



Frequent epidermal growth factor receptor gene mutations in malignant pleural effusion of lung adenocarcinoma

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ABSTRACT: Malignant pleural effusions (MPEs) are often observed in lung cancer, especially adenocarcinoma. Epidermal growth factor receptor (*EGFR*) gene mutations are usually detected in lung adenocarcinoma. The purpose of the present study was to investigate the *EGFR* mutation rate in MPEs of lung adenocarcinoma.

Between June 2005 and December 2006, 136 MPEs from lung adenocarcinoma were collected for *EGFR* mutation detection. In addition, between April 2001 and November 2004, 91 surgically resected specimens of lung adenocarcinoma from patients without MPEs were assessed for *EGFR* mutation.

The *EGFR* mutation rate was significantly higher in the patients with MPEs than in the patients without (68.4% versus 50.5%). The *EGFR* mutation rate in patients with MPEs was not associated with sex, smoking history, age or cancer stage. By multivariate analysis, an age of <65 yrs, never smoking, Eastern Cooperative Oncology Group performance status 0–1, and *EGFR* mutation were significantly associated with a longer overall survival for lung adenocarcinoma patients with MPEs.

The patients with malignant pleural effusions related to lung adenocarcinoma had a higher epidermal growth factor receptor gene mutation rate than the patients from whom surgically resected specimens were taken. Epidermal growth factor receptor tyrosine kinase inhibitors may be the treatment of choice for lung adenocarcinoma with malignant pleural effusions in east Asia.

KEYWORDS: Epidermal growth factor receptor, lung cancer, mutation, pleural effusion

Annually, there are ~1.2 million new cases of lung cancer globally, with ~1.1 million patients dying due to the disease [1]. Platinum-based chemotherapy has a partial response in only 30% of patients with advanced nonsmall cell lung cancer (NSCLC) [2].

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are used in the treatment of NSCLC. EGFR TKIs have a higher response in specific subgroups, including: females; never smokers; adenocarcinoma histology; and east Asians [3–5]. These subgroups also have higher *EGFR* mutation rates [6]. A better response to EGFR TKIs and prolonged survival are related to *EGFR* mutations, including in-frame deletions and point mutations [7–9].

Malignant pleural effusions (MPEs) are often observed in lung cancer, especially adenocarcinoma, because it is a tumour that grows in the periphery of the lung and easily invades the pleural cavity [10]. MPEs also indicate an advanced stage of disease or disease progression.

In total, ~15% of patients have pleural effusion at the initial diagnosis of lung cancer [10]. Thoracentesis is necessary for diagnosis and treatment. MPEs can develop as a direct consequence of cancer cell dissemination into the pleural space; however, the exact mechanisms are not fully understood [11]. MPEs may result from the combination of both decreased capacity of the lymphatics to remove fluid and increased pleural fluid formation [11]. The latter mechanism may be related to an increase in the vascular permeability, and vascular endothelial growth factor (VEGF) plays an important role in increasing the permeability of the vasculature [12]. Median VEGF levels in pleural fluid are higher in MPEs than nonmalignant pleural effusions [13]. In addition to VEGF, other molecular factors or tumour markers can also be detected in MPEs. HSIEH *et al.* [14] demonstrated that pigment epithelium-derived factor and fibrinogen precursors are expressed at lower levels in MPEs than in transudates. DAI *et al.* [15] showed that p53 and K-ras gene mutation patterns are effective markers

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for the detection of recurrent lung carcinoma in cytological specimens of pleural effusions.

MPE is collected *via* very easy techniques, in contrast to other invasive techniques, such as biopsy and surgery, which are necessary to obtain tissue of other metastatic sites. Mutations of *EGFR* can also be detected in pleural effusion samples [16]. *EGFR* mutation screening in pleural effusion is useful for the prediction of the clinical outcome of lung cancer patients treated with gefitinib [16, 17]. In order to understand how frequently *EGFR* mutations occur in MPEs of lung adenocarcinoma, the present authors examined the sequences of exons 18–21 of *EGFRs* in MPEs of lung adenocarcinoma. The mutation rate of MPEs and the mutation rate of surgically resected specimens were then compared. In addition, clinical information was collected to analyse the prognostic factors of overall survival in the patients with lung adenocarcinoma with MPEs.

MATERIALS AND METHODS

Patients and tissue procurement

Between June 2005 and December 2006, 383 consecutive samples of pleural effusion were collected from 273 patients who had received thoracentesis in the chest ultrasonography examination room of the National Taiwan University Hospital (Taipei, Taiwan). This study was approved by the institutional review board of the National Taiwan University Hospital, and all patients had signed an informed consent form before the thoracentesis was performed. The primary malignant tumours or MPEs were confirmed by pathology or cytology reports. Of the 273 patients, 164 had lung adenocarcinoma and the other 109 patients had malignancy other than lung adenocarcinoma or nonmalignancy-related pleural effusions. Among the 164 patients with lung adenocarcinoma, the cytology of pleural effusions was negative in 28 patients. Therefore, 136 MPEs of lung adenocarcinoma were assessed for *EGFR* mutations. These 136 MPEs were obtained before treatment with gefitinib, or erlotinib if the patients received *EGFR* TKI therapy. For comparison, 91 surgically resected specimens of lung adenocarcinoma archived from April 2001 to November 2004 were also retrieved. Informed consent about the use of these specimens for future molecular studies with approval of the institutional review board was obtained before surgery. The retrospective use of archival tissue for *EGFR* gene analysis was approved by the institutional review board of the National Taiwan University Hospital. The clinical information of all the patients was recorded, including age, sex, smoking history, lung cancer stage, performance status (Eastern Cooperative Oncology Group performance status (ECOG PS)), treatment regimens and maximal response. Patients who had smoked <100 cigarettes in their lifetime were categorised as never smokers. Those who smoked cigarettes within 1 yr of the diagnosis were categorised as current smokers. The others were categorised as former smokers.

Collection of pleural effusion fluid and surgically resected specimens

The pleural effusion fluid was collected into heparinised tubes. A 1-mL sample of the fluid was centrifuged at $250 \times g$ for 10 min at room temperature, and the cell pellet frozen. Specimens of lung adenocarcinoma tissue obtained at surgery were immediately

snap-frozen in liquid nitrogen and stored for later use. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and stored at -80°C until RNA extraction was performed. RNA was extracted from cell lysates with a Qiaamp RNA Mini Kit (Qiagen, Hilden, Germany) according to the protocol in the manufacturer's instructions. The RNA obtained was eluted in 50 μL of sterile bi-distilled buffer. The amounts of RNA extracted were measured with spectrophotometry.

PCR amplification and direct sequencing

The four exons (exons 18–21) that code for the tyrosine kinase domain of the *EGFR* gene were amplified with a forward primer (5'-AGCTTGTGGAGCCTCTTACACC-3') and reverse primer (5'-TAAAATTGATTCCAATGCCATCC-3'), as reported in a prior study [8]. The RT-PCR was performed previously as reported [8], using a Qiagen OneStep RT-PCR Kit (Qiagen). The RT-PCR conditions were as previously described [8]. RT-PCR amplicons were purified and sequenced using the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing products underwent electrophoresis on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analysed by basic local alignment search tool against the cDNA sequence of the *EGFR* gene (accession number NM005228) and chromatograms were examined manually. PCR amplicons were sequenced in both sense and antisense directions. *EGFR* mutations detected in the initial round of sequencing were confirmed by subsequent rounds of independent RT-PCR and sequencing reactions. Only specimens in which a mutation was identified in both rounds were recorded as mutation positive. Mutations were also checked against the single nucleotide polymorphism database.

Antitumour therapy and response evaluation of the patients with MPEs of lung adenocarcinoma

The antitumour response of the patients was evaluated by chest radiography every 2–4 weeks and by computed tomography of the disease sites every 8–12 weeks after treatment; this is routine practice in the present authors' institution. For the measurable solid tumours, the unidimension method was used according to the Response Evaluation Criteria in Solid tumours (RECIST) guidelines [18]. Partial response and stable disease were confirmed by a sustained 4-week follow-up. Overall survival after antitumour therapy was calculated from the date of initiation of treatment to the date of death, last follow-up or the final follow-up day of the study, which was September 1, 2007.

Toxicity of *EGFR* TKIs was also recorded, and was graded according to the National Cancer Institute Common Toxicity Criteria Version 3.0.

Statistical analysis

All categorical variables were analysed with Pearson's Chi-squared test, except where a small size required the use of Fisher's exact test. Overall survival was compared by the log-rank test, and multivariate analysis for overall survival was performed using the Cox linear regression method. Two-sided *p*-values <0.05 were considered significant.

RESULTS

Clinical characteristics and EGFR mutations of the surgically resected lung adenocarcinoma patients

In total, 91 surgically resected specimens were assessed for the EGFR mutation (table 1). The median (range) age of the 91 patients was 63.4 (37.5–85.4) yrs. Three stage-IIIB patients had satellite tumour nodules within the same lobe as the primary tumour, and they received lobectomies. Two stage-IV patients, who both had two lung lesions over the right middle lobe and right lower lobe without other distant metastasis lesions, received bi-lobectomies.

In total, 46 out of 91 surgically resected lung adenocarcinoma had EGFR mutations. The EGFR mutation rate was 50.5%. The EGFR mutations included 19 L858R, 23 deletions in exon 19 and four other types (767–769 duplication ASV, P771_H773insYNP+H773Y, K860I+L861Q and L861Q). EGFR mutation rates of the surgically resected lung adenocarcinoma were not significantly different by sex (49% of males *versus* 52.5% of females; $p=0.742$), age ($p=0.746$) or smoking status (54.7% of never smokers *versus* 44.7% of former or current smokers; $p=0.348$; table 1).

Of the 91 patients, 14 took EGFR TKIs after tumour recurrence. Of these, seven patients had EGFR mutations, and the other seven patients were wild type. Five patients with a positive EGFR mutation and three patients of wild type responded to EGFR TKI treatment. However, the patient numbers were too small for further analysis.

Clinical characteristics and EGFR mutations of the lung adenocarcinoma patients with MPE

MPEs were assessed for EGFR mutations in 136 patients (table 2). The median (range) age of the 136 patients was 66.1 (28.7–90.6) yrs. Two (1.5%) patients did not complete staging work-up of lung cancer and they were lost to follow-up.

TABLE 1 Patients' characteristics and epidermal growth factor receptor (EGFR) mutation status of the surgically resected specimens				
Variable	Subjects n	EGFR mutation n	Mutation rate %	p-value
Total	91	46	50.5	
Sex				0.742
Male	51	25	49.0	
Female	40	21	52.5	
Age yrs				0.746
≥65	42	22	52.4	
<65	49	24	49.0	
Smoking				0.348
Never	53	29	54.7	
Current/former	38	17	44.7	
Staging				0.2
I	46	27	58.7	
II	15	4	26.7	
III	28	14	50.0	
IV	2	1	50.0	

In total, 93 out of 136 patients with MPEs of lung adenocarcinoma had positive EGFR mutations. The mutation rate was 68.4%. The EGFR mutations included 50 L858R, 32 deletions in exon 19, and 11 other types. The 11 other types included three G719A, one G719A+S720F, one G719A+S768I, one E746V+L747P, one L747P, one 767–769 duplication ASV, one 768–770 duplication SVD, one L861Q and one R776H+L861Q. EGFR mutation rates of the adenocarcinoma patients with MPEs were not significantly different by sex (64.2% of males *versus* 71.1% of females; $p=0.396$), age ($p=0.734$) or smoking status (70.5% of never smokers *versus* 63.4% of former or current smokers; $p=0.413$; table 2).

Of the 136 patients with MPEs, 111 patients initially had pleural effusions at the diagnosis of lung adenocarcinoma. Of the 111 patients, 77 (68.5%) had EGFR mutations. The mutation rate of initial pleural effusions related to lung adenocarcinoma was similar to the total 136 MPEs (68.5% *versus* 68.4%).

The patients with MPEs of lung adenocarcinoma had a higher EGFR mutation rate than the patients with surgically resected lung adenocarcinoma (68.4% *versus* 50.5%; $p=0.007$; table 3). Interestingly, the mutation rates of deletion in exon 19 in MPEs and surgically resected specimens were not significantly different (23.5% *versus* 25.3%). However, the patients with MPEs of lung adenocarcinoma had a higher L858R mutation rate than the patients with surgically resected lung adenocarcinoma (36.8% *versus* 20.9%; $p=0.011$; table 3).

Response of adenocarcinoma with MPEs treated with EGFR TKI

Of the 136 patients with MPEs of lung adenocarcinoma, 71 had received EGFR TKI treatment (gefitinib 250 mg·day⁻¹ or erlotinib 150 mg·day⁻¹). Two patients were lost to follow-up before treatment and the other 63 patients received other antitumour therapy. Of the 71 patients treated with EGFR TKI, 38 had partial response. Those included one wild type, 23 L858R, 11 deletions in exon 19, one G719A, one L861Q and one

TABLE 2 Patient characteristics and epidermal growth factor receptor (EGFR) mutation status of malignant pleural effusions				
Variable	Subjects n	EGFR mutation n	Mutation rate %	p-value
Total	136	93	68.4	
Sex				0.396
Male	53	34	64.2	
Female	83	59	71.1	
Age yrs				0.734
≥65	73	49	67.1	
<65	63	44	69.8	
Smoking				0.413
Never	95	67	70.5	
Current/former	41	26	63.4	
Staging				0.944
IIIB	26	18	69.2	
IV	108	74	68.5	
Unknown	2	1	50.0	

TABLE 3 The difference in epidermal growth factor receptor (*EGFR*) mutations between adenocarcinoma patients with malignant pleural effusions and surgically resected specimens from other adenocarcinoma patients

	Wild type	L858R	Deletion in exon 19	Others	Total
Malignant pleural effusion	43 (31.6)	50 (36.8)	32 (23.5)	11 (8.1)	136
Surgically resected specimen	45 (49.5)	19 (20.9)	23 (25.3)	4 (4.4)	91

Data are presented as n or n (%). $p=0.017$ for MPE versus surgically resected specimens for positive and negative of *EGFR* mutations; $p=0.011$ for MPE versus surgically resected specimens with L858R mutations.

combined *EGFR* mutation (G719A+S720F; table 4). For the overall survival analysis of the 136 patients with lung adenocarcinoma and MPEs, the median overall survival was longer for patients with *EGFR* mutations than for patients with wild-type *EGFR* (median 21.4 months, 95% confidence interval (CI), 17.9–24.9 months versus 11.5 months, 95% CI 7.4–15.6 months; $p=0.005$ by log-rank test). Multivariate analysis was performed by the Cox regression model for the potential prognostic factors, including: age, smoking status, ECOG PS, *EGFR* mutation (table 5). Lung adenocarcinoma patients with MPEs who were aged <65 yrs ($p=0.011$), who never smoked ($p=0.027$), with ECOG PS 0–1 ($p<0.001$) and *EGFR* mutation ($p=0.001$) were found to be associated with a longer overall survival.

The toxicity of *EGFR* TKIs were distributed from grade 1 to grade 3, and no patients had grade 4 toxicity. The most common toxicity included diarrhoea (24%), skin rash (38%), acne (31%), dry skin (22%), pruritus (22%) and anorexia (20%). Grade 3 events included one diarrhea (1%), one skin rash (1%) and one acne (1%). Neither grade 4 toxicity nor interstitial pneumonitis was recorded.

DISCUSSION

In the present study, patients with lung adenocarcinoma with MPEs had a higher *EGFR* mutation rate than patients with surgically resected adenocarcinoma. The *EGFR* mutation rate in MPEs of lung adenocarcinoma was ~70%, regardless of the patients' sex, smoking status or age.

MPEs are a common clinical problem for patients with lung adenocarcinoma. Thoracentesis may not only relieve dyspnoea [19], but is also an easy way to collect malignant cells from pleural effusion for molecular studies, in contrast to other invasive techniques used to biopsy the primary tumour or its metastases in clinical practice. *EGFR* mutations could be detected in MPEs, and are useful for the prediction of response to gefitinib, as shown in the present study and others [16, 17].

The present study revealed a 68.4% *EGFR* mutation rate in lung adenocarcinoma with MPEs, which is higher than in prior published research. SOH *et al.* [16] reported an *EGFR* mutation rate of 24.5% (13 out of 53) in MPEs related to lung adenocarcinoma. However, only deletions in exon 19 and

TABLE 4 Epidermal growth factor receptor (*EGFR*) mutation status and response of *EGFR* tyrosine kinase inhibitor in patients of malignant pleural effusions

Mutation	Complete response	Partial response	Stable response	Progressive disease	Not evaluable	Total
G719A		1		1		2
L747P				1		1
Del E746_A750		8		1		9
Del L747_P753, insS		1				1
Del E746_T751, insVA		1				1
Del L747_A750, insP		1				1
L858R		23	3	1	2	29
L861Q		1				1
Others						
L858R+T790M				2		2
G719A+S720F		1				1
R776H+L861Q				1		1
L858R+R776G					1	1
Wild type		1	6	12	2	21
Total	0	38	9	19	5	71

Del: deletion; ins: insertion.

TABLE 5 Multivariate analysis of prognostic factors for overall survival of the 136 adenocarcinoma patients with malignant pleural effusions

Factors	Patients n	Median survival months	p-value	
			Univariate analysis	Multivariate analysis
Sex			0.679	
Female	83	19.2		
Male	53	17.0		
Age yrs			0.002	0.011
<65	63	23.1		
≥65	73	12.9		
Smoking			0.025	0.027
Never	95	19.8		
Current/former	41	13.0		
ECOG PS			<0.001	<0.001
0–1	96	21.4		
2–4	40	6.5		
Stage			0.635	
III	26	19.6		
IV	108	19.2		
EGFR			0.005	0.001
Mutation	93	21.4		
Wild type	43	11.5		
EGFR TKI			0.189	
No	65	19.6		
Yes	71	19.8		

ECOG PS: Eastern Cooperative Oncology Group performance status; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor.

L858R were analysed [16]. KIMURA *et al.* [20] demonstrated a 28.2% (11 out of 39) EGFR mutation rate and considered that the lower frequency of EGFR mutation was related to enrolling patients with false-negative results. KIMURA *et al.* [21] showed a 13% (3 out of 23) EGFR mutation rate by direct sequence of EGFR mutations in MPEs related to lung adenocarcinoma in another study, with 9.1% (1 out of 11) in females and 10% (1 out of 10) in never smokers. It should be noted that more current or former smoking patients were enrolled into the KIMURA *et al.* [21] study. The EGFR mutation rate of MPEs ranged 9.1–68.4%. This variability probably reflects methodology, selection of patients, and geographic differences. Besides, the pleural effusion samples obtained in the present study were all positive for malignant cells by cytological examination, and this increases the detection rate of EGFR mutations.

Breast and colon cancers have been established as the models for a multistep oncogenesis process during which the accumulation of genetic mutations results in the development of an invasive phenotype [22, 23]. The EGFR mutation might be involved in the pathogenesis of lung adenocarcinoma. EGFR mutations were more frequent in patients with advanced stage disease (MPE) when compared with more early stages (surgically resectable). The difference of the EGFR mutation rate between the MPEs and the surgically resected lung

adenocarcinoma might reflect the process of carcinogenesis. TANG *et al.* [24] found identical EGFR mutations detected in the normal respiratory epithelium in 43% of patients with EGFR mutant adenocarcinomas. TOMIZAWA *et al.* [25] also demonstrated that EGFR mutations were more frequently observed in the advanced stage of NSCLC. MATSUMOTO *et al.* [26] reported frequent EGFR mutations in brain metastasis of lung adenocarcinoma in a case series of 19 patients. MATSUMOTO *et al.* [26] study showed a 63% EGFR mutation rate, which is similar to the present study in MPEs. The higher incidence of EGFR mutations in MPEs and brain metastasis than in surgically resected lung tumours implies that EGFR mutation is more frequent in patients with advanced stages when compared with earlier stages. Further studies are necessary to address this issue.

The incidence of EGFR mutations in NSCLC ranges from 10% to >50% [17, 27–31]. EGFR mutations of NSCLC are more frequent in females, nonsmokers and in Asian countries [6, 25]. Geographic (ethnic) differences and methodology in detection of EGFR mutations may result in variability. The EGFR mutation rate in surgically resected adenocarcinoma was 36% in Korea, 42–55.6% in Japan and 55% in Taiwan [27, 31, 32]. Reports from Korea and Japan show that the EGFR mutation rates of lung adenocarcinomas are higher in females (females *versus* males: 53.1–76.3% *versus* 22–36%) and never smokers (never smokers *versus* smokers: 57.4–83% *versus* 21–32%) [27–30, 32, 33]. However, HUANG *et al.* [31] showed that the EGFR mutation rate in resected lung adenocarcinoma was not significantly associated with sex (females *versus* males: 54.5% *versus* 55.5%) and smoking history in Taiwan. The present study showed that the EGFR mutation rate was 50.5% in surgically resected specimens of lung adenocarcinoma, and the difference of EGFR mutations between sexes was also not significant. These were consistent with another report from Taiwan [31]. This implies that the Asian population is not so homogeneous with regard to the mutation rate of EGFR and as regards the influence of sex and smoking on the mutation rate.

Among the EGFR TKI-treated patients, the present study showed several different EGFR mutation types other than L858R and deletion in exon 19. The various EGFR mutations did not all have good response to EGFR TKIs. In particular, T790M was found to confer primary and acquired resistance to EGFR TKI [34]. The functional values of these different EGFR mutations are still unclear.

There was one limitation of the present study, that the EGFR copy numbers in the cancer cells of MPEs were not examined. EGFR amplification is also an important predictive factor for NSCLC patients treated with EGFR TKIs [35, 36], although studies have shown that the EGFR mutation, rather than EGFR copy numbers, was a determinant of favourable clinical outcomes in gefitinib-treated patients with NSCLC [37–39]. Another interesting issue is that further studies are necessary to elucidate whether EGFR gene amplification is also a progressive event like the EGFR mutation as shown in the present study.

There have been studies evaluating the high level of VEGF in MPE [13, 40, 41], and VEGF may also have a potential role in the formation of MPE [13]. A case report has demonstrated that

bevacizumab is active in malignant effusion [42]. However, treating malignant effusion with bevacizumab might require higher doses than simply treating the underlying cancer [42]. Therefore, an antiangiogenic therapy for the treatment of MPE may play a important role for future studies.

In the present study, 54% of patients with lung adenocarcinoma with MPEs responded to EGFR TKI treatment, and 74% of patients with EGFR mutations responded to EGFR TKI. Furthermore, EGFR mutation analysis from pleural effusion is feasible in MPEs [8, 39, 43]. The present authors suggest performing EGFR mutation analysis in lung adenocarcinoma with MPEs to select those with the highest response rate to treatment with EGFR TKI. However, future prospective studies are needed to clarify these.

In conclusion, pleural effusion is easy to collect and epidermal growth factor receptor gene mutation analysis from malignant pleural effusions is feasible. The patients with malignant pleural effusions related to lung adenocarcinoma had a higher epidermal growth factor receptor gene mutation rate than the surgically resected specimens. Epidermal growth factor receptor tyrosine kinase inhibitor may be the treatment of choice for lung adenocarcinoma with malignant pleural effusions in east Asia.

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