Survival of lung adenocarcinoma patients with malignant pleural effusion

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ABSTRACT: In the era of targeted therapy, the association between lung adenocarcinoma patient survival and malignant pleural effusions (MPEs) remains unclear. This study investigated the clinical characteristics, survival and epidermal growth factor receptor (EGFR) gene (*EGFR*) mutation status of lung adenocarcinoma patients with MPE.

From June 2005 to December 2010, consecutive pleural effusions were collected prospectively. Patient clinical characteristics, *EGFR* mutation status, and overall survival were analysed.

We collected MPEs from 448 patients in stage IV lung adenocarcinoma at initial diagnosis. Median overall survival for patients with MPEs at initial diagnosis and following disease progression were 14.3 months and 21.4 months, respectively (p=0.001). There were 296 (66.1%) patients harbouring *EGFR* mutations, the mutation rates among patients with an MPE at initial diagnosis and one following disease progression were 68.2% and 56.6%, respectively (p=0.044); the L858R mutation rate was also higher among the former (32.6% *versus* 18.1%; p=0.009). Multivariate analysis revealed that patients who: developed MPEs following disease progression, harboured *EGFR* mutations, and received EGFR-tyrosine kinase inhibitor therapy, had longer overall survival. Patients in stage IV lung adenocarcinoma with MPEs at initial diagnosis have shorter overall survival and higher *EGFR* mutation rate, especially for L858R, than patients who develop MPEs following disease progression.

KEYWORDS: *EGFR* mutation, EGFR-TKIs, gefitinib, lung cancer, pleural effusion

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Pleural effusion is associated with diseases including malignancies, infections, auto-immune diseases and trauma [1]. Carcinomas of the lung, breast and lymphomas frequently cause malignant pleural effusions (MPEs). Lung adenocarcinoma is especially associated with MPEs [2], indicating advanced stage disease or disease progression.

Thoracentesis is necessary for the diagnosis and treatment of MPEs. Cancer cells in MPEs can be collected *via* thoracentesis, instead of through other more invasive procedures, such as biopsy or surgery [3]. Testing cancer cells for epidermal growth factor receptor (EGFR) gene (*EGFR*) mutation status may aid in predicting the response of EGFR-tyrosine kinase inhibitor (TKI) therapy [3]. Our previous reports have demonstrated that direct sequencing using cell-derived RNA from cell pellets of centrifuged MPEs was a sensitive detection method for *EGFR* mutation, without using the complicated procedures necessary to isolate cancer cells [3, 4]. This is because inflammatory and mesothelial cells within MPEs have considerably lower *EGFR* expression in comparison with the overexpression of *EGFR* in nonsmall cell lung cancer (NSCLC) cells [4–6]. The differential expression enriches mutant *EGFR* from tumour cells and minimises the dilution of wild-type *EGFR* content from non-tumour cells [4].

According to the 7th edition of the tumour-node-metastasis (TNM) staging system, the International Association for the Study of Lung Cancer (IASLC) reclassified pleural dissemination from T4 to M1, including malignant pleural or pericardial effusions and pleural nodules [7]. Therefore, NSCLC patients with MPEs at initial diagnosis are now classified as having stage IV disease. Stage IV is further divided into M1a and M1b (distal metastasis), depending on the site of metastasis. Among stage IV patients, it is unknown whether there are differences in the demographics or survival outcomes between patients with MPEs at initial diagnosis and those who develop MPEs following disease progression.

One known predictor for survival is the presence of *EGFR* mutations in NSCLC patients, because they are associated with a higher response rate to EGFR-TKIs [8, 9]. However, acquired resistance develops eventually [10–13]. In approximately half of the NSCLC patients, a secondary *EGFR* mutation, T790M in exon 20, is detected after acquiring resistance to EGFR-TKIs [10, 11]. Although the detection of secondary mutations after acquiring resistance to EGFR-TKIs has been well established [10, 11, 14], whether *EGFR* mutations change after treatment with conventional cytotoxic chemotherapeutic agents is unknown. Furthermore, reports have found that *EGFR* mutation status may differ between paired primary and metastatic tumours [15–17]. Therefore, serial tissue sampling should be conducted to identify changes in *EGFR* mutation status.

This study investigates the clinical characteristics, survival and *EGFR* mutation status of lung adenocarcinoma patients with MPEs.

MATERIALS AND METHODS

Patients and tissue procurement

We consecutively collected pleural fluid samples from patients who received thoracentesis in the chest ultrasonography examination room of the National Taiwan University Hospital (NTUH) from June 2005 to December 2010. This study was approved by the institutional review board (IRB) of the NTUH Research Ethics Committee (IRB approval number: 983700221). Informed consents for the use of specimens in molecular studies were obtained. Cytological examinations of pleural effusions were performed. Malignant cell-positive pleural effusions were diagnosed as MPEs. Pulmonary adenocarcinoma was confirmed by pathology reports for biopsy of the primary tumours or cell blocks of MPEs with positive thyroid transcription factor-1 stains [18]. Cytology-proven MPEs caused by lung adenocarcinoma were all included and analysed for *EGFR* mutations. In our previous studies on *EGFR* mutations, materials from 225 patients were examined [3, 4].

Patient clinical information was recorded, including demographics, treatment regimens and treatment response. Patients who had smoked <100 cigarettes in their lifetimes were categorised as never-smokers [19]. Lung cancer cytology and pathology was classified according to the International Multidisciplinary Classification of Lung Adenocarcinoma criteria [18]. Disease stage was determined according to the IASLC TNM (7th Edn) staging system [7]. The unidimensional method was used to evaluate treatment response according to the Response Evaluation Criteria in Solid Tumours guidelines (version 1.1) [20]. Overall survival was defined as the period from the start date of taking first-line systemic treatment to the date of death.

Collection of pleural effusion fluid and sequencing of EGFR exons 18–21

We collected pleural fluid into heparinised tubes. A 10-mL sample of the fluid was centrifuged at $250 \times g$ for 10 min at 4°C, and the total cell pellets were frozen in RNAlater (Qiagen, Hilden, Germany). The processing of samples (*i.e.* from sampling to freezing) took <2 h as previously reported [4]. RNA was extracted from whole-cell lysate using Tri-reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA) and Qiamp RNA Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA was isolated and stored at -80°C until use.

Spectrophotometry was used to measure the amount of RNA extracted. The Qiagen OneStep reverse transcription (RT)-PCR kit (Qiagen) was used to obtain cDNA from extracted RNA, and exons 18–21 of *EGFR* were amplified. The primers and conditions of RT-PCR have been described previously [3, 4]. PCR amplicons were sequenced using ABI PRISM 3100 or 3700 (Applied Biosystems, Foster City, CA, USA) in both sense and antisense directions.

EGFR *mutation detection of corresponding tumour tissue by DNA sequencing*

For comparison, the use of archival tissue for *EGFR* gene analysis was approved by the NTUH IRB. Tumour specimens, including paraffin blocks of surgical specimens, and fine needle or bronchoscopic biopsies were obtained for mutational analysis. DNA was extracted from tumour samples. Mutational analysis for *EGFR* genes has been described previously [21]; some of the materials have been examined previously and reported in studies of EGFR mutations [15, 21–23].

Statistical analysis

All categorical variables were analysed using Chi-squared tests, except where sample size n<5 required the use of Fisher's exact test. Nonparametric Mann–Whitney U-test was used to compare the median ages of two groups. Overall survival curves were plotted using the Kaplan–Meier method and compared with log-rank test. Multivariate analysis for overall survival was performed using Cox's regression. Two-sided p-values <0.05 were considered significant. All analyses were performed using Statistical Package for the Social Sciences (SPSS) software (version 17.0 for Windows; SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics of lung adenocarcinoma patients with MPEs

Of the 1400 pleural effusions collected, 890 MPEs were confirmed by cytological examination (online supplementary material, fig. S1). The 803 MPE samples from lung adenocarcinomas were analysed for *EGFR* mutation. Because 26 samples had insufficient RNA for RT-PCR and sequencing, 777 (96.8%) MPEs were sequenced for *EGFR* status. The 777 MPEs were obtained from 494 patients. The numbers and timing of MPE sampling between treatments was shown in the online supplementary table S1. The distribution of the lung cancer staging at initial diagnosis was 46 in stage I–III and 448 in stage IV. There were 713 MPEs caused by lung adenocarcinoma obtained from 448 patients with stage IV disease at initial diagnosis of NSCLC.

Of these, 365 (81.5%) had MPEs at initial diagnosis, and 83 (18.5%) developed MPEs following disease progression. There were 244 (54.5%) females and 329 (73.4%) never-smokers. Other clinical characteristics are presented in table 1. Patients with MPEs at initial diagnosis were older (p=0.002) and had poorer Eastern Cooperative Oncology Group performance status (ECOG PS) (p<0.001) than patients with MPEs after disease progression.

In addition, patients were divided into stages M1a and M1b; patient characteristics are demonstrated in table 2. Patients in stage IV M1b had poorer ECOG PS (p=0.024), more lung metastasis (p<0.001), and pericardial effusion (p=0.018). More

TABLE 1	Clinical characteristics of stage IV lung adenocarcinoma patients			
	All patients	**MPEs at initial diagnosis**	**MPEs following disease progression**	**p-value**
Total	448	365	83	
Age years	66.6 (27.9–95.5)	67.9 (27.9–95.5)	61.5 (28.7–92.2)	0.002[#]
Sex				0.354
Female	244	195 (53.4)	49 (59.0)	
Male	204	170 (46.6)	34 (41.0)	
Smoking status				0.265
Never-smokers	329	264 (72.3)	65 (78.3)	
Smokers	119	101 (27.7)	18 (21.7)	
ECOG PS				<0.001
0–1	354	276 (75.6)	78 (94.0)	
2–4	94	89 (24.4)	5 (6)	
Stage IV				0.126
M1a	179	152 (41.6)	27 (32.5)	
M1b	269	213 (58.4)	56 (67.5)	
EGFR				0.044
Wild-type	152	116 (31.8)	36 (43.4)	
Mutation	296	249 (68.2)	47 (56.6)	

Data are presented as n, median (range) or n (%), unless otherwise stated. MPE: malignant pleural effusion; ECOG PS: Eastern Cooperative Oncology Group performance status; EGFR: epidermal growth factor receptor gene. [#]: Mann–Whitney U-test.

pleural tumour seeding at initial diagnosis was noted in patients in stage IV M1a than those in stage IV M1b (p=0.002).

Detection of EGFR mutations from MPEs in stage IV lung adenocarcinoma patients

Of the total 713 samples of MPEs related to lung adenocarcinoma from 448 patients, direct sequencing using cell-derived RNA as the template were used to identify EGFR mutations. Overall, 296 (66.1%) patients had cancer cells harbouring EGFR mutations. Females (72.1% *versus* male 58.8%; p=0.003) and never-smokers (70.8% *versus* smokers 52.9%; p<0.001) had higher EGFR mutation rates.

Patients with MPEs at initial diagnosis had a higher rate of EGFR mutation than those with MPEs following disease progression (68.2% *versus* 56.6%; p=0.044) (table 1). Patients with MPEs at initial diagnosis also had a higher L858R mutation rate (32.6% *versus* 18.1%; p=0.009) (table 3). The proportion of presence of deletion in exon 19 (Del-19) mutation did not differ between the two groups (26.6% *versus* 25.3%) (p=0.812).

Overall survival of patients with stage IV lung adenocarcinoma at diagnosis

The median overall survival for the 448 patients was 16 months. Patients with MPEs at initial diagnosis had shorter overall survival (median 14.3 months) than those with MPEs following disease progression (median 21.4 months; p=0.001) (fig. 1a). Patients whose tumour cells harboured EGFR mutations (median 17.4 months) had longer overall survival than patients whose tumour cells harboured wild-type EGFR (median 10.9 months; p=0.005) (fig. 1b). In addition, patients aged <65 years, never-smokers, patients with better performance status (ECOG PS 0–1), patients with M1a disease status, and patients with EGFR-TKI therapy had longer overall survival (table 4).

To clarify the interaction between EGFR mutation and the timing of MPE development, overall survival of patients whose tumour cells harboured EGFR mutations and those with wild-type EGFR was plotted. Each group was further stratified by the timing of MPE development. Among patients whose tumour cells harboured EGFR mutations, those with MPEs following disease progression (median 27.1 months) had longer overall survival than those with MPEs at initial diagnosis (median 16.3 months; p=0.003) (fig. 1c). In patients whose tumour cells harboured wild-type EGFR, those with MPEs following disease progression (median 16.8 months) also had longer overall survival than those with MPEs at initial diagnosis (median 8.3 months; p=0.021) (fig. 1d).

EGFR mutation status and the use of EGFR-TKI therapy both had an effect on overall survival. Longest overall survival was among patients with EGFR mutations and EGFR-TKI use (18.9 months), followed sequentially by those with wild-type EGFR and who used EGFR-TKI therapy (13.8 months), those with wild-type EGFR and no EGFR-TKI therapy (7.1 months), and those with EGFR mutations and no EGFR-TKI therapy (6.3 months; p<0.001) (fig. 2). For patients with wild-type EGFR, patients without EGFR-TKI therapy were more likely to be smokers (53.5% *versus* 30.3%; p=0.008), to show brain metastasis (25.6% *versus* 11.9%; p=0.038) and adrenal gland metastasis (20.9% *versus* 6.4%; p=0.009) than those using EGFR-TKI therapy (online supplementary data table S2). However, the difference in survival was not statistically significant between patients with and without EGFR-TKI therapy (p=0.065).

To investigate the impact of the timing of MPE development, patients were stratified into stage IV M1a and M1b. Each group was further stratified by the time at which an MPE developed. In patients with stage IV M1a disease, there was no

TABLE 2	Clinical characteristics of stage IV lung adenocarcinoma patients according to M1a and M1b division			
	All patients	**M1a**	**M1b**	**p-value**
Total	448	179	269	
Age years	66.6 (27.9–95.5)	68.0 (28.7–89.6)	66.1 (27.9–95.5)	0.378[#]
Sex				0.147
Female	244	90 (50.3)	154 (57.2)	
Male	204	89 (49.7)	115 (42.8)	
Smoking status				0.439
Never-smokers	329	135 (75.4)	194 (72.1)	
Smokers	119	44 (24.6)	75 (27.9)	
ECOG PS				0.024
0–1	354	151 (84.4)	203 (75.5)	
2–4	94	28 (15.6)	66 (24.5)	
Metastatic sites				
Lung	215	62 (34.6)	153 (56.9)	<0.001
Pleural effusion	365	152 (84.9)	213 (79.2)	0.126
Pleural seeding	148	74 (41.3)	74 (27.5)	0.002
Pericardial effusion	24	4 (2.2)	20 (7.4)	0.018[¶]
Bone	204	0	204 (75.8)	<0.001
Brain	84	0	84 (31.2)	<0.001
Liver	57	0	57 (21.2)	<0.001
Adrenal gland	40	0	40 (14.9)	<0.001
Other	25	0	25 (9.3)	<0.001
MPE				0.126
At initial diagnosis	365	152 (84.9)	213 (79.2)	
Following disease progression	83	27 (15.1)	56 (20.8)	
EGFR				0.506
Wild-type	152	64 (35.8)	88 (32.7)	
Mutation	296	115 (64.2)	181 (67.3)	

Data are presented as n, median (range) or n (%), unless otherwise stated. ECOG PS: Eastern Cooperative Oncology Group performance status; MPE: malignant pleural effusion; *EFGR*: epidermal growth factor receptor. [#]: Mann–Whitney U-test; [¶]: Fisher's exact test.

difference in overall survival between those with MPEs at initial diagnosis (20.9 months) and those with MPEs following disease progression (24 months; p=0.511) (fig. 3a). In patients with stage IV M1b disease, those with MPEs at initial diagnosis (11.8 months) had shorter overall survival than those with MPEs following disease progression (21.4 months; p<0.001) (fig. 3b).

Multivariate analysis using the Cox regression model showed that having MPEs at initial diagnosis (hazard ratios 1.65; p<0.001), M1b (HR 1.79; p<0.001) and ECOG PS 2–4 (HR 2.22; p<0.001) was statistically significantly associated with shorter overall survival. Presence of *EGFR* mutation (HR 0.76; p=0.015) and the use of EGFR-TKI therapy (HR 0.51; p<0.001) were associated with longer overall survival (table 4).

TABLE 3	Differences in epidermal growth factor receptor gene (*EGFR*) mutation rates determined from malignant pleural effusions of stage IV adenocarcinoma at initial diagnosis and following disease progression				
MPE	**Wild-type**	**Del-19**	**L858R**	**Others**	**Total**
At initial diagnosis	116 (31.8)	97 (26.6)	119 (32.6)	33 (9.0)	365
Following disease progression	36 (43.4)	21 (25.3)	15 (18.1)	11 (13.3)	83
Total	152 (33.9)	117 (26.1)	134 (29.9)	45 (10.0)	448

Data are presented as n (%) or n. Each patient is included only once. No patients had different *EGFR* mutations in several malignant pleural effusion (MPE) samples, except acquired T790M *EGFR* mutation. Del-19: deletion in exon 19. MPEs at initial diagnosis *versus* MPEs after disease progression for wild-type and *EGFR* mutations, p=0.044. MPEs at initial diagnosis *versus* MPEs after disease progression for different mutation status, p=0.033. MPEs at initial diagnosis *versus* MPEs after disease progression for L858R *versus* non-L858R (including wild-type, Del-19 and other types), p=0.009.
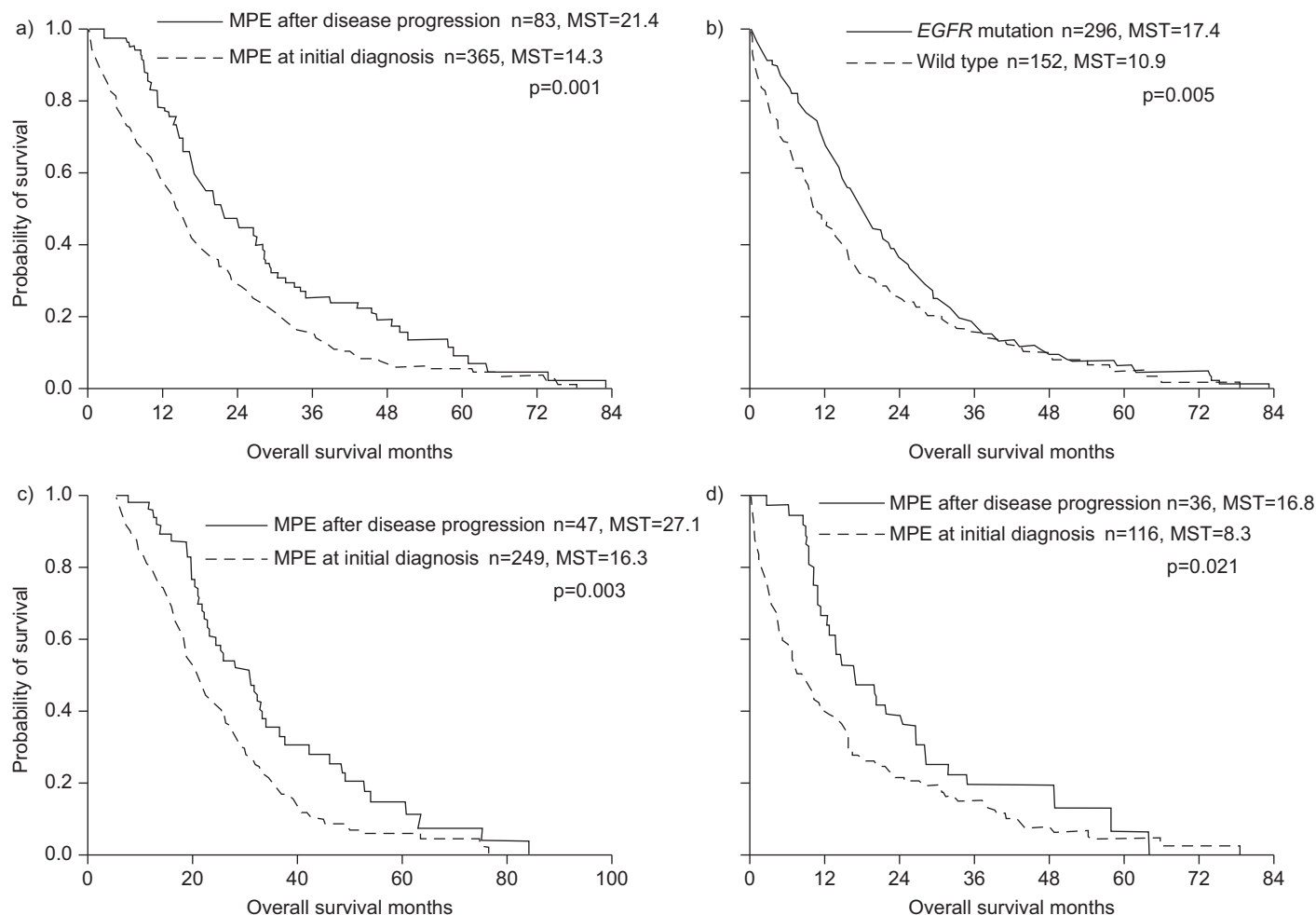
FIGURE 1. Kaplan–Meier curves of overall survival were constructed based on a) the time at which a malignant pleural effusion (MPE) developed, and b) epidermal growth factor receptor gene (*EGFR*) mutation sequencing results. The overall survival curves of stage IV lung adenocarcinoma patients with MPEs at initial diagnosis and those with MPEs following disease progression were plotted separately for patients with c) *EGFR* mutations and d) wild-type *EGFR*. MST: median survival time (months). p-values were calculated using the log-rank test.

No change in EGFR *mutations after conventional chemotherapy*

There were 177 patients from whom two or more MPE samples (range 2–8) were obtained, and all MPE samples from the same patient were analysed for *EGFR* mutations. Among these, between serial samplings, 81 received chemotherapy only. The results of *EGFR* mutation analysis were the same among multiple MPEs from the same patient.

Of the 81 patients receiving chemotherapy only, the median duration between the first and the last MPE sampling was 204 days (range 21–1781 days). 49 patients received one regimen of chemotherapy, 20 patients received two regimens, eight patients received three regimens and four patients received four regimens between serial MPE samplings. The chemotherapy regimens used between serial MPE is shown in the online supplementary data table S3.

EGFR mutation status of the 81 patients included 35 wild-type, 19 Del-19, 18 L858R, two Del-19+T790M, two L858R+T790M, one P772_H773insYNP+H773Y, one R776H+L861Q, one L858R+ E709G, one L858R+A871E and one G719D+L861Q. No acquired

mutation or change of the *EGFR* mutation was detected after chemotherapy, irrespective of response to the cytotoxic agents.

Incidence of T790M before and after EGFR-TKI treatment

MPEs were sampled from 317 patients before they underwent EGFR-TKI treatment. *De novo* T790M mutations were detected in seven (2%) patients by direct sequencing. All seven patients had concomitant L858R mutations. Of these, six patients received EGFR-TKI (four gefitinib and two erlotinib). All six patients had progressive disease, with median progression-free survival after EGFR-TKI therapy of 1.6 months (range 0.2–2.8 months).

99 patients with acquired resistance to EGFR-TKIs had MPEs sampled after EGFR-TKI therapy. Secondary T790M mutations were detected in 48 (48.5%) patients. In 23 (47.9%) out of 48 patients, this secondary mutation was detected in conjunction with L858R mutations, and in 25 (52.1%) patients, it was detected in conjunction with Del-19. Among the 48 patients with secondary T790M mutations, 16 patients underwent MPE sampling before EGFR-TKI treatment. No primary T790M mutations were detected in EGFR-TKI-naïve MPEs.

TABLE 4	Factors affecting overall survival of stage IV lung adenocarcinoma patients with malignant pleural effusions (MPEs)				
	Patients	Overall survival months	Univariate analysis	Multivariate analysis	
			p-value	HR (95% CI)	p-value
Sex					
Female	244	15.6			
Male	204	15.5	0.440	1.00 (0.77–1.30)	0.974
Age					
<65 years	203	18.4			
≥65 years	245	13.2	0.004	1.13 (0.92–1.40)	0.243
Smoking					
Never-smokers	329	16.5			
Smokers	119	12.8	0.009	1.08 (0.81–1.46)	0.597
ECOG PS					
0–1	354	17.4			
2–4	94	6.6	<0.001	2.22 (1.71–2.87)	<0.001
Stage IV					
M1a	179	20.9			
M1b	269	13.8	<0.001	1.79 (1.43–2.24)	<0.001
MPE					
Following disease progression	83	21.4			
At initial diagnosis	365	14.3	0.001	1.65 (1.26–2.15)	<0.001
EGFR					
Wild-type	152	10.9			
Mutation	296	17.4	0.005	0.76 (0.61–0.95)	0.015
EGFR-TKI therapy					
No	80	6.8			
Yes	368	16.8	<0.001	0.51 (0.37–0.69)	<0.001

Data are presented as n, unless otherwise stated. HR: hazard ratio; 95% CI: 95% confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; EGFR: epidermal growth factor receptor; *EGFR*: epidermal growth factor receptor gene; TKI: tyrosine kinase inhibitor.
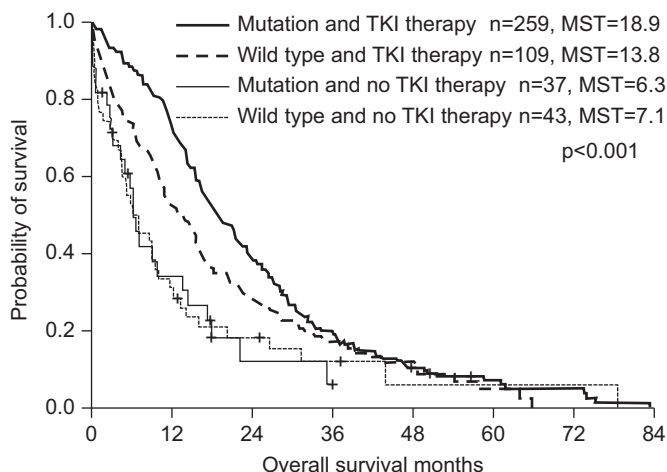

FIGURE 2. Kaplan–Meier survival curve of overall survival for stage IV lung adenocarcinoma patients with malignant pleural effusions. Epidermal growth factor receptor (EFGR) gene (*EGFR*) mutation-positive patients undergoing EGFR-tyrosine kinase inhibitor (TKI) therapy had longer overall survival than wild-type patients without EGFR-TKI therapy, *EGFR* mutation-positive patients without EGFR-TKI therapy, and wild-type patients without EGFR-TKI use (p<0.001). MST: median survival time (months). p-value was calculated using the log-rank test.

Between the patients with (n=48) and without (n=51) secondary T790M mutations, there were no significant differences in clinical characteristics, including sex, age, smoking, ECOG PS, using gefitinib or erlotinib, stage M1a or M1b, the timing of MPE development and prior chemotherapy use (online supplementary data table S4).

Correlation of the mutation analyses between the MPE and the tumour specimens

There were 82 paired MPEs and corresponding tumour specimens. After exclusion of five tumour specimens because of poor quality DNA for *EGFR* mutation analysis, 62 (80.5%) of the 77 paired samples showed concordant *EGFR* mutation; 15 (19.5%) patients had discordant results between MPEs and corresponding tumour specimens (table 5).

DISCUSSION

This large prospective cohort study of MPEs derived from lung adenocarcinoma showed that the clinical characteristics of stage IV lung adenocarcinoma patients with MPEs at initial diagnosis were different from those who developed MPEs following disease progression. *EGFR* mutation rate, especially L858R, was higher in patients with MPEs at initial diagnosis; although all patients had stage IV disease, overall survival for
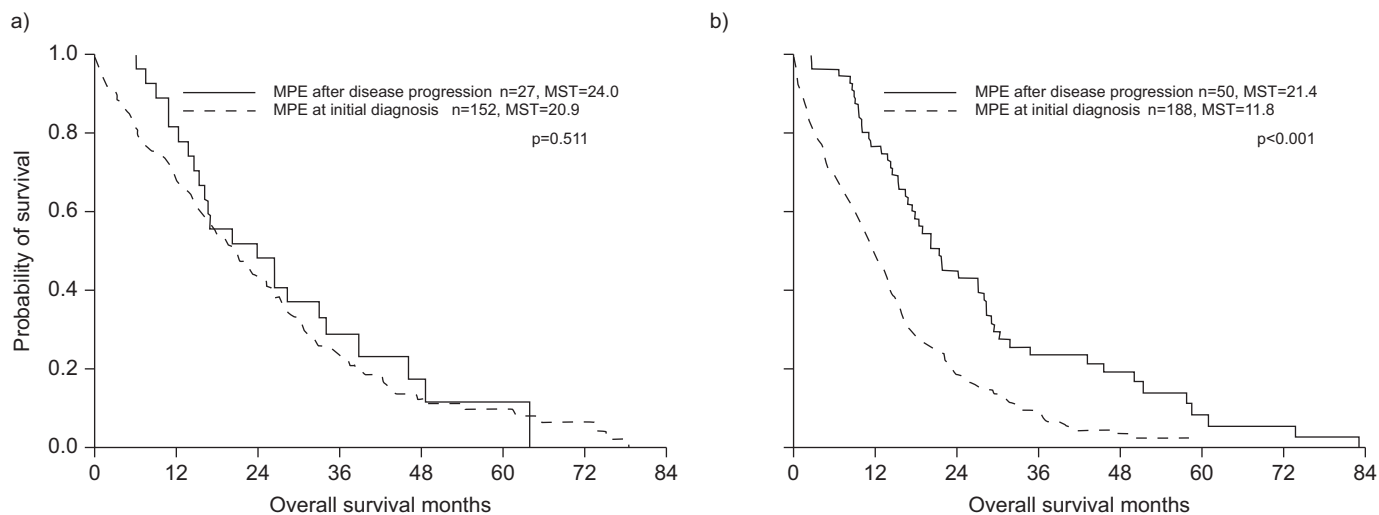
FIGURE 3. Kaplan–Meier survival curves of overall survival for lung adenocarcinoma patients in a) stage IV M1a and b) stage IV M1b. Presence of malignant pleural effusion (MPE) at initial diagnosis or following disease progress was plotted. MST: median survival time (months). p-values were calculated using the log-rank test.

patients with MPEs at initial diagnosis was shorter. Conventional chemotherapy did not change the mutation status or induce *EGFR* mutations.

Compared with tumour specimens, mutation analyses using MPEs had a high concordance rate (table 5). Thoracentesis is an easy procedure for obtaining cells to confirm the presence of MPE. Lung adenocarcinoma patients with MPEs had a higher rate of *EGFR* mutations [3], and a previous report also showed

TABLE 5	Comparison of results of epidermal growth factor receptor gene (*EGFR*) mutation analysis between malignant pleural effusions (MPE) and tumour specimens		
	EGFR mutation analysis		Patients
	MPE	Tumour specimens	
Concordance			62 (80.5)
	Wild-type	Wild-type	24 (31.2)
	Del-19	Del-19	12 (15.6)
	L858R	L858R	18 (23.4)
	Insertion in exon 20	Insertion in exon 20	4 (5.2)
	G719A+S720F	G719A+S720F	1 (1.3)
	G719A+T790M	G719A+T790M	1 (1.3)
	L858R+E709G	L858R+E709G	1 (1.3)
	L858R+A871E	L858R+A871E	1 (1.3)
Discordance			15 (19.5)
	Del-19	Wild-type	7 (9.1)
	L858R	Wild-type	4 (5.2)
	L861Q+G719S	Wild-type	1 (1.3)
	L861Q+E746G	Wild-type	1 (1.3)
	Wild-type	Del-19	1 (1.3)
	Wild-type	L858R	1 (1.3)
Total			77 (100)

Data are presented as n (%), unless otherwise stated.

that direct sequencing using cell-derived RNA was very sensitive and favourable for *EGFR* mutation detection in lung adenocarcinoma MPEs [4]. If tumour specimens were used, less than half of the patients would have adequate specimens to obtain a molecular profile [9, 24]. In addition, because DNA from cancer cells makes only a small fraction of total DNA, using specimens with high proportion of non-neoplastic cells, such as biopsy samples, lowers the sensitivity for mutation analysis [25]. Furthermore, patients are more likely to accept repeated thoracentesis rather than re-biopsy of the primary tumour to detect molecular changes, making thoracentesis an important procedure in monitoring disease status for personalising treatment for lung cancer.

Although the presence of an MPE is a significant prognostic factor for advanced-stage NSCLC patients [26, 27], no studies on the effects of the time at which an MPE developed on the overall survival of a patient have been conducted. In this study, stage IV lung adenocarcinoma patients who developed MPEs following disease progression showed longer overall survival than those with MPEs at initial diagnosis. This effect is observed in patients with *EGFR* mutations and wild-type *EGFR*. However, the difference was only statistically significant in patients with distal metastasis (M1b). For patients without distal metastasis (M1a) there was no difference. In the present study, patients with MPEs at initial diagnosis of stage IV lung adenocarcinoma had overall survival of 16.2 months, which was longer than the previously reported 6.5–8 months [27–29]. The longer overall survival in the present study may result from four possible reasons. First, only patients of adenocarcinoma, which had higher *EGFR* mutations, were included in this study, and those with MPEs had a higher *EGFR* mutation rate [3]. Most patients of the previous studies were from Western countries, where the *EGFR* mutation rate is low. Secondly, patients in previous studies were enrolled before the approval of EGFR-TKI therapy. In this study, a high proportion 368 (82.1%) out of 448 patients received EGFR-TKI treatment. TAKANO *et al.* [30] showed that patients with lung cancer treated after the approval of gefitinib had longer overall survival than those treated before gefitinib

approval. Thirdly, overall survival in phase III trials of EGFR-TKI in Asians ranged from 18.8 months to 22.3 months [31, 32]. Ethnic difference might be responsible for the difference in overall survival. Finally, a selection bias of patient collection could have been introduced into the study. The patients may have a better performance status because they were able to have thoracentesis in the chest ultrasonography examination room.

We previously showed that patients with MPEs associated with lung adenocarcinoma had a higher EGFR mutation rate than that observed in surgically resected specimens [3]. With more patients enrolled in the present study, we found that the overall EGFR mutation rate was similar to that in our previous report [3]. However, patients with MPEs at initial diagnosis had a significantly higher rate of mutation than patients with MPEs after disease progression; in particular, the L858R mutation rate was significantly higher. In our previous study, a higher L858R mutation rate was also observed in MPEs from lung adenocarcinoma than in surgically resected samples. From these observations, it has been postulated that EGFR mutation is an early event in the pathogenesis of lung adenocarcinoma [33]. L858R may play a role in the development of MPE among lung adenocarcinoma patients. Further studies are necessary to clarify the relationship between EGFR mutations and metastasis of lung adenocarcinoma.

Gene mutation could result in acquired resistance to target therapy drugs. In chronic myeloid leukaemia and gastrointestinal stromal tumours, T315I, which causes a protein structural change, is considered to be one of the most common imatinib resistance mutations in BCR-ABL [34–36]. For breast cancer, XIA et al. [37] found that acquired resistance to lapatinib, a potent ErbB2 TKI, occurred when cell survival regulation is changed to depend on both oestrogen receptor and ErbB2. All these acquired resistance-associated mutations developed after patients received target therapy. For NSCLC, the mutations T790M, D761Y and L747S are all considered to be associated with acquired resistance to EGFR-TKIs [10, 38, 39]. For cytotoxic drugs, studies have shown cancers to develop drug resistance. After acquiring resistance to cytotoxic agents, there were several changes in cancer cells, including gene amplification, gene rearrangements, epigenetic changes, and microRNA changes [40]. Apart from one conference poster demonstrating that chemotherapy could influence EGFR mutation status [41], no drug resistance through gene mutation induced by cytotoxic drugs has been documented. Comparing paired cancer cells before and after chemotherapy from MPEs, we found that cytotoxic chemotherapy did not alter EGFR mutation status. This implies that after chemotherapy, EGFR mutant lung cancer cells preserve the sensitive mutations and, therefore, cell response to EGFR-TKI. This finding is consistent with previous reports showing that response rate and progression-free survival in patients with sensitive EGFR mutations were similar whether EGFR-TKI was used as first-line or second-line therapy [22, 42]. A large prospective study would further clarify this effect.

The secondary T790M mutation was reported to occur in 50% of patients with EGFR mutations after EGFR-TKI treatment [11, 43, 44]. In the present study, the secondary T790M mutation rate was 48.5%, which was consistent with the previously reported rate. However, we still cannot predict who will develop secondary T790M mutations after acquired resistance to EGFR-TKIs based on clinical features.

For patients with wild-type EGFR, although our study showed patients undergoing EGFR-TKI therapy had longer survival than those without EGFR-TKI therapy, it did not reach statistical significance (p=0.065). The difference may result from the following reasons. First, the BR.21 study showed a survival benefit of erlotinib in NSCLC patients in all statuses, including patients with wild-type EGFR [45]. A phase-II trial showed that the overall survival after erlotinib treatment was 9.2 months in patients with wild-type EGFR who had received chemotherapy previously [46]. The present study showed that the median overall survival after EGFR-TKI treatment was 8.3 months (95% CI 4.3–12.3 months) in patients with wild-type EGFR, which is compatible with previous reports [46]. Secondly, in the present study, patients with wild-type EGFR and no EGFR-TKI therapy were more likely to be smokers and to have brain metastasis and adrenal gland metastasis than those with wild-type EGFR and EGFR-TKI therapy. Finally, the response rate of EGFR-TKI therapy in patients with wild-type EGFR was 10–20% in previous reports [8, 12, 32, 47, 48].

The present study has some limitations. First, patients enrolled in the present study were all Taiwanese, among whom a high EGFR mutation rate, even among smokers, has been documented [49]. Using resected lung adenocarcinoma samples, HUANG et al. [50] also showed that the EGFR mutation rate was not associated with sex or smoking status in Taiwan. Because of the ethnic uniformity of the study population, the findings of the present study might not be general to other racial or ethnic groups. Secondly, some patients with tumours harbouring EGFR mutations did not receive EGFR-TKI treatment in the present study because EGFR mutation analysis was not routinely performed in clinical practice at the time of sample collection.

In conclusion, stage IV lung adenocarcinoma patients with MPEs at initial diagnosis have a higher EGFR mutation rate, especially for L858R, and shorter overall survival than patients who develop MPEs following disease progression. EGFR mutations and EGFR-TKI therapy are associated with longer overall survival in lung adenocarcinoma patients with MPEs.

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