Original Article

Vimentin expression status is a potential biomarker for brain metastasis development in *EGFR*-mutant NSCLC patients

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Background: Despite advances in systemic therapy and improvements in survival for advanced epidermal growth factor receptor (*EGFR*) mutant non-small cell lung cancer (NSCLC), brain metastasis (BM) remains a poor outcome. Previous studies on risk factors for BM occurrence included unselected patients and biomarker prediction of BM in these populations were not well studied. We aimed to identify the role of epithelial mesenchymal transition (EMT) marker and clinical factors predicting BM in *EGFR*-mutant NSCLC patients.

Methods: Advanced *EGFR*-mutant NSCLC patients in the King Chulalongkorn Memorial Hospital from January 2013 to December 2017 were included. Vimentin expression was assessed by immunohistochemistry. The correlation between vimentin expression and factors associated with BM occurrence was analyzed by univariate and multivariate analyses.

Results: 304 patients were enrolled. Of these, 149 patients (49%) developed BM. In multivariate analysis, the occurrence of BM was associated with age <60 years, metastatic disease at diagnosis, and 3 or more metastatic sites. Moreover, positive vimentin expression was also found more common in patients with BM than those without BM (52.4% *vs.* 27.6%, respectively) and predicted overall BM development in *EGFR*-mutant patients (OR 2.53, 95% CI, 1.11–5.77; P=0.027). Overall survival (OS) was shorter in vimentin^{positive} group than in vimentin^{negative} group. Median OS was 20.0 months (95% CI, 14.51–25.51) and 30.9 months (95% CI, 20.99–40.84), respectively (HR, 1.57; P=0.04).

Conclusions: Younger patients with *EGFR*-mutant NSCLC who had high disease burden were more likely to develop BM. Vimentin served as a biomarker for predicting BM and poor prognostic factor in *EGFR*-mutant patients. EMT pathway may be considered as a therapeutic target in these high-risk populations.

Keywords: Non-small cell lung cancer (NSCLC); epidermal growth factor receptor (EGFR) mutation; epithelial mesenchymal transition (EMT); vimentin, brain metastasis (BM)

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Introduction

Non-small cell lung cancer (NSCLC) poses a major health problem throughout the world and also in Thailand (1,2). Only 19% of all patients with lung cancers are alive 5 years or more after diagnosis (3). Despite newer targeted agents improving the systemic control of malignancy and hence survival, the incidence of brain metastasis (BM) has still increased. Approximately 10% of NSCLC patients develop BM at presentation, and approximately 40% of all patients develop BM subsequently (4). Incidence is higher in patients, especially whose cancers harbor epidermal growth factor receptor (*EGFR*) mutation, in whom up to 50–60% will develop BM over the course of their disease (5-8). The association between the *EGFR* mutation status and BM in patients with NSCLC has been reported (5,8). Patients with *EGFR* mutations were more likely to develop BM than those with *EGFR* wild type, especially during the course of the disease (9). It remains unclear whether this is because these patients have longer survival times and thus, more time to develop BM, whether there are selective pressure and poor central nervous system (CNS) penetration of systemic therapies, or whether these mutation-driven cancers have biologic features that predispose towards progression and growth within the CNS. Previous studies on risk factors for the development of BM in NSCLC including younger age (8,10-13), female gender (12), non-squamous cell carcinoma (11-14), and more advanced in tumor and nodal stage (8,10,11,14) have been reported, however, most of these studies included unselected patients with NSCLC. Biomarker prediction for BM in these populations is not well studied.

Several studies suggested that molecular factors play an important role in contributing to BM, such as genes involved in cell adhesion, extravasation, metabolism, and cellular signaling (15). Epithelial mesenchymal transition (EMT), a process by which epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties to become mesenchymal stem cells, play a role in the initiation of metastasis. Accumulating evidence has indicated that vimentin is critical for the progression and prognosis of lung cancer (16). Furthermore, activation of *EGFR* expression promoted EMT phenotype in various cancer cell lines, including lung cancer (17,18). Although the correlation between *EGFR* mutation and BM has been widely studied (9,13,19,20), data concerning the association of EMT status and BM development are scare and underlying mechanisms of BM progression in these patients remain poorly understood.

Therefore, we aimed to identify the factors associated with BM in *EGFR*-mutant NSCLC and identify the patients at higher risk for BM development for earlier detection and treatment as well as characterizing the role of EMT marker as a biomarker that can predict the occurrence of BM in *EGFR*-mutant NSCLC. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-1020).

Methods

Study population

This retrospective study enrolled patients who were diagnosed with recurrent/metastatic NSCLC at King Chulalongkorn Memorial Hospital (KCMH) over a period of 5 years (January 1, 2013, to December 31, 2017) and had complete patient medical records on key exposure and outcome variables. The main inclusion criteria were adults aged 18 or older with cytology or histologically confirmed NSCLC who had *EGFR* testing results. The patients were excluded if they had more than one primary cancer, unknown *EGFR* mutation status, had anaplastic lymphoma kinase (ALK) rearrangement or other mutations, and incomplete follow up data. The presence of BM was confirmed by brain radiography, either by computed tomography (CT) or magnetic resonance imaging (MRI). Patients were categorized into initial BM (inBM) if BM was identified at presentation and subsequent BM (subBM) if BM was identified in patients who had negative brain imaging at baseline and were imaged to identify BM when BM associated neurologic symptoms/signs occurred or BM found during or after treatment. The variables include age, gender, Eastern Cooperative Oncology Group (ECOG) performance status (PS), smoking status, histology, initial stage at diagnosis, number of metastatic sites, *EGFR* mutation subtypes, and treatment history were collected. *EGFR* mutations (including G719X in exon 18, exon 19 deletion, T790M in exon 20, and L858R and L861Q in exon 21) were performed by cobas® *EGFR* Mutation Test v2 kit according to the manufacture's protocol. This study was approved by the Institutional Review Board of the Faculty of Medicine at Chulalongkorn University. (No. 267/62). For this retrospective study, the written informed consent from patients was waived per the IRB, and the study was performed following the Health Insurance Portability and Accountability Act and the Declaration of Helsinki (as revised in 2013).

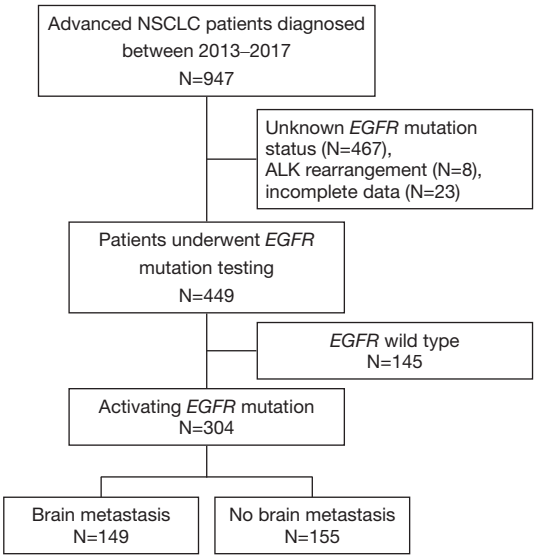

Figure 1 Flow chart of study. Of the 449 non-small cell lung cancer (NSCLC) patients, 304 patients harbored activating epidermal growth factor receptor *(EGFR)* mutation were included in the final analysis.

Immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) tumor samples from the histopathological files of the Department of Pathology, KCMH were retrospectively analyzed. Two-micron thick FFPE tissue sections on charged glass slides were prepared per standard protocol for IHC. Epitope retrieval was performed on the Dako PT link (Dako, Denmark) and immunostaining was performed using automated staining systems, DakoAutostainer Link48 (Dako, Denmark) with antibodies against vimentin antibody (Monoclonal Mouse anti-Human Vimentin, clone V9, RTU, Cat no; IR630, Dako Denmark). In accordance with similar thresholds used in previous studies (21), a value of ≥10% positive tumor cells independent of intensity was chosen to define positive expression of vimentin . Briefly, the staining intensity was determined by cytosolic staining for vimentin. Vimentin expression that was equal to or more than 10% of tumor cells with cytoplasmic staining intensity of +1, +2 or +3 was categorized as positive. All slides were evaluated by a pathologist (K.R) who was blinded from patient outcomes.

Statistical analysis

Categorical variables were summarized by frequencies and percentages while continuous variables were reported by the median and interquartile range (IQR). Clinicopathologic factors and treatment outcomes were analyzed in correlation with BM status using Chi-square or Fisher exact test as appropriate. The univariate and multivariate analysis assessed factors associated with the development of BM and analyzed by odds ratio (OR). Overall survival (OS) was defined from the date of recurrent or metastatic NSCLC diagnosis to the date of death or the last contact. Patients who were not deceased were censored on December 31, 2019. Time-to-event was analyzed using the Kaplan-Meier method and was compared between groups by the log-rank test. Hazard ratios (HR) and corresponding 95% confidence intervals (95% CI) were calculated. The P value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, California, USA) and SPSS 23.0 (SPSS Inc, Chicago, Illinois, USA).

Results

Patient characteristics of the study population

449 patients were identified. Of these, 304 patients (67.7%) who had *EGFR* mutations were analyzed (*Figure 1*). Baseline characteristics are summarized in *Table 1*. The median age of the patients was 63 years (IQR 55–70.8), mostly female (65.8%), good ECOG PS 0 to 1 (86.8%) and never smokers (76.6%). The majority of the patients were diagnosed as adenocarcinoma (93.8%), metastatic disease at

Table 1 Baseline characteristics in *EGFR*-mutant patients (N=304)

Characteristics	All (N=304)	no BM (N=155)	inBM (N=73)	subBM (N=76)	P values
Age at diagnosis, n (%)					<0.001*
<60 years	115 (37.8)	41 (35.7)	37 (32.2)	37 (32.2)	
≥60 years	189 (62.2)	114 (60.3)	36 (19.0)	39 (20.6)	
Median (IQR)	63 (55-70.8)	66 (59-73)	59 (51.5-67)	60 (53-67)	
Gender, n (%)					0.622
Male	104 (34.2)	49 (47.1)	27 (26.0)	28 (26.9)	
Female	200 (65.8)	106 (53.0)	46 (23.0)	48 (24.0)	
ECOG PS, n (%)					0.021*
0–1	245 (86.6)	132 (53.9)	56 (22.9)	57 (23.3)	
≥2	38 (13.4%)	17 (44.7)	13 (34.2)	8 (21.1)	
Missing	21	6	4	11	
Smoking status, n (%)					0.617
Never	196 (76.6)	104 (53.1)	48 (24.5)	44 (22.4)	
Current/former	60 (23.4)	29 (48.3)	15 (25.0)	16 (26.7)	
Missing	48	22	10	16	
Histology, n (%)					0.044*
Adenocarcinoma	285 (93.8)	149 (52.3)	64 (22.5)	72 (25.3)	
Non adenocarcinoma	19 (6.2)	6 (31.6)	9 (47.4)	4 (21.1)	
Stage at diagnosis, n (%)					0.095
Recurrent	58 (19.1)	37 (63.8)	10 (17.2)	11 (19.0)	
Metastatic	246 (80.9)	118 (48.0)	63 (25.6)	65 (26.4)	
Number of metastatic site(s), n (%)					<0.001*
1–2 sites	228 (75.2)	124 (54.4)	41 (18.0)	63 (27.6)	
≥3 sites	75 (24.8)	31 (41.3)	31 (41.3)	13 (17.3)	
Missing	1	0	1	0	
EGFR mutation subtypes, n (%)					0.11
Del19	154 (50.7)	84 (54.5)	33 (21.4)	37 (24.0)	
L858R	123 (40.4)	62 (50.4)	28 (22.8)	33 (26.8)	
Others[†]	27 (8.9)	9 (33.3)	12 (44.4)	6 (22.2)	
T790M status, n (%)[‡]					0.296
Positive	63 (52.5)	36 (57.1)	9 (14.3)	18 (28.6)	
Negative	57 (47.5)	26 (45.6)	14 (24.6)	17 (29.8)	
Number of systemic treatment(s), n (%)					0.007*
Supportive care	12 (3.9)	8 (66.7)	4 (33.3)	0	
1-2 regimens	204 (67.1)	111 (54.4)	53 (26.0)	40 (19.6)	
3 regimens or more	88 (28.9)	36 (40.9)	16 (18.2)	36 (40.9)	

Table 1 (*continued*)

Table 1 (*continued*)

Characteristics	All (N=304)	no BM (N=155)	inBM (N=73)	subBM (N=76)	P values
EGFR TKI treatment, n (%)					0.462
Yes	269 (88.5)	135 (50.1)	63 (23.4)	71 (26.4)	
No	35 (11.5)	20 (57.1)	10 (28.6)	5 (14.3)	
Type of EGFR TKI treatment at first treatment, n (%)					0.008*
1st generation	246 (91.4)	130 (52.8)	52 (21.1)	64 (26.0)	
2nd generation	17 (6.3)	3 (17.6)	7 (41.2)	7 (41.2)	
3rd generation	6 (2.2)	2 (33.3)	4 (66.7)	0	
Line of EGFR TKI treatment, n (%)					0.014*
First line	190 (70.6)	101 (53.2)	49 (25.8)	40 (21.1)	
Second or later line	79 (29.4)	34 (43.0)	14 (17.7)	31 (39.2)	

†, Other *EGFR* mutation subtypes included exon 21 insertion (n=8), L861G (n=6), G719X (n=5), S768I (n=1) and any *EGFR* mutations (n=7), respectively. ‡, Only 120 patients who progressed after *EGFR* TKIs treatment were further tested for secondary T790M mutation; *, P<0.05. EGFR, epidermal growth factor receptor; BM, brain metastasis; inBM, initial brain metastasis; subBM, subsequent brain metastasis; IQR, interquartile range; ECOG PS, Eastern Cooperative Oncology Group Performance Status; TKI, tyrosine kinase inhibitor.

diagnosis (80.9%), and 1 or 2 metastatic sites (75.2%). Exon 19 deletion (n=154) and L858R mutation (n=123) were the most common *EGFR* mutation subtypes, accounting for 91.1% of the patients. Other *EGFR* mutation subtypes included exon 21 insertion (n=8), L861G (n=6), G719X (n=5), S768I (n=1) and any *EGFR* mutations (n=7), respectively. Sixty-seven percent of patients received 1 or 2 lines of systemic treatment for the advanced stage of the disease. Of these, 269 patients (88.5%) received *EGFR*-TKIs during the course of the disease and 190 patients (70.6%) were treated with *EGFR*-TKIs as first-line treatment. 91.4% of patients received 1st generation *EGFR* TKI as the first *EGFR* TKI treatment. A total of 120 patients (45.6%) who progressed after *EGFR*-TKIs treatment were further tested for secondary T790M mutation and 63 patients (52.5%) were found to have the T790M mutation. Of these, 58 patients received osimertinib, 3rd generation of *EGFR* TKIs, as subsequent treatment.

Factors associated with the development of BM in patients with EGFR-mutant NSCLC

The median follow-up was 46.42 months (95% CI, 41.34–51.51), 73 patients (24%) experienced BM at diagnosis and 76 patients (25%) developed subBM. Baseline characteristics are reported in *Table 1*.

The clinicopathological factors that were significantly associated with the overall occurrence of BM included age <60 years (OR 2.74, 95% CI, 1.69–4.43, P<0.001), metastatic disease at diagnosis (OR 1.91, 95% CI, 1.05–3.45, P=0.032), and 3 or more metastatic sites (OR 1.69, 95% CI, 0.99–2.87, P=0.05). Multivariate analyses showed that only age <60 years was statistically significantly associated with BM occurrence. However, there was no difference in BM occurrence between the exon 19 deletion and L858R mutation (*Table 2*).

Interestingly, factors associated with the development of BM in patients with *EGFR*-mutant NSCLC differed between those who experienced BM at diagnosis and those who developed BM subsequently. *EGFR*-mutant patients who had inBM were more likely to be younger (<60 years) (OR 2.85, 95% CI, 1.59–5.11, P<0.001), with non-adenocarcinoma histology (OR 3.49, 95% CI, 1.19–10.21, P=0.022), 3 or more metastatic sites (OR 3.02, 95% CI, 1.64–5.56, P<0.001) and had uncommon *EGFR* mutation subtype (OR 3.19, 95% CI, 1.27–7.96, P=0.013) compared to patients without BM. Multivariate analyses revealed that younger patients (<60 years) (OR 2.81, 95% CI, 1.51–5.24, P=0.001) and higher disease burden (≥3 metastatic sites) (OR 3.00, 95% CI, 1.57–5.74, P=0.001) were statistically significantly associated with inBM development. While only age <60 years (OR 2.63, 95% CI, 1.47–4.69, P=0.001) was associated with subBM compared to those without BM (*Table 3*).

Table 2 Univariate and multivariate analysis for clinicopathological factors associated with brain metastasis in *EGFR*-mutant patients

Covariate†	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Age (<60/≥60)	2.74 (1.69–4.43)	<0.001*	2.74 (1.67–4.49)	<0.001*
Gender (Male/female)	1.26 (0.78–2.03)	0.331		
ECOG PS (≥2/0–1)	1.44 (0.72–2.86)	0.295		
Smoking (current-former/never)	1.20 (0.67–2.15)	0.522		
Histology (Non-ADC/ADC)	2.37 (0.87–6.42)	0.089	1.99 (0.69–5.70)	0.199
Stage at diagnosis (M1/M0)	1.91 (1.05–3.45)	0.032*	1.83 (0.98–3.41)	0.058
No. metastatic site (≥3/<3)	1.69 (0.99–2.87)	0.050*	1.65 (0.95–2.87)	0.075
EGFR subtype (Del19/L858R)	1.18 (0.73–1.90)	0.493		
EGFR subtypes (others/common)	2.22 (0.96–5.13)	0.060	1.95 (0.80–4.73)	0.137

†, Category after the slash (/) was set as reference category. *, P<0.05. EGFR, epidermal growth factor receptor; ADC, adenocarcinoma; ECOG PS, Eastern Cooperative Oncology Group Performance Status; M1, metastatic disease; M0, recurrent disease; OR, odds ratio.

Systemic treatment may contribute to the occurrence of subBM in patients with EGFR-mutant NSCLC

We analyzed whether systemic treatment is associated with subBM occurrence. Among 231 patients with *EGFR*-mutated NSCLC without BM at diagnosis, 223 patients (96.5%) received systemic treatment for the advanced stage of the disease. Of these, 206 patients (89.2%) received *EGFR*-TKIs during the course of the disease and 141 patients (68.4%) were treated with *EGFR*-TKIs as first-line treatment. 54 of 101 patients who progressed after *EGFR*-TKIs treatment were found to have secondary T790M mutation and 57 patients received 3rd generation of *EGFR* TKIs as subsequent treatment.

Patients who received 3 lines or more of systemic treatment (OR 2.84, 95% CI, 1.58–5.12, P<0.001) and did not receive *EGFR*-TKI as first-line treatment (OR 2.30, 95% CI, 1.25–4.23, P=0.007) were associated with subBM. However, these treatment factors were not statistically significantly associated with subBM occurrence after adjusting for other clinicopathological factors (Table S1). Outcomes of *EGFR*-TKI were also analyzed by the time to subBM (TTSBM). Treatment of *EGFR*-TKIs had longer time to subBM than those patients who did not receive *EGFR*-TKIs (median TTSBM was 51.78 months *vs.* 26.61 months, P=0.002; Figure S1). Cox regression analysis was performed on the factors that would correlate with TTSBM. Multivariate analyses revealed that the treatment of *EGFR* TKIs could delay the occurrence of subBM more than those who did not receive *EGFR*-TKIs (HR 2.18, 95% CI, 1.18–4.02, P=0.013; Table S2).

Outcome of BM in patients with EGFR-mutant NSCLC

At data cut-off on December 31, 2019, the median follow-up time was 46.42 months (95% CI, 41.34–51.51) and 80 patients (26.3%) survived to the last contact. The median OS of the overall study cohort was 22.97 months (95% CI, 20.98–24.95). Patients with BM had a significantly shorter OS than patients without BM (median OS was 22.44 months (95% CI, 19.76–25.12) *vs.* 24.18 months (95% CI, 20.41–27.95), respectively; HR 1.48; 95% CI, 1.14–1.93, P=0.004; *Figure 2A*).

Of the 304 patients with *EGFR*-mutant NSCLC, 268 patients (88.2%) received *EGFR* TKIs whereas 36 patients (11.8%) did not. Patients without BM who received *EGFR* TKIs had longer survival than those with BM who received *EGFR* TKIs, and those with/without BM but not received *EGFR* TKIs (median OS were 25.13, 22.96, 12.22 and 14.39 months, respectively; P<0.001; *Figure 2B*).

Vimentin expression status as one of EMT marker predicts the development of BM in patients with EGFR-mutant NSCLC

To validate whether vimentin expression status as a predictive marker for BM occurrence, we next analyzed vimentin expression by IHC on 190 available tumor specimens according to *EGFR* mutation status. Baseline characteristics of these 190 patients are listed in Table S3.

Table 3 Univariate and multivariate analysis for the occurrence of BM at diagnosis and subsequent BM in *EGFR* mutant patients

	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Initial BM occurrence covariate[†]				
Age (<60/≥60)	2.85 (1.59–5.11)	<0.001*	2.81 (1.51–5.24)	0.001*
Gender (Male/female)	1.27 (0.70–2.27)	0.422		
ECOG PS (≥2/0–1)	1.80 (0.82–3.95)	0.142		
Smoking (current-former/never)	1.12 (0.55–2.28)	0.753		
Histology (Non-ADC/ADC)	3.49 (1.19–10.21)	0.022*	2.55 (0.76–8.49)	0.127
Stage at diagnosis (M1/M0)	1.97 (0.92–4.23)	0.080	2.03 (0.87–4.72)	0.101
No. metastatic site (≥3/<3)	3.02 (1.64–5.56)	<0.001*	3.00 (1.57–5.74)	0.001*
EGFR subtype (Del19/L858R)	1.15 (0.63–2.10)	0.649		
EGFR subtypes (others/common)	3.19 (1.27–7.96)	0.013*	2.36 (0.85–6.57)	0.098
Subsequent BM occurrence covariate[†]				
Age (<60/≥60)	2.63 (1.48–4.68)	0.001*	2.63 (1.47–4.69)	0.001*
Gender (Male/female)	0.79 (0.44–1.41)	0.429		
ECOG PS (≥2/0–1)	1.09 (0.44–2.66)	0.851		
Smoking (current-former/never)	1.30 (0.64–2.63)	0.460		
Histology (Non-ADC/ADC)	1.38 (0.37–5.04)	0.626		
Stage at diagnosis (M1/M0)	1.85 (0.88–4.87)	0.101	1.84 (0.86–3.92)	0.111
No. metastatic site (≥3/<3)	0.82 (0.40–1.68)	0.599		
EGFR subtype (Del19/L858R)	1.21 (0.68–2.14)	0.517		
EGFR subtypes (others/common)	1.39 (0.47–4.06)	0.547		

[†], Category after the slash (/) was set as reference category. *, P<0.05. BM, brain metastasis; EGFR, epidermal growth factor receptor; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; OR, odds ratio.

Overall, the mean of vimentin expression in our study was 28.5% and the distribution of vimentin expression according to BM status and *EGFR* mutation status was shown in Figure S2. Using a value of ≥10% positive tumor cells, vimentin expression was detected in 83 patients (43.7%) and was found more common in patients with BM than those without BM (53.6% *vs.* 33.3%, respectively; P=0.005). Although vimentin expression was similar between mutated-*EGFR* (40.5%) and those with wild-type *EGFR* (49.3%), respectively, we found a significant association between vimentin expression and the occurrence of BM in patients with *EGFR*-mutant NSCLC (52.4% *vs.* 27.6%, respectively; P=0.006), but not in those with wild type *EGFR* (55.9% *vs.* 42.9%, respectively; P=0.28; Figure S3).

Using multivariate analysis in patients with *EGFR*-mutant NSCLC, the occurrence of BM was significantly associated with the expression of vimentin (OR 2.53, 95% CI, 1.11–5.77; P=0.027; *Table 4*). Moreover, vimentin expression also was statistically significantly associated with subBM occurrence (OR 3.06, 95% CI, 1.15–8.11, P=0.025) and there was a trend of association with inBM occurrence (OR 2.69, 95% CI, 0.88–8.17, P=0.08; *Table 4*). Conversely, there was no association were identified between the vimentin expression and the BM occurrence in those with wild-type *EGFR* (Table S4).

Furthermore, additional analysis using other methods for cut-off point of vimentin expression by receiver operating

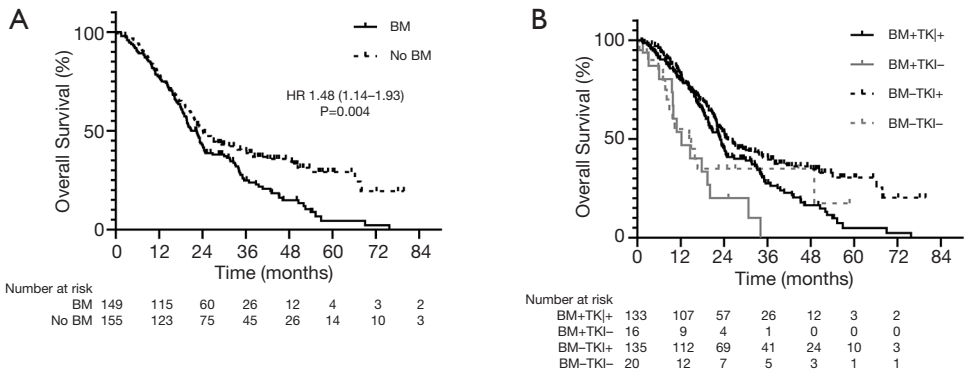

Figure 2 Outcome in patients with epidermal growth factor receptor *(EGFR)*-mutant NSCLC. (A) overall survival (OS) by Kaplan-Meier analysis according to brain metastasis (BM) status (B) OS by Kaplan-Meier analysis according to BM status and treatment with *EGFR* TKIs [patients with BM who received *EGFR* TKIs (BM+TKI+), patients with BM but did not receive *EGFR* TKIs (BM+TKI–), patients without BM who received *EGFR* TKIs (BM–TKI+), and patients without BM but did not receive *EGFR* TKIs (BM–TKI–)].

characteristic (ROC) analysis was also done. The area under the curve (AUC) was 0.604 (95% CI, 0.523–0.685) and the cut-off values were 22.5%. Consistent with our results that using cut-off point at 10% of positive cells, vimentin expression in *EGFR*-mutant but not WT significantly correlated with occurrence of BM (Figure S4, Table S5).

Taken together, these findings indicate that vimentin expression plays a role in the initiation of metastasis and promotes BM occurrence especially in patients with *EGFR*-mutant NSCLC, and maybe serves as a potential biomarker for predicting BM occurrence in these patients.

Prognostic role of vimentin expression in patients with NSCLC

To determine the prognostic role of vimentin expression, we next analyzed the correlation of vimentin expression and OS according to BM and *EGFR* mutation status.

In the overall population regardless of BM status, tumors with positive vimentin expression tended to have shorter OS compared to those with negative expression. Median OS was 19.7 months (95% CI, 14.23–25.19) in vimentin^{positive} and 22.7 months (95% CI, 20.43–24.98) in vimentin^{negative}, respectively (P=0.193). However, tumors with vimentin^{positive} correlated with survival according to *EGFR* mutation status. In the *EGFR*-mutant group, tumors with vimentin^{positive} had a significantly shorter OS than those with negative expression (median OS was 20.0 months (95% CI, 14.51–25.51) *vs.* 30.9 months (95% CI, 20.99–40.84), respectively; HR 1.57; P=0.04). (*Figure 3A*) While there was a similar OS between positive- and negative-vimentin expression in the wild type-*EGFR* group. [median OS was 14.29 months (95% CI, 10.16–18.43 months) *vs.* 11.86 months (95% CI, 7.17–16.55 months), respectively; HR 0.70; P=0.18; *Figure 3B*]. Moreover, in *EGFR*-mutant group, patients with BM and vimentin^{positive} was the worse OS compared to patients with BM and vimentin^{negative}, and patients without BM and positive/negative vimentin expression (median OS were 16.27, 23.76, 50.40 and 38.05 months, respectively; P<0.001).

Discussion

Our study found a high incidence of BM in patients with *EGFR*-mutant NSCLC (49%). Similar results were reported by previous studies (40–64%) (8,18,22-24). Furthermore, *EGFR* mutation not only was associated with overall BM but also predicted subBM (9,18,19,22). As previously mentioned, studies on risk factors for the development of BM in NSCLC have been reported (5,6-8,10-14,25-27), however, most of these studies included unselected patients with NSCLC and studies in *EGFR*-mutant patients were not well evaluated. Our study focused on the factors for the BM occurrence in patients with *EGFR*-mutant NSCLC and found the difference of risk factors between patients who experienced BM at diagnosis and patients who developed BM subsequently. In *EGFR*-mutant patients who had inBM were more likely to be younger (<60 years), had non-adenocarcinoma histology, high disease burden (≥3 metastatic sites), and uncommon *EGFR* mutation subtype whereas only age <60 years was associated with subBM compared to patients without BM. Moreover, systemic

Table 4 Vimentin expression is associated with the occurrence of BM in patients with *EGFR*-mutant NSCLC (N=121)

	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Overall BM occurrence covariate[†]				
Age (<60/≥60)	2.51 (1.15–5.48)	0.021*	2.71 (1.16–6.30)	0.020*
Gender (Male/female)	1.68 (0.76–3.73)	0.197		
ECOG PS (≥2/0–1)	2.03 (0.77–5.31)	0.147		
Smoking (current-former/never)	1.46 (0.55–3.84)	0.436		
Histology (Non-ADC/ADC)	2.85 (0.28–28.20)	0.370		
Stage at diagnosis (M1/M0)	2.48 (1.06–5.80)	0.035*	1.65 (0.65–4.16)	0.283
No. metastatic site (≥3/<3)	3.12 (1.25–7.77)	0.014*	3.27 (1.21–8.77)	0.019*
EGFR subtype (Del19/L858R)	0.55 (0.26–1.17)	0.120		
EGFR subtypes (others/common)	1.16 (0.29–4.56)	0.828		
Vimentin (positive/negative)	2.88 (1.35–6.16)	0.006*	2.53 (1.11–5.77)	0.027*
Initial BM occurrence covariate[†]				
Age (<60/≥60)	2.75 (1.07–7.01)	0.034*	3.06 (1.02–9.18)	0.045*
Gender (Male/female)	2.09 (0.81–5.39)	0.125		
ECOG PS (≥2/0–1)	1.90 (0.61–5.87)	0.264		
Smoking (current-former/never)	1.46 (0.46–4.68)	0.518		
Histology (Non-ADC/ADC)	4.07 (0.35–46.83)	0.260		
Stage at diagnosis (M1/M0)	2.63 (0.87–7.92)	0.085	1.40 (0.39–5.07)	0.600
No. metastatic site (≥3/<3)	6.25 (2.22–17.57)	0.001*	7.56 (2.35–24.27)	0.001*
EGFR subtype (Del19/L858R)	1.13 (0.47–2.75)	0.785		
EGFR subtypes (others/common)	2.07 (0.48–8.96)	0.327		
Vimentin (positive/negative)	3.00 (1.19–7.52)	0.019*	2.69 (0.88–8.17)	0.080
Subsequent BM occurrence covariate[†]				
Age (<60/≥60)	2.31 (0.92–5.78)	0.072	2.25 (0.84–6.02)	0.106
Gender (Male/female)	1.36 (0.52–3.55)	0.522		
ECOG PS (≥2/0–1)	2.18 (0.70–6.83)	0.178		
Smoking (current-former/never)	1.46 (0.46–4.68)	0.518		
Histology (Non-ADC/ADC)	1.78 (0.10–29.45)	0.687		
Stage at diagnosis (M1/M0)	2.36 (0.84–6.68)	0.103		
No. metastatic site (≥3/<3)	1.38 (0.43–4.41)	0.578		
EGFR subtype (Del19/L858R)	0.40 (0.16–0.99)	0.046	0.38 (0.14–0.97)	0.044*
EGFR subtypes (others/common)	0.42 (0.45–3.94)	0.449		
Vimentin (positive/negative)	2.78 (1.14–6.81)	0.024*	3.06 (1.15–8.11)	0.025*

[†], Category after the slash (/) was set as reference category. *, P<0.05. BM, brain metastasis; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; OR, odds ratio.

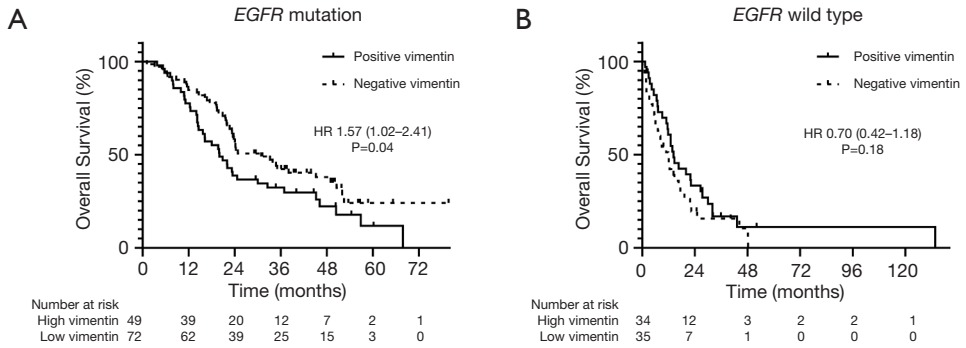

Figure 3 Prognostic role of vimentin expression in patients with NSCLC. OS by Kaplan-Meier analysis according to vimentin expression in (A) *EGFR*-mutant patients and (B) *EGFR* wild type patients

treatment may contribute to subBM in these patients. The high frequency of subBM in *EGFR*-mutated NSCLC can be mainly attributed to better response to systemic treatment which leads to longer survival and thus, probably increases the risk of subBM development. Our study also found an association between subBM occurrence and patients who received multiple lines of systemic treatment. We found that *EGFR*-TKIs could delay the occurrence of subBM more than patients who did not receive *EGFR*-TKIs.

It remains unclear whether the *EGFR*-mutation-driven cancers have biologic features that predispose towards progression and growth within the CNS. Accumulating evidence has indicated that vimentin is critical for the progression and prognosis of lung cancer (16) and preclinical studies suggested that the activation of *EGFR* expression promoted EMT phenotype in various cancer cell lines, including lung cancer (17,18). Activating *EGFR* mutation enhances cell mobility and promotes vimentin expression, a hallmark of mesenchymal cells. The analyses of tumor samples revealed the association between *EGFR* mutation status and vimentin expression (18). Recently, AXL, a receptor tyrosine kinase belonging to the TAM (TYRO3/AXL/MER) family, and its ligand GAS6, growth arrest-specific gene 6, has been reported to have a potential key role in various processes, including epithelial to mesenchymal transition (28,29). High expression of AXL/GAS6 has been found to be poor prognostic biomarker for NSCLC patients with BM. However, the role of EMT marker in BM from patients with *EGFR*-mutant NSCLC and its potential prognostic importance have not been well identified. Herein, the analyses of tumor samples from our cohort supported the correlation of vimentin expression and BM occurrence in patients with *EGFR*-mutated NSCLC. Overexpression of vimentin has been observed in nearly 45% of patients in this study and found more common in patients with BM than those without BM. We also found the association between vimentin expression and the occurrence of BM especially in patients with *EGFR*-mutant NSCLC and conferred worse survival outcome in these patients. To best of our knowledge, this is the first study to identify the vimentin expression as one potential biomarker for poor outcome of BM among patients with *EGFR*-mutant NSCLC. Because this biomarker can be readily established in clinical practice using widely available IHC methods with reasonable cost, our findings may have clinical implications as this potential biomarker can predict BM occurrence and serve as a therapeutic target in these patients.

There are several limitations to our study. First, patient selection and information bias may have occurred due to the retrospective nature and single center setting of the study. Second, there was a different strategy to define patients with BM due to a lack of routine brain imaging in asymptomatic patients. Third, the possibility of vimentin expression discordance between the primary and metastatic sites may influence our results. There was a various cut-off point of IHC interpretation for vimentin expression when comparing our result to other studies. Therefore, interpretation should be done more carefully for future study. Finally, there are several markers related to EMT process, including epithelial markers (E-cadherin, N-cadherin), transcription factors that repress E-cadherin expression (Snail, Twist) and mesenchymal markers (vimentin) (30). Combination assessment of these EMT-related biomarkers to explore the clinical significance of distinct EMT phenotype in these patients would add additional information.

In conclusion, younger patients with *EGFR*-mutant

NSCLC who had high disease burden were more likely to development of BM. Vimentin serves as a biomarker predicting BM and poor prognostic factor in *EGFR*-mutant patients. Our findings may have important implications for treatment and follow-up strategies in these high-risk patients. Vimentin may be a prognostic factor and therapeutic target for BM in patients with *EGFR* mutant NSCLC.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/tlcr-20-1020

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tlcr-20-1020

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr-20-1020). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Institutional Review Board of the Faculty of Medicine at Chulalongkorn University. (No. 267/62). For this retrospective study, the written informed consent from patients was waived per the IRB, and the study was performed following the Health Insurance Portability and Accountability Act and the Declaration of Helsinki (as revised in 2013).

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Supplementary

Table S1 The association of systemic treatment and subsequent BM occurrence among 231 patients with *EGFR*-mutated NSCLC without BM at diagnosis

Covariate[†]	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Age (<60/≥60)	2.63 (1.48–4.68)	0.001*	3.22 (1.68-6.15)	<0.001*
Gender (male/female)	0.79 (0.44–1.41)	0.429		
ECOG PS (≥2/0–1)	1.09 (0.44–2.66)	0.851		
Smoking (current-former/never)	1.30 (0.64–2.63)	0.460		
Histology (non-ADC/ADC)	1.38 (0.37–5.04)	0.626		
Stage at diagnosis (M1/M0)	1.85 (0.88–4.87)	0.101	1.58 (0.71-3.53)	0.257
No. metastatic site (≥3/<3)	0.82 (0.40–1.68)	0.599		
EGFR subtype (Del19/L858R)	1.21 (0.68–2.14)	0.517		
EGFR subtypes (others/common)	1.39 (0.47–4.06)	0.547		
T790M status (negative/positive)[‡]	1.48 (0.65–3.35)	0.350		
No. lines of treatment (≥3/1–2)	2.84 (1.58–5.12)	<0.001*	1.82 (0.89-3.73)	0.101
TKIs (no/yes)	1.55 (0.73–3.27)	0.251		
TKIs as first treatment (no/yes)	2.30 (1.25–4.23)	0.007*	1.77 (0.86-3.67)	0.119
Generation of TKIs(first/others)	2.32(0.65–8.34)	0.197		
First generation of TKIs (gefitinib/erlotinib)	1.02 (0.52–2.01)	0.955		
Subsequent 3rd generation TKIs (no/yes)^	1.14(0.18–7.40)	0.889		

[†], Category after the slash (/) was set as reference category. [‡], Only 101 patients who progressed after *EGFR*-TKIs treatment were further tested for secondary T790M mutation. *, P<0.05; ^, 58 patients received 3rd generation of *EGFR* TKIs as subsequent treatment. BM, brain metastasis; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; OR, odds ratio.

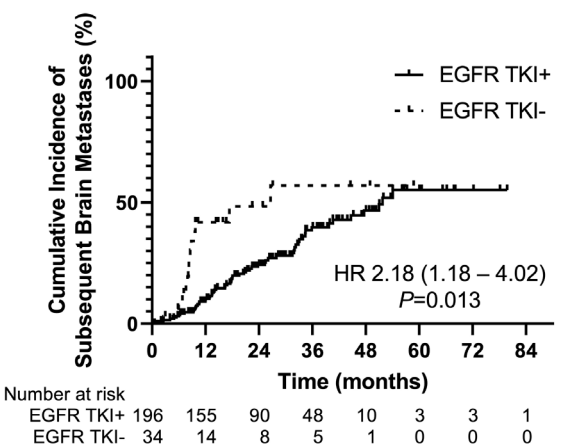

Figure S1 Estimated cumulative incidence curves illustrating subsequent brain metastasis over time according to *EGFR* TKIs treatment. EGFR, epidermal growth factor receptor.

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Table S2 Factors associated with time to subsequent BM (TTSBM) among 231 patients with *EGFR*-mutated NSCLC without BM at diagnosis

Covariate[†]	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (<60/≥60)	1.89 (1.21–2.97)	0.005*	1.66 (1.03–2.66)	0.036*
Gender (male/female)	1.41 (0.89–2.26)	0.147		
ECOG PS (≥2/0–1)	1.57 (0.74–3.31)	0.237		
Smoking (current-former/never)	1.77 (0.99–3.15)	0.053		
Histology (Non-ADC/ADC)	1.62 (0.59–4.46)	0.352		
Stage at diagnosis (M1/M0)	2.37 (1.24–4.50)	0.009*	2.51 (1.31–4.79)	0.006*
No. metastatic site (≥3/<3)	1.13 (0.62–2.06)	0.695		
EGFR subtype (Del19/L858R)	0.83 (0.52–1.32)	0.430		
EGFR subtypes (others/common)	2.26 (0.97–5.27)	0.059		
T790M status (negative/positive)[‡]	1.60 (0.81–3.13)	0.173		
No. lines of treatment (≥3/1-2)	1.43 (0.90–2.25)	0.127		
TKIs (no/yes)	2.45 (1.37–4.41)	0.003*	2.18 (1.18–4.02)	0.013*
TKIs as first treatment (no/yes)	1.27 (0.79–2.04)	0.323		
Generation of TKIs (first/others)	1.74 (0.70–4.37)	0.235		
First generation of TKIs (gefitinib/erlotinib)	1.04 (0.59–1.85)	0.886		
Subsequent 3rdgeneration TKIs (no/yes)^	1.19 (0.85–1.68)	0.304		

[†], Category after the slash (/) was set as reference category. [‡], Only 101 patients who progressed after *EGFR*-TKIs treatment were further tested for secondary T790M mutation. *, P<0.05; ^, 58 patients received 3rd generation of *EGFR* TKIs as subsequent treatment. BM, brain metastasis; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; HR, hazard ratio.

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Table S3 Baseline characteristics for the 190 available tumor specimens for vimentin expression by IHC according to *EGFR* mutation status

Characteristics	*EGFR* mutation (N=121)	*EGFR* wild type (N=69)
Age at diagnosis, median (IQR)	63.8 (55.3–73.1)	61.1 (54.4–67.5)
Gender, n (%)		
Male	36 (29.8%)	47 (68.1%)
Female	85 (70.2%)	22 (31.9%)
ECOG PS, n (%)		
0–1	93 (80.9%)	56 (84.8%)
≥2	22 (19.1%)	10 (15.2%)
Missing	6	3
Smoking status, n (%)		
Never	84 (80.0%)	21 (35.0%)
Current/former	21 (20.0%)	39 (65.0%)
Missing	16	9
Histology, n (%)		
Adenocarcinoma	117 (96.7%)	59 (85.5%)
Non adenocarcinoma	4 (3.3%)	10 (14.5%)
Stage at diagnosis, n (%)		
Recurrent	31 (25.6%)	33 (47.8%)
Metastatic	90 (74.4%)	36 (52.2%)
Number of metastatic site(s), n (%)		
1–2 sites	92 (76.0%)	60 (89.6%)
≥3 sites	29 (24.0%)	31 (10.4%)
EGFR mutation subtypes, n (%)		
Del19	62 (51.2%)	N/A
L858R	50 (41.3%)	
Others	9 (7.4%)	
Number of systemic treatment(s), n (%)		
Supportive care	0	20 (29%)
1–2 regimens	85 (70.2%)	38 (55.1%)
3 regimens or more	36 (29.8%)	11 (15.9%)
Brain metastases, n (%)		
Brain metastases	63 (52.1%)	34 (49.3%)
No brain metastases	58 (47.9%)	35 (50.7%)
Vimentin expression, n (%)		
Positive	49 (40.5%)	34 (49.3%)
Negative	72 (59.5%)	35 (50.7%)

EGFR, epidermal growth factor receptor; IQR, interquartile range; ECOG PS, Eastern Cooperative Oncology Group Performance Status.

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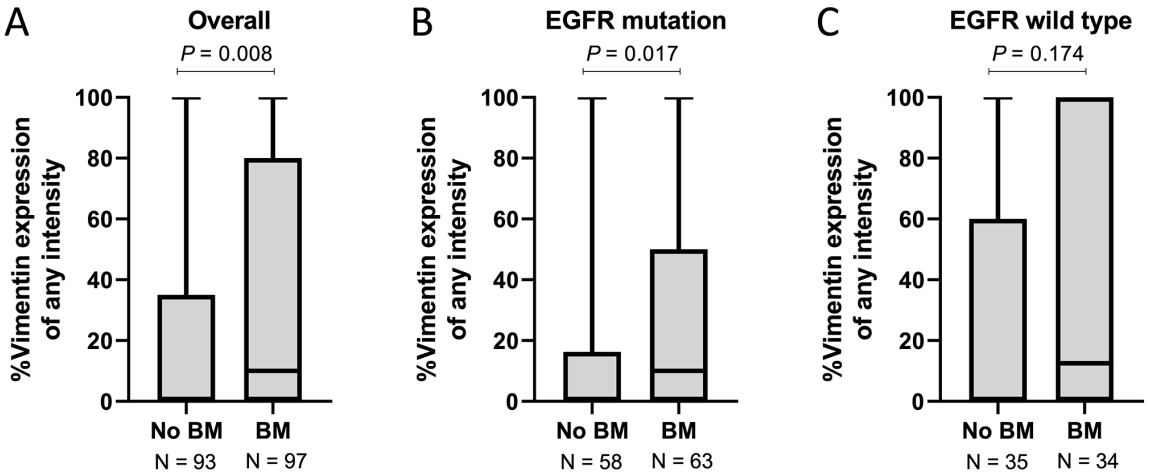

Figure S2 Distribution of vimentin expression according to BM status and *EGFR* mutation status in patients with NSCLC. BM, brain metastasis; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

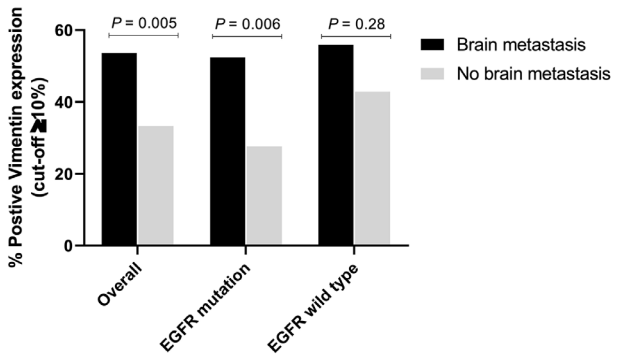

Figure S3 Correlation between vimentin expression according to BM status and *EGFR* mutation status in patients with NSCLC. BM, brain metastasis; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

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Table S4 Vimentin expression is associated with occurrence of BM in patients with wild-type *EGFR* (N=69)

Overall BM occurrence covariate[†]	Univariate	
	OR (95% CI)	P value
Age (<60/≥60)	0.66 (0.26–1.71)	0.394
Gender (Male/female)	1.64 (0.59–4.58)	0.343
ECOG PS (≥2/0–1)	2.69 (0.63–11.49)	0.181
Smoking (current-former/never)	2.88 (0.95–8.72)	0.062
Histology (Non-ADC/ADC)	1.66 (0.42–6.50)	0.466
Stage at diagnosis (M1/M0)	0.84 (0.33–2.17)	0.722
No. metastatic site (≥3/<3)	0.40 (0.07–2.23)	0.295
Vimentin (positive/negative)	1.68 (0.65–4.37)	0.281

[†], Category after the slash (/) was set as reference category. BM, brain metastasis; EGFR, epidermal growth factor receptor; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; OR, odds ratio.

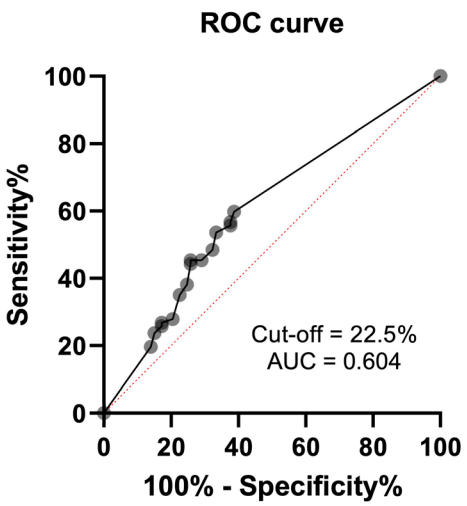

Figure S4 The cut-off value of vimentin expression using ROC curve analysis. ROC, receiver operating characteristic.

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Table S5 Vimentin expression and occurrence of BM using the cut-off value of vimentin expression by ROC analysis

	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Overall populations covariate†				
Age (<60/≥60)	1.46 (0.81–2.62)	0.210		
Gender (Male/female)	1.49 (0.84–2.65)	0.177		
ECOG PS (≥2/0–1)	2.21 (1.00–4.91)	0.051	2.31 (0.92–5.79)	0.076
Smoking (current-former/never)	1.73 (0.91–3.28)	0.095	1.82 (0.92–3.60)	0.085
Histology (Non-ADC/ADC)	1.80 (0.58–5.59)	0.309		
Stage at diagnosis (M1/M0)	1.56 (0.85–2.85)	0.152		
No. metastatic site (≥3/<3)	0.97 (0.93–4.17)	0.078	2.42 (1.03–5.69)	0.042*
Vimentin (positive/negative)	2.39 (1.29–4.41)	0.005*	2.39 (1.21–4.70)	0.012*
Mutant *EGFR* covariate†				
Age (<60/≥60)	2.51 (1.15–5.48)	0.021*	2.93 (1.25–6.86)	0.013*
Gender (Male/female)	1.68 (0.76–3.73)	0.197		
ECOG PS (≥2/0–1)	2.03 (0.77–5.31)	0.147		
Smoking (current-former/never)	1.46 (0.55–3.84)	0.436		
Histology (Non-ADC/ADC)	2.85 (0.28–28.20)	0.370		
Stage at diagnosis (M1/M0)	2.48 (1.06–5.80)	0.035*	1.71 (0.69–4.26)	0.250
No. metastatic site (≥3/<3)	3.12 (1.25–7.77)	0.014*	3.27 (1.21–8.80)	0.019*
Vimentin (positive/negative)	3.07 (1.37–6.87)	0.006*	2.92 (1.22–6.98)	0.016*
Wild-type *EGFR* covariate†				
Age (<60/≥60)	0.66 (0.26–1.71)	0.394		
Gender (Male/female)	1.64 (0.59–4.58)	0.343		
ECOG PS (≥2/0–1)	2.69 (0.63–11.49)	0.181		
Smoking (current-former/never)	2.88 (0.95–8.72)	0.062		
Histology (Non-ADC/ADC)	1.66 (0.42–6.50)	0.466		
Stage at diagnosis (M1/M0)	0.84 (0.33–2.17)	0.722		
No. metastatic site (≥3/<3)	0.40 (0.07–2.23)	0.295		
Vimentin (positive/negative)	1.70 (0.65–4.49)	0.282		

†, Category after the slash (/) was set as reference category. *, P<0.05. BM, brain metastasis; ROC, receiver operating characteristic; ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; M1, metastatic disease; M0, recurrent disease; EGFR, epidermal growth factor receptor; OR, odds ratio.

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