and there was no significant difference in prognosis between five

cases with EGFR gain and 15 cases without EGFR gain (data not shown).

The significance of HER-2 and/or EGFR status in primary and metastatic NSCLC is the possibility of immunotherapy targeted to theses biomarkers. Indeed, monoclonal antibody such as trastuzumab has been widely used to HER-2 overexpressing breast cancer. EGFR TKI such as gefitinib is the candidate for EGFR immunotherapy, and in clinical trial study, gefitinib gave a rise to a greater treatment response rate and survival time in NSCLC with EGFR expression than NSCLC without EGFR expression [12, 15, 16]. In addition, an increase in survival time was noted in metastatic NSCLC [14]. Irrespective of the method to evaluate EGFR status, NSCLC with EGFR positivity showed better response rate to EGFR TKI. However, EGFR gene copy index measured by FISH showed a stronger association with treatment response rate than EGFR protein expression measured by IHC [12, 15]. HER-2 status in NSCLC is important for not only the possibility to use monoclonal antibody such as trastuzumab, but also the possibility for crosstalk with EGFR. NSCLC with coexpression to EGFR and HER-2 showed greater treatment response rate and survival time to gefitinib therapy than NSCLC without coexpression to EGFR and HER-2 through increased sensitivity to EGFR TKI [13, 18]. Therefore, when the use of EGFR TKI is considered, not only EGFR status but also HER-2 status should be evaluated in NSCLC. One of the important factors to be considered in the EGFR TKI therapy for metastatic NSCLC in the brain is the permeability of blood brain barrier, which shows slight differences according to the type of EGFR TKI. For instance, as reported in the articles on mice experiments, gefitinib cannot easily permeate through the blood brain barrier [22], whereas erlotinib can pass through more easily due to the relatively higher serum concentration [23].

In conclusion, the subgroup of brain metastatic NSCLC showed HER-2 or/and EGFR overexpression. Because brain metastatic NSCLC demonstrated different expression pattern of theses biomarkers, particularly EGFR when compared to primary NSCLC, we carefully suggest that HER-2 and EGFR statuses might be evaluated in brain metastatic NSCLC for targeted monotherapy.

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