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Impact of *EGFR* Mutation Detection Methods on the Efficacy of Erlotinib in Patients with Advanced *EGFR*-Wild Type Lung Adenocarcinoma

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Abstract

Introduction: Methods used for *epidermal growth factor receptor (EGFR)* mutation testing vary widely. The impact of detection methods on the rates of response to EGFR-tyrosine kinase inhibitors (TKIs) in *EGFR*-wild type (wt) lung adenocarcinoma patients is unknown.

Methods: We recruited the Group-I patients to evaluate the efficacy of erlotinib in patients with *EGFR*-wt lung adenocarcinoma by either direct sequencing (DS) or mutant type-specific sensitive (MtS) methods in six medical centers in Taiwan. Cross recheck of *EGFR* mutations was performed in patients who achieved objective response to erlotinib and had adequate specimens. The independent Group-II lung adenocarcinoma patients whose *EGFR* mutation status determined by DS were recruited to evaluate the potential limitations of three MtS methods.

Results: In Group-I analysis, 38 of 261 *EGFR*-wt patients (14.6%) achieved partial response to erlotinib treatment. Nineteen patients (50.0%) had adequate specimens for cross recheck of *EGFR* mutations and 10 of them (52.6%) had changes in *EGFR* mutation status, 5 in 10 by DS and 5 in 9 by MtS methods originally. In Group-II analysis, 598 of 996 lung adenocarcinoma patients (60.0%) had detectable *EGFR* mutations. The accuracy rates of the three MtS methods, MALDI-TOF MS, Scorpions ARMS and Cobas, were 87.8%, 86.8% and 85.8%, respectively.

Conclusions: A significant portion of the erlotinib responses in *EGFR*-wt lung adenocarcinoma patients were related to the limitations of detection methods, not only DS but also MtS methods with similar percentages. Prospective studies are needed to define the proper strategy for *EGFR* mutation testing.

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Introduction

In recent years, epidermal growth factor receptor (EGFR)-targeted therapy has emerged as a novel and effective strategy in lung cancer management with major benefits in patients with *EGFR* activating mutations. Not only in front line but also in subsequent therapy, EGFR-tyrosine kinase inhibitors (TKIs) in comparison with chemotherapy have demonstrated significantly higher response rate and longer progression-free survival (PFS) in patients with *EGFR*-mutant non-small cell lung cancer (NSCLC) [1]. Moreover, EGFR-TKIs therapy is associated with a better quality of life [2–4]. Therefore, many studies suggested EGFR-TKI as the first line therapy for *EGFR*-mutant NSCLC patients [5,6].

Despite the close association between *EGFR* mutations and EGFR-TKIs responsiveness, NSCLC patients, who had no detectable *EGFR* mutations, have been reported to benefit from the EGFR-TKIs [7–9] and erlotinib remains an important second-line treatment option in the clinical practice guidelines for NSCLC, irrespective of biological characteristics [10–12]. A pooled analysis, which included three Phase III randomized controlled trials that compared the efficacy of erlotinib with other therapies in *EGFR*-wild type (*EGFR*-wt) NSCLC patients, also suggested a significant benefit of erlotinib treatment [13]. However, various *EGFR* mutation detection methods were used in studies regarding the efficacy of erlotinib in *EGFR*-wt NSCLC and their false negative rates have been suspected to be a possible reason for the responses to EGFR-TKIs in patients without detectable *EGFR* mutations [14,15].

Direct sequencing (DS) can detect all existing mutations but is limited by its lower sensitivity [16]. Mutant type-specific sensitive (MtS) methods, such as the protein nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp or Scorpions amplification refractory mutation system (ARMS) can detect specific and known mutations but not rare mutations [17]. The results of studies that investigated the association between discrepant *EGFR* mutation results by different methods and the outcomes of EGFR-TKIs treatment were inconsistent [18,19]. The impact of detection methods on the rates of response to EGFR-TKIs in *EGFR*-wt lung adenocarcinoma patients is unknown. We conducted this study to evaluate the impact of detection methods on the efficacy of erlotinib in patients with advanced *EGFR*-wt lung adenocarcinoma.

Materials and Methods

Patients

We recruited two independent groups of patients for participation in this study. From August 2005 to March 2013, we evaluated the efficacy of erlotinib in lung adenocarcinoma patients (Group – I) with *EGFR*-wt status assessed by regular methods (either DS or MtS methods) used in six participating medical centers in Taiwan (Taichung Veterans General Hospital, (TCVGH) Taipei Veterans General Hospital, Chang Gung Memorial Hospital (CGMH), Kaohsiung Medical University Hospital, National Taiwan University Hospital Yunlin Branch and Far Eastern Memorial Hospital). Inclusion criteria for Group-I patients were advanced lung adenocarcinoma without detectable *EGFR* mutations (exon 18, 19, 20 and 21) at initial molecular analysis, a history of erlotinib treatment for more than 7 days and clinically measurable disease. Patients were excluded if they had other active malignancy, incomplete data records or received other treatments concurrently. All patients received erlotinib at a daily dose of 150 mg initially. TNM (tumor, node, and metastases) staging was done according to the 7th edition of the American Joint Committee for Cancer (AJCC) staging system [20].

From January 2000 to June 2013 we evaluated consecutive lung adenocarcinoma patients of any stage who were treated in TCVGH and CGMH (Group-II). We assessed their *EGFR* mutation status by DS and calculated the number of *EGFR* mutations that would not be detected by three MtS methods. This study was approved by the institutional review boards of the participating institutions, including Institutional Review Board of Taichung Veterans General Hospital, Institutional Review Board of Taipei Veterans General Hospital, Chang Gung Medical Foundation Institutional Review Board, Kaohsiung Medical University Chung-Ho Memorial Hospital Institutional Review Board, National Taiwan University Hospital Research Ethics Committee and Far Eastern Memorial Hospital Research Ethics Review Committee. Written informed consent for genetic testing and clinical data records was obtained from all patients.

Data records and response evaluation

Clinical data for analysis included age, gender, Eastern Cooperative Oncology Group performance status (ECOG PS), tumor stage, prior chemotherapies, smoking status, *EGFR* detection methods and erlotinib treatment history. The adverse events associated with erlotinib treatment including interstitial lung disease and grade 3–4 hepatotoxicity were recorded. Chest computed tomographies, including the liver and adrenal glands, and other required imaging studies for response evaluation were reviewed by two chest physicians. Unidimensional measurements as defined by Response Evaluation Criteria in Solid Tumors version 1.1 were used in this study [21]. The objective response rate (ORR), disease control rate (DCR), PFS and overall survival (OS) of erlotinib treatment were assessed.

EGFR mutation tests

For the Group-I patients, several molecular tests, including DS, PNA-LNA PCR clamp, Scorpions ARMS (EGFR RGQ PCR Kit) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) were used for *EGFR* mutation analysis [9,22–24], which depended on the laboratory facilities of participating institutions. As for DS, PNA-LNA PCR clamp and MALDI-TOF MS methods, DNA was extracted from the tumors for *EGFR* mutation analysis as previously described [9,24] and the detection spectrum of PNA-LNA PCR clamp and MALDI-TOF MS is summarized in Table S1. As for Scorpions ARMS, commercialized kit was used and samples were processed according to the manufacturer's protocol [25]. We defined PNA-LNA PCR clamp, Scorpions ARMS and MALDI-TOF MS as the MtS methods to be compared with DS for evaluation of the influence of detection methods on the efficacy of erlotinib treatment. For the Group-II patients, we assessed their *EGFR* mutation status by DS and calculated the number of *EGFR* mutations that would not be detected by MALDI-TOF MS and two other commercialized methods, Scorpions ARMS [25] and Cobas *EGFR* Mutation Test [26].

Statistical methods

Univariate analysis of ORR and DCR were performed using Fisher's exact test to evaluate the effects of clinical factors relating to patients' characteristics and *EGFR* detection methods. Multivariate analyses of ORR and DCR were performed using logistic regression model. The Kaplan–Meier method was used to estimate PFS and OS. Differences in survival time in regard to *EGFR* detection methods were analyzed using the log-rank test. Multivariate analyses of PFS and OS were performed using Cox

proportional hazard model. All statistical tests were done with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Two-tailed tests and p values <0.05 for significance were used.

Results

Efficacy and adverse effects of erlotinib in lung adenocarcinoma patients without detectable EGFR mutations at initial molecular testing

A total of 261 patients were included in Group-I and the baseline characteristics are shown in Table S2. The median age was 62 years, 162 patients (62.1%) were male, 138 patients (52.9%) were non-smokers and 174 patients (66.7%) had ECOS PS 0–1. Initial *EGFR* mutation status was assessed by DS in 191 patients (73.2%) and by MtS methods in 70 patients (26.8%).

Thirty-eight patients achieved partial response (PR) and 52 had stable disease. No patient achieved complete response. The ORR and DCR were 14.6% and 34.5%, respectively. The responses and survival analysis are summarized in Table 1. The median PFS and OS were 1.9 (95% CI 1.7–2.1) and 8.3 (95% CI 5.9–10.7) months respectively. The 1-year survival rate was 25.7%. PFS and OS were significantly longer in patients with disease control than in those with progressive disease (both $P<0.001$).

Results of univariate analysis of ORR are shown in Table 2. There was no significant association between the erlotinib treatment responses and patients' age, gender, smoking status and ECOG PS. Furthermore, the ORR of patients whose *EGFR* mutation status was assessed by DS and by MtS methods were comparable (14.1 vs. 15.7%, $P = 0.843$). No covariate reached the significance level to enter the multivariate logistic regression model.

Kaplan–Meier curve of PFS in regard to detection methods is shown in Figure 1. There was no significant difference in PFS between patients with *EGFR*-wt tumors assessed by DS and by MtS methods (2.0 vs. 1.9 months, $P = 0.855$) and similar survival periods were noted in OS analysis (8.3 vs. 10.9 months, $P = 0.782$). Patients' characteristics other than detection methods did not correlate significantly with PFS and OS (data not shown) and no covariates reached the significance level to enter the multivariate Cox proportional hazard model.

As for adverse events, interstitial lung disease occurred in 2 patients (0.8%) and 8 patients (3.1%) had grade 3–4 hepatotoxicity. None of these adverse events led to death.

Cross recheck of EGFR mutation status in EGFR-wt patients with objective responses to erlotinib treatment

Thirty-eight of 261 patients (14.6%) in Group-I achieved objective responses to erlotinib treatment. Nineteen of them (50.0%) had adequate specimens for *EGFR* mutation status cross recheck. Initial molecular testing was performed by DS in 10 patients (52.6%) and by MtS methods in 9 patients (47.4%). Patients with *EGFR*-wt mutation status assessed by DS were rechecked by MtS methods and vice versa. In this study, the MtS method used for the recheck was MALDI-TOF MS.

Results of *EGFR* mutation status recheck are summarized in Table 3. Of 10 patients with *EGFR*-wt mutation status assessed by DS, 5 patients (50.0%) were found to have *EGFR* mutations by MALDI-TOF MS, including 2 with Del E746_A750, 1 with L858R and 2 with complex mutations, Del E746_A750/T790M and L858R/T790M. Of 9 patients with *EGFR*-wt mutation status assessed by MtS methods, 5 patients (55.6%) were found to have *EGFR* mutations by DS, including 4 with exon 19 deletions (Del L745_A750>R, Del K746_T751>VP, Del L747-A750>P and Del L747_T751>N) and 1 with I706T, a point mutation at exon 18. Of theses mutations, only Del L747_A750>P can be detected by available MtS methods. *EGFR* mutation status of patient S4 was assessed as wild type by PNA-LNA PCR clamp in September 2011. Our laboratory facility was not able to detect Del L747_A750>P until September 2013 when we added new

Table 1. Efficacy of erlotinib in 261 lung adenocarcinoma patients without detectable *EGFR* mutations at initial molecular testing.

Best Response			
	Patient No. (%)		
Complete response (CR)	0 (0)		
Partial response (PR)	38 (14.6)		
Objective response rate	38 (14.6)		
(ORR = CR + PR)			
Stable disease (SD)	52 (19.9)		
Disease control rate	90 (34.5)		
(DCR = CR + PR + SD)			
Progressive disease (PD)	171 (65.5)		
Survival			
	Median PFS (m)[a] (95% CI)	**Median OS (m)[b] (95% CI)**	**1-year survival rate (%)[b]**
PR	11.0 (8.1–13.9)	32.6 (26.5–38.7)	-
SD	5.8 (3.4–8.2)	18.4 (7.4–29.3)	-
DC	8.4 (7.2–9.6)	30.0 (15.9–44.0)	-
PD	1.4 (1.2–1.6)	4.9 (3.7–6.1)	-
Total	1.9 (1.7–2.1)	8.3 (5.9–10.7)	25.7

PFS, progression-free survival; OS, overall survival; PR, partial response; SD, stable disease; DC, disease control; PD, progressive disease.
[a]38 patients are still under erlotinib treatment without PD.
[b]102 patients are still alive.
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Table 2. Univariate analysis of objective response rate of erlotinib treatment in lung adenocarcinoma patients without detectable *EGFR* mutations at initial molecular testing.

	Patient No.	ORR (%)	P value
Gender			0.209
Male	162	12.3	
Female	99	18.2	
Age (yrs)			0.478
≤ 65	158	13.3	
> 65	103	16.5	
ECOG PS			0.358
0–1	174	16.1	
≥ 2	87	11.5	
Smoking			0.113
NS	138	18.1	
C/FS	123	10.6	
EGFR methods			0.843
Direct sequencing	191	14.1	
Sensitive methods[a]	70	15.7	

ORR, objective response rate; ECOG PS, Eastern Cooperative Oncology Group performance status; NS, nonsmoker; C/FS, current or former smoker; EGFR, epidermal growth factor receptor.
[a]Include Scorpions ARMS, MALDI-TOF MS and PNA-LNA PCR clamp methods.
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mutation detection probes. In total, 10 of 19 patients (52.6%) had changes in *EGFR* mutation status.

Using independent direct sequencing cohort to evaluate the limitations of three mutant type-specific sensitive methods

Results of the analysis of Group-I patients showed that both DS and MtS methods were unable to detect a significant portion of *EGFR*-mutations. As DS is well known by its low detection sensitivity, which may miss up to 20–25% *EGFR* mutations in comparison with varied MtS methods [16,18,19], we focused on how many *EGFR* mutations would not be detected by MtS methods. Therefore, we recruited the independent Group-II patients, whose *EGFR* mutation status was assessed by DS method, to evaluate the potential limitations of MtS methods.

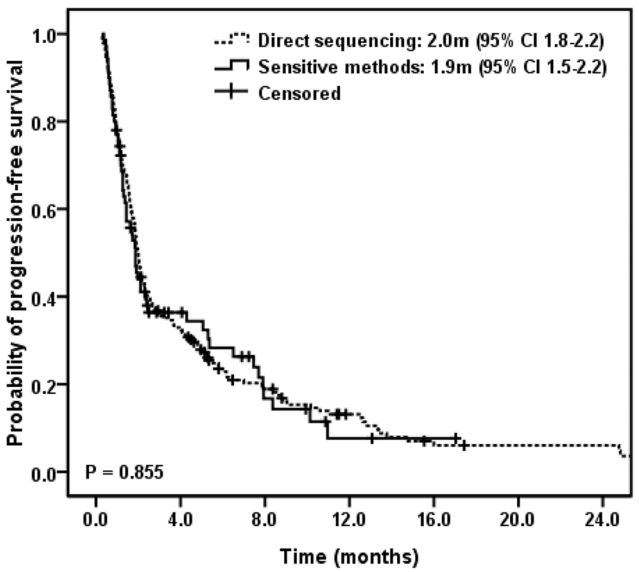

Figure 1. Kaplan-Meier plot showing progression-free survival according to different *EGFR* mutation detection methods.
doi:10.1371/journal.pone.0107160.g001

In total, 996 consecutive lung adenocarcinoma patients were included in Group-II and the baseline characteristics are shown in Table S3. We used the database to evaluate the detectability of three MtS methods, including MALDI-TOF MS, which has been established at National Taiwan University Center of Genomic Medicine as one of our standard *EGFR* detection methods and two commercialized methods, Scorpions ARMS and Cobas *EGFR* Mutation Test.

Figure 2 shows that 598 of the 996 patients (60.0%) had detectable *EGFR* mutations. The exon 19 deletions (41.6%) and L858R (42.0%) were the major mutation types. Complex mutations of any combinations were categorized into the group "others". In the detectability analysis, we defined fully and partly detectable as full spectrum of mutation(s) or only part of complex mutations could be detected respectively. Detection rate is the percentage of fully plus partly detectable mutations and the accuracy rate is the percentage of fully detectable mutations. As shown in Figure 2, MALDI-TOF MS, Scorpions ARMS and Cobas could not detect or only partly detected the mutation types in 73, 79 and 85 patients respectively. The detection rates of MALDI-TOF MS, Scorpions ARMS and Cobas were 92.8%, 92.8% and 91.8% and the accuracy rates of the three methods were be 87.8%, 86.8% and 85.8% respectively as disclosed in Table 4. Table S4 shows the full *EGFR* mutation spectrum of Group-II patients. It also indicated the detectability of three MtS methods and the mutations associated with disease control in response to EGFR-TKIs therapy according to treatment history at our facilities and the DNA-Mutation Inventory to Refine and Enhance Cancer Treatment (DIRECT) database [27].

Table 3. Results of *EGFR* mutation status recheck in part of patients with objective response to erlotinib treatment.

	Demographic data				Cross recheck of *EGFR* mutations		Efficacy of erlotinib	
Pt	**Age**	**Gender**	**Smoking**	**ECOG**	**Method**	**Result**	**Prior C/T**	**PFS (m)**
Direct sequencing group								
D1	82	M	CS	1	MS	Del E746_A750	1	8.6
D2	60	M	NS	1	MS	Del E746_A750	0	12.8
D3	61	M	FS	1	MS	Unfound	1	5.0
D4	73	F	NS	3	MS	Unfound	0	15.5[b]
D5	77	M	NS	1	MS	Unfound	1	11.0
D6	82	M	FS	2	MS	Unfound	1	2.9[b]
D7	84	M	NS	2	MS	Unfound	0	5.8[b]
D8	64	F	NS	2	MS	Del E746_A750/T790M	0	5.3[b]
D9	82	M	CS	2	MS	L858R	1	5.1[b]
D10	49	F	NS	1	MS	L858R/T790M	2	8.4
Sensitive method group								
S1	77	F	NS	1	DS	Del L747_T751>N	1	13.1[b]
S2	73	M	FS	1	DS	Del K745_A750>R	1	2.4[b]
S3	58	F	NS	2	DS	Del E746_T751>VP	1	5.1
S4	62	F	NS	3	DS	Del L747_A750>P[a]	3	6.5
S5	61	F	NS	1	DS	I706T	2	7.9
S6	65	F	NS	1	DS	Wild type	1	7.7
S7	77	M	CS	1	DS	Wild type	0	6.9
S8	65	M	NS	2	DS	Wild type	2	4.1[b]
S9	59	F	NS	1	DS	Wild type	1	10.1

EGFR, epidermal growth factor receptor; ECOG, Eastern Cooperative Oncology Group performance status; C/T, chemotherapy; PFS, progression-free survival; CS, current smoker; FS, former smoker; NS, non-smoker; MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; DS, direct sequencing.

[a]*EGFR* mutation status of patient S4 was analyzed as wild type by PNA-LNA PCR clamp in September 2011 and our facility was able to detect Del L747_A750>P since September 2013 by adding new mutation detection probes.

[b]Still under erlotinib treatment without progression.

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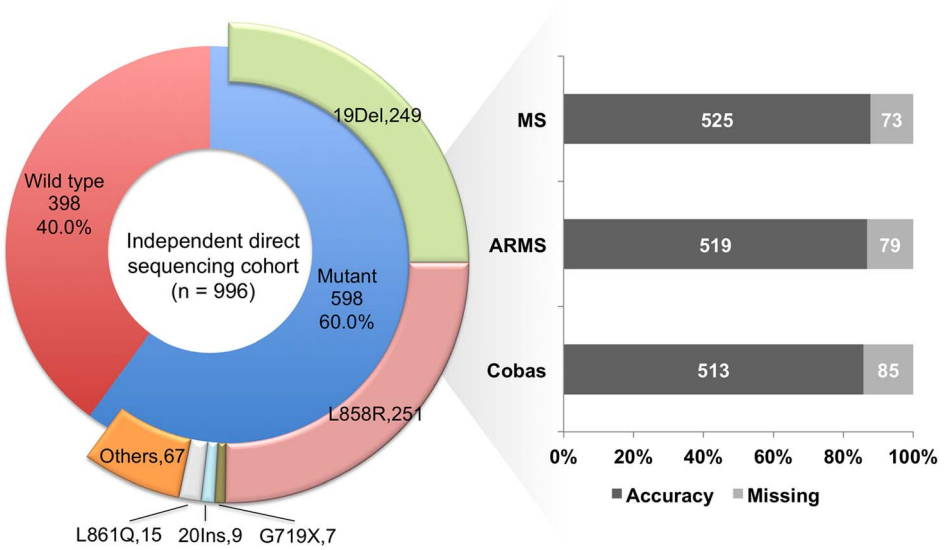

Figure 2. Detectability analysis of various mutant type-specific sensitive methods in an independent direct sequencing cohort (complex mutations were categorized into the group "others"; "missing" indicated partly detectable plus undetectable *EGFR* mutations; MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; ARMS, Scorpions amplification refractory mutation system).
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Discussion

A subset of patients who do not harbor *EGFR* mutations could benefit from EGFR-TKIs treatment and a pooled analysis by Lindeman et al. showed an 11% ORR of EGFR-TKIs in patients with *EGFR*-wt NSCLC assessed by various detection methods [28]. Similar to this result, our study showed that ORR of erlotinib in Group-I patients was 14.6%. By the cross recheck of *EGFR* mutation status, we found that more than half of the erlotinib responders actually harbored *EGFR* mutations and both DS and MtS methods were unable to detect a significant portion of *EGFR*-mutations.

In 2011, Naoki et al. compared the detection sensitivity of DS and PCR-invader method and reported that *EGFR* mutations were detected in 52% of the samples with PCR-invader method but only 35% of the samples by DS [16]. Similar results have been reported when DS was compared with other MtS methods [14,29]. In the present study, 5 of 10 erlotinib responders (50.0%), who had *EGFR*-wt tumors by DS, were found to harbor *EGFR* mutations by MALDI-TOF MS. These results provided evidence that the relative low sensitivity of DS could account for some of the responses to erlotinib in patients without detectable *EGFR* mutations.

In the present study, we divided patients into DS and MtS groups depending on which methods used at initial molecular testing. As the relative low sensitivity of DS has been suspected to be a possible reason for the responses to EGFR-TKIs in patients without detectable *EGFR* mutations [14,15], a better outcome would be expected in the group detected by DS because it could miss more *EGFR*-mutant patients. However, in the present study, we found that neither responsiveness nor survival time correlated significantly with detection methods. These results suggested that

Table 4. *EGFR* mutation detectability of various mutant type-specific sensitive methods in an independent cohort analyzed by direct sequencing (a total of 996 patients, of whom 598 harbored *EGFR* mutations).

	MS	ARMS	Cobas
Detectability, n			
Fully detectable[a]	525	519	513
Partly detectable[b]	30	36	36
Undetectable	43	43	49
Detection rate, (%)	92.8 (555/598)	92.8 (555/598)	91.8 (549/598)
(Fully + Partly detectable)			
Accuracy rate (%)	87.8 (525/598)	86.8 (519/598)	85.8 (513/598)
(Fully detectable)			

EGFR, epidermal growth factor receptor; MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; ARMS, Scorpions amplification refractory mutation system.
[a]Fully detectable: full spectrum of mutation(s) could be detected correctly.
[b]Partly detectable: part of complex mutations could be detected.
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there might be also limitations in MtS methods that could potentially lead to failure to detect *EGFR* mutations in some patients.

MtS methods have higher detection sensitivity but might not be able to detect rare and unknown mutations. In contrast to DS, only a few studies have focused on the impact of the limitations of MtS methods [17,30]. Though the exon 19 deletions and L858R account for the majority of *EGFR* mutations, patients harboring other uncommon mutations could also benefit from EGFR-TKIs therapy [31]. Yang et al. has suggested that the absence of an *EGFR* mutation, as determined by methods that only detect known mutations, should not be used as an exclusion criterion for the EGFR-TKIs therapy [17]. In the present study, 5 of 9 erlotinib responders (55.6%), who had *EGFR*-wt tumors by MtS methods, were found to possess *EGFR* mutations by DS. Moreover, analysis of an independent DS cohort showed that about 8% of *EGFR* mutations might be undetectable by MtS methods and the accuracy rates would be less than 90%. Moreover, a significant portion of these uncommon mutations is associated with disease control in response to EGFR-TKIs therapy. According to the Catalogue of Somatic Mutations in Cancer (COSMIC) (v66) database, 9.5% (524 of 5544 reported cases) of the *EGFR* mutations in lung adenocarcinoma may be undetected by modern MtS methods [32], a finding which was similar to that of our study. Our study highlighted the limitations of MtS methods.

A recently published molecular testing guideline for *EGFR* mutations suggested that laboratories are strongly encouraged to use sensitive methods that are able to detect mutations in specimens with as little as 10% cancer cells [28], However, our results indicated that both DS and MtS methods have strengths and weaknesses and could potentially miss part of *EGFR*-mutant patients. Recently, Er et al. also focused on this issue and suggested that all samples should be screened by MtS methods first and if the mutation is detected, the results could be reported directly. If no mutation can be found, the samples should be rechecked by DS. The results indicated that combination strategy as real-time PCR screening followed by DS could increase the *EGFR* mutation detection rate by 4% [30]. In regions with higher frequency of *EGFR* mutations, there could miss more *EGFR*-mutant patients as the similar false negative rates of detection methods. The cost-effectiveness should also be considered in determining which strategy is suitable for clinical settings.

In the present study, there were 9 patients, who were really *EGFR*-wt by both DS and MtS methods, achieved PR to erlotinib treatment. One possible reason for the response to erlotinib in *EGFR*-wt NSCLC is that erlotinib might target pathways related to antitumor activity other than the *EGFR* mutations because objective responses to erlotinib have been independently observed in *EGFR*-wt NSCLC patients, not only in those with adenocarcinoma but also in the squamous cell carcinoma subgroup, which usually has a low *EGFR* mutation rate [9,33]. Previous studies have suggested potential mechanisms to explain the erlotinib activities in *EGFR*-wt lung cancers, such as *EGFR* copy numbers [34], mutations in other exons of the *EGFR* gene [35], cancerous inhibitor of protein phosphatase 2A (CIP2A) pathway [36] and VeriStrat status [37]. Further studies are needed to define the underlying mechanisms.

Recent advances in sequencing methods, such as next-generation technologies, could provide a rapid, multiplexed, ultrasensitive and high throughput detection of *EGFR* and other actionable mutations [38]. Furthermore, a recent study by Couraud et al. suggested the potential utility of using next-generation sequencing to non-invasively screen actionable mutations in plasma cell-free DNA in lung cancer patients [39]. These results may provide another aspect on future targeted molecular therapy.

In conclusion, a significant portion of the erlotinib responses in lung adenocarcinoma patients without detectable *EGFR* mutations was related to the limitations of detection methods. We further highlighted that not only DS but also MtS methods were unable to detect *EGFR* mutations in some patients. Prospective studies are needed to define the proper strategy for *EGFR* mutation testing in order to enable more patients to undergo EGFR-TKIs therapy, which should take balance between the cost-effectiveness and detection sensitivity.

Supporting Information

Table S1 *EGFR* mutations detected by PNA-LNA PCR clamp and MALDI-TOF MS.
(PDF)

Table S2 Demographic data of the Group-I patients.
(PDF)

Table S3 Demographic data of the Group-II patients.
(PDF)

Table S4 *EGFR* mutation spectrum, detectability and responses to EGFR-TKIs treatment of an independent direct sequencing cohort.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: YMC GCC. Performed the experiments: CRT MHT SLY KYS. Analyzed the data: MSH C-Y. Chen C-Y. Chang TYY KCC KHH. Contributed reagents/materials/analysis tools: CWW CTY. Contributed to the writing of the manuscript: JST CLW.

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