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Frequent *EGFR* mutations in malignant pleural effusion of lung adenocarcinoma

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Abbreviations: *EGFR*, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; MPE, malignant pleural effusion; CXR, chest radiography; CT, computed tomography; MRI, magnetic resonance imaging; ECOG PS, Eastern Cooperative Oncology Group performance status; PR, partial response; SD, stable disease; PD, progressive disease; del, deletion; ins, insertion;

Short title: Frequent *EGFR* mutations in malignant pleural effusion

Brief statement about the impact of the paper: Our study shows that lung adenocarcinoma with malignant pleural effusion has a higher rate of *EGFR* mutations than surgically resected adenocarcinoma. EGFR TKI may be the choice of treatment for lung adenocarcinoma with MPE in East Asia.

Abstract

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

Materials and Methods: 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.

Results: The *EGFR* mutation rate was higher in the patients with MPEs than in the patients whose surgically resected specimens (68.4% vs. 50.5%, p=0.007). The *EGFR* mutation rate in patients with MPEs was not associated with gender, smoking history, age or cancer stage. By multivariate analysis, an age of less than 65 (p=0.011), never smoking (p=0.027), ECOG PS 0-1 (p<0.001), and *EGFR* mutation (p=0.001) were associated with a longer overall survival for lung adenocarcinoma patients with MPEs.

Conclusion: The patients with MPEs related to lung adenocarcinoma had a higher *EGFR* mutation rate than the surgically resected specimens. EGFR TKIs may be the treatment of choice for lung adenocarcinoma with MPEs in East Asia.

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

Introduction

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

EGFR tyrosine kinase inhibitors (EGFR TKIs) are used in the treatment for 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 tumor that grows in the periphery of the lung and easily invades the pleural cavity [10]. MPEs also indicate an advanced stage of the disease or disease progression. About 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 might 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]. VEGF levels in pleural fluid have higher median levels in MPEs than non-malignant pleural effusions [13]. In addition to VEGF, other molecular factors or tumor markers can also be detected in MPEs. Hsieh et al. showed that

pigment epithelium-derived factor and fibrinogen precursors are expressed at lower levels in malignant pleural effusions than in transudates [14]. Dai et al. showed that p53 and K-ras gene mutation patterns are effective markers for the detection of recurrent lung carcinoma in cytologic specimens of pleural effusions [15].

Malignant pleural effusion is very easy to collect in contrast to other invasive techniques, such as biopsy and surgery, which are necessay to get the 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 to predict 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, we examined the sequences of exon 18-21 of *EGFR*s in MPEs of lung adenocarcinoma. We then compared the mutation rate of MPEs and the mutation rate of surgically resected specimens. In addition, we collected clinical information to analyze 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, we consecutively collected 383 samples of pleural effusion from 273 patients who had received thoracentesis in the chest ultrasonography examination room of the National Taiwan University Hospital. This study was approved by the Institutional Review Board (IRB) of the National Taiwan University Hospital (NTUH), and all patients had signed an informed consent form before the thoracentesis was performed. The primary malignant tumors or MPEs were confirmed by pathology or cytology reports. Of the 273 patients, 164 patients had lung adenocarcinoma and the other 109 patients had malignancy other than lung adenocarcinoma or non-malignancy 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 we also retrieved 91 surgically resected specimens of lung adenocarcinoma archived from April 2001 to November 2004. Informed consent about the use of these specimens for future molecular studies with approval of the IRB was obtained before surgery. The retrospective use of archival tissue for *EGFR* gene analysis was approved by institutional review board of NTUH. 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 categorized as never smokers.

Those who smoked cigarettes within 1 year of the diagnosis were categorized as current smokers. The others were categorized 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 g for 10 min at room temperature, and the cell pellets were 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, Ohio) and stored at -80 °C until RNA extraction. RNA was extracted from cell lysates with a Qiamp 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.

Polymerase chain reaction amplification and direct sequencing

The four exons (exon 18-21) that code for the TK domain of the *EGFR* gene were amplified with forward primer (5'-AGCTTGTGGAGCCTCTTACACC-3') and reverse primer (5'-TAAAATTGATTCCAATGCCATCC-3') as reported in a prior study [8]. The reverse transcription polymerase chain reaction (RT-PCR) was performed as reported [8], using a Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The RT-PCR conditions were as have been previously described [8]. RT-PCR amplicons were purified and sequenced using the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI

PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST (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. Epidermal growth factor receptor 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 SNP database.

Anti-tumor therapy and response evaluation of the patients with MPEs of lung adenocarcinoma

The anti-tumor response of the patients was evaluated by chest radiography (CXR) every 2-4 weeks and by computed tomography (CT) of the disease sites every 8-12 weeks after treatment; this is routine practice in our institution. For the measurable solid tumors, the unidimension method was used according to the "Response Evaluation Criteria in Solid Tumors (RECIST)" guidelines [18]. Partial response (PR) and stable disease (SD) were confirmed by a sustained 4-week follow-up. Overall survival after anti-tumor 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 1st September 2007.

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

Statistical analysis

All categorical variables were analyzed with Pearson's χ^2 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 less than 0.05 were considered significant. All analyses were performed using SPSS software (version 13.0 for Windows; SPSS Inc.).

Results

The clinical characteristics and EGFR mutations of the surgically resected lung adenocarcinoma patients

Ninety-one surgically resected specimens were assessed for *EGFR* mutation (Table 1). The median age of the 91 patients was 63.4 years (range, 37.5-85.4 years). Three stage IIIB patients had satellite tumor nodule(s) within the same lobe as the primary tumor, and they received lobectomies. Two stage IV patients, who both had two lung lesions over the RML and RLL without other distant metastasis lesions, received bi-lobectomies.

Forty-six of the total 91 surgically resected lung adenocarcinoma had *EGFR* mutations. The *EGFR* mutation rate was 50.5%. The *EGFR* mutations included 19 L858R, 23 deletion in exon 19 and four other types (767-769 dup ASV, 771-773 dup NPH, K860I + L861Q, and L861Q). *EGFR* mutation rates of the surgically resected lung adenocarcinoma were not significantly different in gender (49% of males vs 52.5% of fenales, p=0.742), age (p=0.746) or smoking status (54.7% of never smokers vs 44.7% of former or current smokers, p=0.348) (Table 1).

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

The 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 age of the 136 patients was 66.1 years (range, 28.7-90.6 years). Two patients (1.5%) did not complete staging work-up of lung cancer and they were lost to follow-up.

Ninety-three of the total 136 patients with MPEs of lung adenocarcinoma had positive *EGFR* mutations. The mutation rate was 68.4%. The *EGFR* mutations included fifty L858R, thirty-two deletion in exon 19 and eleven other types. The eleven other types included three G719A, one G719A+S720F, one G719A+S768I, one E746V+L747P, one L747P, one 767-769 dup ASV, one 768-770 dup SVD, one L861Q and one R776H+L861Q. *EGFR* mutation rates of the adenocarcinoma patients with MPEs were not significantly different in gender (64.2% of males vs 71.1% of females, p=0.396), age (p=0.734) or smoking status (70.5% of never smokers vs 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. Seventy-seven of the 111 patients (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% vs. 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% vs. 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% vs 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% vs. 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 patients had received EGFR TKI treatment (gefitinib 250 mg/day or erlotinib 150 mg/day). Two patients were lost to follow-up before treatment and the other 63 patients received other anti-tumor therapy. Of the 71 patients treated with EGFR TKI, 38 patients had partial response. Those included one wild type, twenty-three L858R, and eleven deletion in exon 19 one G719A, one L861Q and one combined *EGFR* mutation (G719A + S720F) (Table 4). For the overall survival analysis of the 136 patients with lung adenocarcinoma with MPEs, the median overall survival was longer for patients with *EGFR* mutations than for patients with wild type of *EGFR* (median: 21.4 months (95% CI, 17.9-24.9 months) vs. 11.5 months (95% CI, 7.4–15.6 months); p=0.005, by the 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). We found that lung adenocarcinoma patients with MPEs who were aged less than 65 (p=0.011), who never smoked (p=0.027), with ECOG PS 0-1 (p<0.001), and *EGFR* mutation (p=0.001) were 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 diarrhea (24%), skin rash (38%), acne (31%), dry skin (22%), purities (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

We demonstrated that the 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 around 70% regardless of the patients' gender, smoking status or age.

Malignant pleural effusions are a common clinical problem for patients with lung adenocarcinoma. Thoracentesis may not only relieve dyspnea [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 tumor or its metastases in clinical practice. *EGFR* mutations could be detected in MPEs, and it is useful to predict response to gefitinib as shown in this study and others [16, 17].

Our study revealed a 68.4% *EGFR* mutation rate in lung adenocarcinoma with MPEs, and this result is higher than in prior published research. Soh et al. reported an *EGFR* mutation rate of 24.5% (13 of 53) in MPEs related to lung adenocarcinoma [16]. However, only deletion in exon 19 and L858R were analyzed [16]. Kimura et al. showed a 28.2% *EGFR* mutation rate (11 of 39) and they considered that the lower frequency of *EGFR* mutation was related to enrolling patients with false-negative results [20]. Kimura et al. showed a 13% *EGFR* mutation rate (3/23) by direct sequence of *EGFR* mutations in MPEs related to lung adenocarcinoma in another study, with 9.1% in females (1 of 11) and 10% in never smokers (1/10) [21]. It should be noted that more current or former smoking patients were enrolled into their study. The *EGFR* mutation rate of MPEs ranged from 9.1% to 68.4%. This variability likely reflects methodology, selection of patients, and geographic differences. Besides, our pleural effusion samples 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 the accumulation of genetic mutations resulting 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 (malignant pleural effusion) when compared with more early stages (surgically resectable). The difference of *EGFR* mutation rate between the MPEs and the surgically resected lung adenocarcinoma might reflect the process of carcinogenesis. Tang et al. found identical *EGFR* mutations detected in the normal respiratory epithelium in 43% of patients with *EGFR* mutant adenocarcinomas [24]. Tamizawa et al. also demonstrated that *EGFR* mutations were more frequently observed in the advanced stage of NSCLC [25]. Matsumoto et al. reported frequent *EGFR* mutations in brain metastasis of lung adenocarcinoma in a case series of 19 patients [26]. Matsumoto's study showed a 63% *EGFR* mutation rate [26], and this rate is similar to our study in MPEs. The higher incidence of *EGFR* mutations in MPEs and brain metastasis than surgically resected lung tumors implies that *EGFR* mutations is more frequent in patients with advanced stages when compared with more early stages. Further studies are necessary to address this issue.

The incidence of *EGFR* mutations in NSCLC ranges from 10% to over 50% [17, 27-31]. *EGFR* mutations of NSCLC are more frequent in females, non-smokers 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 vs. males = 53.1-76.3% vs. 22-36%) and never smokers (never smokers vs. smokers = 57.4-83% vs. 21-32%) [27-30,

32, 33]. However, Huang et al. showed that the *EGFR* mutation rate in resected lung adenocarcinoma was not significantly associated with gender (females vs. males = 54.5% vs. 55.5%) and smoking history in Taiwan [31]. Our study showed that the *EGFR* mutation rate was 50.5% in surgically resected specimens of lung adenocarcinoma, and the difference of *EGFR* mutations between genders 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 gender and smoking on the mutation rate.

Among the EGFR TKI treated patients, our study showed several different *EGFR* mutation types other than to L858R and deletion in exon 19. The vasious *EGFR* mutations did not all have good response to EGFR TKIs. Especially, T790M was known 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 our study that we did not examine the *EGFR* copy numbers in the cancer cells of MPEs. *EGFR* amplification is also an important predictive factor of NSCLC patients treated with EGFR TKIs [35, 36], although Sasaki et al.'s and Soh et al.'s studies both showed that the *EGFR* mutation, rather than *EGFR* copy numbers, was a determinant of favorable 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 *EGFR* mutation as shown in our study.

There have been studies about the high level of VEGF in malignant pleural effusion [13, 40, 41], and VEGF may have a potential role in the formation of malignant pleural effusion, too [13]. There is a case report that shows that bevacizumab is active in malignant effusion [42]. However, treating malignant

effusion with bevacizumab might require higher dosages than simply treating the underlying cancer [42]. So, an antiangiogenic therapy in treating malignant pleural effusion may play a important role for future studies.

From our study, 54% 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]. We suggest performing *EGFR* mutation analysis in lung adenocarcinoma with MPEs to select those with highest response rate to treatment of EGFR TKI. However, we still need future prospective studies to clarify these.

In conclusion, pleural effusion is easy to collect and *EGFR* mutation analysis from MPE is feasible. The patients with MPEs related to lung adenocarcinoma had a higher *EGFR* mutation rate than the surgically resected specimens. EGFR TKIs may be the treatment of choice for lung adenocarcinoma with MPEs in East Asia.

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Table 1 Patients' characteristics and *EGFR* mutation status of the surgically resected specimens

Variable		Patient No.	*EGFR* mutation	Mutation rate	*p* value
Total No.		91	46	50.5 %	
Sex					
	Male	51	25	49.0%	0.742
	Female	40	21	52.5%	
Age					
	>=65y/o	42	22	52.4%	0.746
	<65y/o	49	24	49.0%	
Smoking					
	Never	53	29	54.7%	0.348
	Current/Former	38	17	44.7%	
Staging					
	I	46	27	58.7%	0.2
	II	15	4	26.7%	
	III	28	14	50.0%	
	IV	2	1	50.0%	

EGFR = epidermal growth factor receptor

Table 2 **Patients' characteristics and *EGFR* mutation status of malignant pleural effusions**

Variable		Patient No.	*EGFR* mutation	Mutation rate	*p* value
Total No.		136	93	68.4%	
Sex					
	Male	53	34	64.2%	0.396
	Female	83	59	71.1%	
Age					
	>=65y/o	73	49	67.1%	0.734
	<65y/o	63	44	69.8%	
Smoking					
	Never	95	67	70.5%	0.413
	Current/ Former	41	26	63.4%	
Staging					
	IIIB	26	18	69.2%	0.944
	IV	108	74	68.5%	
	Unknown	2	1	50.0%	

EGFR = epidermal growth factor receptor

Table 3 **The difference in *EGFR* mutations between adenocarcinoma with malignant pleural effusions and adenocarcinoma of surgically resected specimens.**

	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

p=0.017 as compared with the *EGFR* mutation rates of the different *EGFR* mutation types in MPEs and surgically resected specimens.
p=0.011 as compared with the L858R mutation rates in MPEs and surgically resected specimens.

Table 4 ***EGFR*** **mutation status and response of EGFR TKI in patients of malignant pleural effusions.**

Mutation	CR	PR	SD	PD	inevaluable	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

CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease

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

Factors	Patient Number	Median OS (months)	Univariate analysis	Multivariate analysis
			p value	*p* value
Gender				
Female	83	19.2	0.679	
Male	53	17.0		
Age (>=65 vs <65)				
<65	63	23.1	0.002	0.011
>=65	73	12.9		
Smoking				
Never	95	19.8	0.025	0.027
Current/Former	41	13.0		
ECOG PS				
0-1	96	21.4	<0.001	<0.001
2-4	40	6.5		
Stage				
III	26	19.6	0.635	
IV	108	19.2		
EGFR				
Mutation	93	21.4	0.005	0.001
Wild type	43	11.5		
EGFR TKI				
No	65	19.6	0.189	
Yes	71	19.8		

EGFR = epidermal growth factor receptor, TKI = tyrosine kinase inhibitor, ECOG PS= Eastern Cooperative Oncology Group performance status, OS = overall survival