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Afatinib is effective in the treatment of lung adenocarcinoma with uncommon *EGFR* p.L747P and p.L747S mutations

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ABSTRACT

Objectives: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are used as first-line standard treatment for advanced lung adenocarcinoma with mutant *EGFR*. Nevertheless, few studies have demonstrated the efficacy of first- and second-generation EGFR TKIs in patients harboring the uncommon p.L747P and p.L747S mutations in exon 19 of *EGFR*.

Materials and methods: From 2005–2018, we identified patients with lung adenocarcinoma with *EGFR* p.L747P or p.L747S mutations using DNA and cDNA sequencing or commercial kits and recorded their clinical data. Published data pertaining to these mutations were also reviewed.

Results: Twelve eligible patients were enrolled at National Taiwan University Hospital (NTUH), and ten additional patients were identified in published literature. In NTUH cohort, the direct DNA sequencing had a 60.0% (3 of 5 patients) false-negative rate, and use of commercial kits all caused misidentification of *EGFR* p.L747P or p.L747S. Of the 7 patients receiving EGFR TKI treatment, five stage-IV lung adenocarcinoma patients that received afatinib had a 80.0% objective response rate (ORR), while two patients administered gefitinib or erlotinib showed a 0% ORR. The median progression-free survival (PFS) rates were 11.97 and 0.92 months (P = 0.012) for afatinib and gefitinib/erlotinib, respectively. No patients (0%) acquired p.T790 M resistance after failure of afatinib (n = 3). Of 10 patients harboring *EGFR* p.L747P from published literature, six patients used first-generation EGFR TKIs as treatment also showed 0% ORR and 1.00 month median PFS.

Conclusions: Patients with the uncommon *EGFR* mutations p.L747P and p.L747S could be incorrectly classified as having wild-type *EGFR* or a 19DEL when using direct DNA sequencing or commercial kits. Moreover, use of afatinib may provide significantly improved PFS in patients with advanced lung adenocarcinoma with one of these two *EGFR* mutations.

1. Introduction

Use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) has proven effective therapy for advanced lung adenocarcinoma with mutant *EGFR* [1–4]. The exon 21 p.L858R mutation and exon 19 deletions (19DEL) are the most common mutations and are sensitive to EGFR TKIs [5–7]. However, uncommon *EGFR* mutations have shown various responses to different EGFR TKIs [8–10]. An irreversible ErbB family blocker, afatinib, was found to be more effective against uncommon *EGFR* mutations than the first-generation TKIs gefitinib and erlotinib [8–10]. In addition, our previous real-world study of afatinib showed nearly equal objective response rates (ORRs) and progression-free survival (PFS) in patients with subgroups of common and uncommon mutations, excluding the primary resistant exon 20 insertion [11].

Some commercial methods, such as the therascreen® EGFR RGQ PCR kit (Qiagen), cobas® EGFR mutation test (Roche), and MassARRAY® genotyping (Sequenom) methods, are recommended and widely used for *EGFR* mutation testing in clinical practice because of their rapid turn-around time and minimal tumor content required [1,12]. These targeted methods for *EGFR* testing are designed to detect the most common *EGFR* mutations, such as 19DEL and p.L858R, and less

Abbreviations: 19DEL, exon 19 deletions; CI, confidence interval; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; NGS, next-generation sequencing; ORR, objective response rate; OS, overall survival; PFS, progression-free survival

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common but reported mutations, including p.G719X, p.T790M, p.L861Q, etc. [13] However, some activating somatic but uncommon mutations in the *EGFR* gene could not be detected by using these commercial kits [13]. Recently, Walsh et al. [17] reported that a patient with early-stage lung adenocarcinoma was incorrectly diagnosed as having 19DEL when tested by therascreen® and cobas®. Using next-generation sequencing (NGS), the patient was confirmed to harbor the p.L747 P mutation in the *EGFR* gene [12]. The p.L747P results from codon 747 of the exon 19 with a 2 bp mutations (c.2239_2240 TT > CC), and this mutation drives the oncogenesis the same as other activating common *EGFR* mutations. The limited number of case reports demonstrated that lung adenocarcinoma patients harboring p.L747 P showed various responses to EGFR TKIs treatment [14–20]. Meanwhile, p.L747S (c.2240 T > C) had been reported to be the acquired resistant mutation to gefitinib, but was sensitive to erlotinib treatment [21]. Analyzing the three-dimensional structure of the EGFR kinase, Leu747 is located at the end of strand β3 connecting to the C-helix and part of hydrophobic residues to stabilize the inactive function of the kinase [22]. Theoretically, the substitutions of L747 to Pro or Ser with minimal structure change could cause a constitutional activity of EGFR kinase and are sensitive to EGFR TKIs treatment [22], but various responses to TKIs were observed from above clinical case reports.

In the well-known clinical trials LUX-Lung 2, LUX-Lung 3, and LUX-Lung 6, 75 (12%) patients had uncommon *EGFR* mutations among 600 patients treated with afatinib. None of them had the *EGFR* p.L747P or p.L747S mutation [10]. The effectiveness of EGFR TKIs in patients with these two rare point mutations in exon 19 of *EGFR* is seldom discussed. Therefore, we retrospectively identified patients with either of these two uncommon mutations treated at National Taiwan University Hospital (NTUH), and we simultaneously reviewed published cases by searching PubMed. We further investigated the clinical characteristics and outcomes of these patients to evaluate the efficacy of EGFR TKIs in treating such patients.

2. Materials and methods

2.1. Patients and data collection

We systematically retrieved data on patients with lung adenocarcinoma who harbored *EGFR* mutations and were treated at NTUH, a tertiary hospital in Taipei, Taiwan, from 2005 to 2018, and we determined whether these patients had *EGFR* p.L747P or p.L747S mutations. The Research Ethics Committee of NTUH approved this study. Written informed consent was not required because this was a retrospective review. The clinical characteristics, imaging data, and medical records of the 12 enrolled patients were also collected.

We also reviewed the published literature to identify lung adenocarcinoma cases with *EGFR* p.L747P or p.L747S mutations. Key words, such as *EGFR* uncommon mutations, *EGFR* exon 19 mutation, and *EGFR* p.L747P or p.L747S mutations, were used to search for studies in PubMed. Relevant information was extracted from published papers [12,14–21,23,24].

2.2. Data on EGFR mutation status

Information on the *EGFR* mutation status of NTUH patients with lung adenocarcinoma was obtained through various methods and reviewed via electronic medical records. Some lung adenocarcinoma patients underwent biopsies at other hospitals before referral to NTUH for further treatment. In these referred cases, *EGFR* mutations were identified by therascreen® EGFR RGQ PCR. In other cases, paraffin-embedded specimens were sent to the Department of Pathology at NTUH and were subjected to direct DNA sequencing or MassARRAY® genotyping (Sequenom) at the National Taiwan University Centers of Genomics and Precision Medicine for *EGFR* mutation detection using procedures as previously reported [25,26]. Briefly, the direct DNA sequencing used specific primers to amplify the Exons 18–21 of the *EGFR* by nested polymerase-chain-reaction (PCR), which applied primer sequences as previous reported [26], and the products were analyzed by bidirectional sequencing and compared with GenBank-archived human EGFR sequence (accession number AY588246). Lastly, some patients underwent surgery, echo-guided biopsy, computed tomography (CT)-guided biopsy, bronchoscopic biopsy, or thoracocentesis. Fresh lung cancer specimens from these procedures were collected. RNA was extracted using a QIAamp RNA Mini Kit (Qiagen, Hilden, Germany), based on the manufacturer's protocol as previously described [6]. The quantity and quality of extracted RNA were measured by spectrophotometry. OneStep RT-PCR Kit (Qiagen) was applied for reverse transcription polymerase chain reaction (RT-PCR), and Exons 18–21 of *EGFR* were amplified with a sense primer (5'-GGA-TCG-GCC-TCT-TCA-TGC-3') and an antisense primer (5'-TAA-AAT-TGA-TTC-CAA-TGC-CAT-CC-3'). The BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for sequencing of cDNA amplicons, and the automatic ABI PRISM 3700 genetic analyser (Applied Biosystems) analyzed the final sequencing products [27–29]. Furthermore, the results of obtained forward and reverse sequences were both analyzed, and two independent reviewers manually checked and confirmed the data of chromatograms. p.L747P or p.L747S mutations detected were further confirmed by the second independent RT-PCR and sequencing reactions.

EGFR mutations in the cases retrieved from published studies were detected according to the methods described in the relevant studies, including DNA direct sequencing, commercial kits for *EGFR* detection, and NGS [12,14–21,23,24].

2.3. Treatment responses and survival analysis

Tumor responses to each EGFR TKI treatment were evaluated, either by primary care physicians or in follow-up imaging studies, including chest radiographs every 2–4 weeks and chest CT scans every 2–3 months, according to the Response Evaluation Criteria in Solid Tumors (version 1.1) [30]. PFS was determined in patients that received EGFR TKIs only, such as gefitinib, erlotinib, or afatinib, without concomitant anti-cancer drugs. Overall survival (OS) was defined as the time from confirmation of the diagnosis of stage IV lung cancer or tumor recurrence after curative intent therapy to death from any cause.

2.4. Statistical analysis

Survival analyses were performed using the Kaplan–Meier method, and log-rank tests were used to compare the PFS and OS between subgroups of patients treated with first- or second-generation EGFR TKIs. PFS and OS are presented as median values with 95.0% confidence intervals (CIs). All statistical analyses were performed using Statistical Package for the Social Sciences 18.0 (SPSS Inc., Chicago, IL, USA). STATA 14.0 (StataCorp, College Station, TX, USA) was used to plot PFS and OS. A two-sided P value < 0.05 was viewed statistically significant.

3. Results

3.1. Demographics of enrolled patients

From June 2005 to June 2018 at NTUH, total 3648 patients were diagnosed with lung adenocarcinoma and their tissue specimens or malignant pleural effusion undergoing DNA or cDNA Sanger sequencing for *EGFR*, and 2031 (55.7%) patients tested positive for *EGFR* mutations. Of these EGFR mutant lung adenocarcinoma patients, only 12 patients (0.59%) with lung adenocarcinoma harboring uncommon *EGFR* p.L747 P or p.L747S mutations were identified and retrieved. Patient median age was 64.5 (range, 36–93) years. Eight patients (66.7%) were male, and six (50.0%) were never-smokers (Table 1).

Table 1
Clinical characteristics of patients from NTUH and published data harboring *EGFR* p.L747 P or p.L747S mutations.

Characteristic	NTUH	Published data
Number of patients	12	10
Age (years), median (range)	64.5 (36–93)	61.0 (44–78)
Sex, *n* (%)		
Male	8 (66.7)	3 (33.3)
Female	4 (33.3)	6 (66.7)
Smoking status, *n* (%)		
Never smoker	6 (50.0)	4 (57.1)
Current/ex-smoker	6 (50.0)	3 (42.9)
Clinical stage at diagnosis, *n* (%)		
Stage IB	0	2 (20.0)
Stage IIA	2 (16.7)	0
Stage IIIA	1 (8.3)	0
Stage IV	9 (75.0)	8 (80.0)
Treatment for stage IV disease, *n* (%)		
Gefitinib	1 (11.1)	5 (62.5)
Erlotinib	1 (11.1)	1 (12.5)
Afatinib	5 (55.6)	1 (12.5)
Chemotherapy	2 (22.2)	0
Unspecified EGFR TKI	0	1 (12.5)

Most patients were diagnosed at advanced stages, including one (8.3%) at stage IIIA and nine (75.0%) at stage IV. Of the nine stage-IV patients, one (11.1%) patient was treated with gefitinib, one (11.1%) patient was treated with erlotinib, five (55.6%) received afatinib, and two (22.2%) underwent chemotherapy. Detailed information on these 12 patients is presented in Table 2. Eleven patients were found to have p.L747 P or p.L747S by cDNA Sanger sequencing, and three patients were identified by DNA sequencing. For 9 patients, paired analyses, incorporating cDNA sequencing as well as another detection method, were available for comparison. In five cases (case 3, 8, 10, 11 and 12 in Table 2), 19DEL rather than *EGFR* p.L747 P or p.L747S was detected by MassARRAY® or Therascreen®. Notably, two specimens (case 3 and 12 in Table 2) were both detected as L747-P753 > Q, a subtype of 19DEL, by MassARRAY®, and were finally identified to have c.2239_2240 TT > CC (p.L747 P) by cDNA Sanger sequencing. Another patient (case 10 in Table 2) with p.L747 P was detected as DEL L747-T751 in the *EGFR* gene by MassARRAY®, but cDNA Sanger sequencing disclosed c.2239_2240 TT > CC. Two patients (case 8 and 11 in Table 2) identified as Del in exon 19 by the method of Therascreen® was found to have c.2239_2240 TT > CC (p.L747 P) by cDNA Sanger sequencing. Among the other five patients, who were tested by direct DNA sequencing of *EGFR*, two showed an *EGFR* c.2239_2240 TT > CC (p.L747 P) mutation, consistent with the results of cDNA Sanger sequencing. However, three patients confirmed as having an *EGFR* c.2239_2240 TT > CC (p.L747 P) mutation by cDNA Sanger sequencing were misidentified as wild-type *EGFR* by DNA sequencing. In summary, direct DNA sequencing had a 60.0% (3/5) false-negative rate, and all 5 p.L747 P or p.L747S mutations were undetected or misclassified by commercial kits among the paired *EGFR* results.

Reviewing the published literature [12,14–21,23,24], we identified 11 patients with lung adenocarcinoma harboring p.L747 P (n = 10) or p.L747S (n = 1) mutations. One case was excluded because p.L747S was acquired in addition to the original p.L858R mutation after acquiring resistance to gefitinib [24]. Therefore, data for ten cases were retrieved and incorporated into our study. The median age of these ten patients was 61.0 (range, 44–78) years. Six patients (66.7%) were female, and 4 (57.1%) were never-smokers (Table 1). Eight of the ten patients (80.0%) were diagnosed at stage IV, and two (20.0%) had stage IB lung adenocarcinoma. Of the eight stage-IV patients, six were treated with first-generation EGFR TKIs, including five (62.5%) that received erlotinib and one (12.5%) treated with gefitinib, while one (12.5%) was administered with afatinib and one (12.5%) was treated with an unspecified EGFR TKI. Relevant information for these ten patients is provided in Table 3. All were found to have p.L747 P by DNA direct sequencing and NGS. As in the NTUH cases, among 5 tests of the four patients, no *EGFR* p.L747 P mutation was detected by MassARRAY® or therascreen®.

3.2. Clinical outcomes of patients

Of the nine stage-IV lung adenocarcinoma patients from NTUH, seven patients received EGFR TKIs (Table 2). Before 2014, two patients received one of the first-generation EGFR TKIs as first-line treatment. The ORR was 0% (0 of 2 patients), and both patients showed progressive disease in response to gefitinib or erlotinib. After 2014, afatinib was approved by the Taiwan Food and Drug Administration as a first-line treatment for *EGFR* mutant lung adenocarcinoma. Five patients diagnosed after 2014 were administered afatinib as first-line treatment. Of these, one patient developed interstitial pneumonitis within 30 days of receiving afatinib, and the ORR was 80% (4 of 5 patients), with four patients showing partial response to afatinib. Of these 7 patients receiving EGFR TKI treatment, the median PFS was 11.97 months (95.0% CI: 6.37–17.57) in the afatinib group (n = 5), compared with 0.92 month in the gefitinib/erlotinib group (n = 2) (P = 0.012) (Fig. 1). The median OS of patients receiving afatinib was longer than that of patients who received gefitinib/erlotinib, but this difference was not statistically significant (median: 16.62 [95.0% CI: 3.05–30.19] *vs.* 5.90 months, respectively; P = 0.290) (Fig. 2). Lastly, three patients with *EGFR* p.L747 P or p.L747S mutations underwent re-biopsies after developing acquired resistance to afatinib as first-line treatment. None (0%, 0 of 3 patients) were found to have acquired the p.T790 M *EGFR* mutation.

Of the six patients identified from the published literature that received first-generation EGFR TKI treatment, two patients showed stable disease and four patients exhibited progressive disease after gefitinib or erlotinib treatment; thus, the ORR to first-generation EGFR TKIs in these cases was also 0% (0 of 6 patients). The median PFS was 1.00 month (95.0% CI: 0.12–1.88), and the median OS was 12.00 [95.0% CI: 2.40–21.60]. In addition, one patient receiving second-generation EGFR TKI, afatinib, showed stable disease without progression for 24 months (Table 3).

4. Discussion

The uncommon mutations p.L747 P and p.L747S in exon 19 of *EGFR* are seldom discussed. Of 12 patients in our NTUH cohort study, 66.7% were male (n = 9) and 50.0% were current-smokers (n = 6). The presences of common or uncommon *EGFR* mutations are associated with female gender and a never-smoking history [6,31,32], which are distinct from the clinical features of this cohort. To the best of our knowledge, the clinical effectiveness of the irreversible ErbB TKI afatinib has never been comprehensively assessed as a treatment for lung adenocarcinoma harboring the *EGFR* p.L747P or p.L747S mutations. In our cohort study, two patients taking gefitinib or erlotinib as first-line treatment also showed progressive disease. Remarkably, the other five patients using afatinib as first-line treatment showed a favorable ORR (80%). Comparing the effectiveness of gefitinib/erlotinib and afatinib in the treatment of adenocarcinoma with p.L747P or p.L747S mutations in *EGFR*, PFS was significantly improved in those receiving afatinib. The median PFS was 11.97 months, which is comparable to that for the common *EGFR* mutations [10,16].

Six cases with p.L747P mutations from the published literature showed the similar treatment effectiveness with first generation EGFR TKIs comparing with our cohort study. None of six patients receiving gefitinib or erlotinib had responsive disease (2 with stable disease and 4 with progressive disease) and the PFS was relatively short (ranged from 0.5 to 7 months).

Identification of somatic *EGFR* mutations has been integrated with the diagnosis of lung adenocarcinoma in therapeutic protocols [33].

Table 2
Detailed information for 12 patients from NTUH harboring *EGFR* p.L747P or p.L747S mutations.

	Age (y)/ Sex	SS	Year of Dx	Hx	cS	Quality of tissues (% of tumor cells)	Results of *EGFR* mutation testing				EGFR TKI	Response to TKI	PFS after TKI (months)	Second biopsy after TKI failure	p.T790M in rebiopsy specimen
							DNA Sanger sequencing	MassARRAY® genotyping	Therascreen® EGFR RGQ PCR	cDNA Sanger sequencing					
1	64/F	NS	2011	AD	IIA	N/A				c.2239_2240 TT > CC (p.L747 P)	None	–			
2	76/M	AS	2011	ADS	IIA	30-40%	c.2239_2240 TT > CC (p.L747 P)			c.2239_2240 TT > CC (p.L747 P)	None	–			
3	65/M	AS	2017	AD	IIIA	N/A		c.2239_2258 > CA (L747-P753 > Q)		c.2239_2240 TT > CC (p.L747 P)	None	–			
4	93/M	AS	2014	AD	IV	30-40%	Wild type			c.2239_2240 TT > CC (p.L747 P)	None	–			
5	72/M	NS	2015	AD	IV	10-15%	c.2239_2240 TT > CC (p.L747 P)				None	–			
6	63/M	NS	2006	AD	IV	N/A	Wild type			c.2239_2240 TT > CC (p.L747 P)	Gefitinib	PD	0.9	No	–
7	36/M	AS	2008	AD	IV	N/A	Wild type			c.2239_2240 TT > CC (p.L747 P)	Erlotinib	PD	2.9	No	–
8	69/M	NS	2014	AD	IV	N/A			19DEL	c.2239_2240 TT > CC (p.L747 P)	Afatinib	PR	12.0	Yes	No
9	82/M	NS	2014	AD	IV	N/A				2155 G > A (p.G719S) + c.2240 T > C (p.L747S)	Afatinib	PR	14.7	No	–
10	58/F	NS	2016	AD	IV	N/A		c.2240_2254del15 (DEL L747-T751)		c.2240 T > C (p.L747S) + c.2250_2264del15 (DEL A750-K754)	Afatinib	PR	9.0	Yes	No
11	49/M	AS	2016	AD	IV	N/A			19DEL	c.2239_2240 TT > CC (p.L747 P)	Afatinib	PR	19.8	Yes	No
12	61/F	AS	2017	AD	IV	5-10%	c.2239_2240 TT > CC (p.L747 P)	c.2239_2258 > CA (L747-P753 > Q)		c.2239_2240 TT > CC (p.L747 P)	Afatinib	NE	1.0		

AD, lung adenocarcinoma; ADS, lung adenosquamous carcinoma; AS, active-smoker; cS, clinical stage; Dx, diagnosis; EGFR, epidermal growth factor receptor; Hx, histology; PR, partial response; F, female; M, male; N/A, not available; NE, non-evaluable; NS, never-smoker; PD, progressive disease; PFS, progression-free survival; SD, stable disease; SS, smoking status; TKI, tyrosine kinase inhibitor.

Table 3
Detailed information for 10 lung adenocarcinoma patients in published literature harboring *EGFR* p.L747P mutation.

	Age (y)/ Sex	Ethnicity	SS	Year of report	Hx	cS	Results of *EGFR* mutation testing				EGFR TKI	Response to TKI	PFS after TKI (months)	Reference
							DNA Sanger sequencing	Cobas®/ MassARRAY®	therascreen® EGFR RGQ PCR	NGS				
1	N/A	N/A	AS	2014	AD	IB	c.2239_2240 TT > CC (p.L747 P)	19DEL (cobas®)	19DEL	c.2239_2240 TT > CC (p.L747 P)	–	–	–	12
2	78/M	Japanese	AS	2014	AD	IB	c.2155 G > A (p.G719S) + c.2239_2240 TT > CC (p.L747 P)				–	–	–	23
3	48/F	N/A	NS	2015	AD	IV		Wild type (MassARRAY®)		c.2239_2240 TT > CC (p.L747 P)	N/A	–	–	24
4	59/F	Caucasian	N/A	2012	AD	IV	c.2239_2240 TT > CC (p.L747 P)				Gefitinib	SD	6	14
5	76/F	N/A	NS	2015	AD	IV	c.2237_2238AA > TT (p.E746 V) + c.2239_2240 TT > CC (p.L747 P)				Gefitinib	SD	7	15
6	66/M	Chinese	N/A	2015	AD	IV	c.2239_2240 TT > CC (p.L747 P)				Gefitinib	PD	0.5	16
7	69/F	Japanese	NS	2016	AD	IV	c.2239_2240 TT > CC (p.L747 P)		19DEL		Gefitinib	PD	1.6	17
8	61/M	Chinese	AS	2016	AD	IV	c.2239_2240 TT > CC (p.L747 P)			c.2239_2240 TT > CC (p.L747 P)	Erlotinib	PD	1	18
9	54/F	Chinese	NS	2018	AD	IV				c.2239_2240 TT > CC (p.L747 P)	Gefitinib	PD	1	19
10	44/F	Chinese	N/A	2018	AD	IV			Wild type	c.2239_2240 TT > CC (p.L747 P)	Afatinib	SD	24	20

AD, lung adenocarcinoma; AS, active-smoker; cS, clinical stage; EGFR, epidermal growth factor receptor; Hx, histology; PR, partial response; F, female; M, male; N/A, not available; NGS, next-generation sequencing; NS, never-smoker; PD, progressive disease; PFS, progression-free survival; SD, stable disease; SS, smoking status; TKI, tyrosine kinase inhibitor.

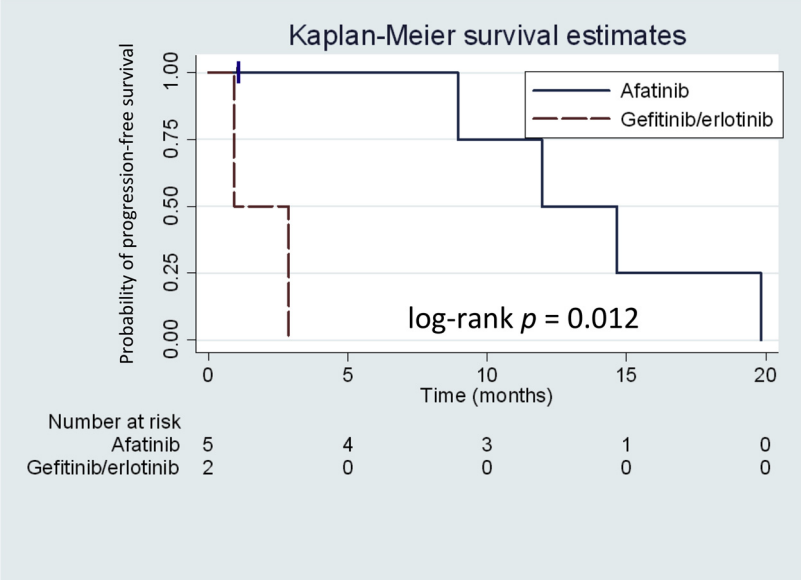

Fig. 1. Kaplan–Meier progression-free survival (PFS) curves according to tyrosine kinase inhibitor use. Patients receiving afatinib or gefitinib/erlotinib are represented by solid blue and dashed red lines, respectively.

Commercial kits, including the therascreen® EGFR RGQ PCR kit, cobas® EGFR mutation test, and MassARRAY® genotyping, are commonly applied in clinical practice in most hospitals, and the results of such tests are used to guide physicians in treating patients. However, the sensitivity and accuracy of *EGFR* mutation detection using these methods should be considered and the results cautiously interpreted, as the misidentification or false-negative detection of *EGFR* mutations would provide incorrect information for the guidance of clinical management. Our previous research showed that RNA-based sequencing was more sensitive and accurate than direct genomic DNA sequencing or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of genomic DNA [28]. Therefore, the results of RNA-based sequencing for *EGFR* detection could be viewed as the reference data in this study. From our combined study of NTUH patients and those identified from the literature, eight patients who were previously screened using commercial kits, namely the therascreen® and MassARRAY®, showed misclassification of p.L747P or p.L747S mutations as 19DEL mutations or wild-type *EGFR*. The misidentification of

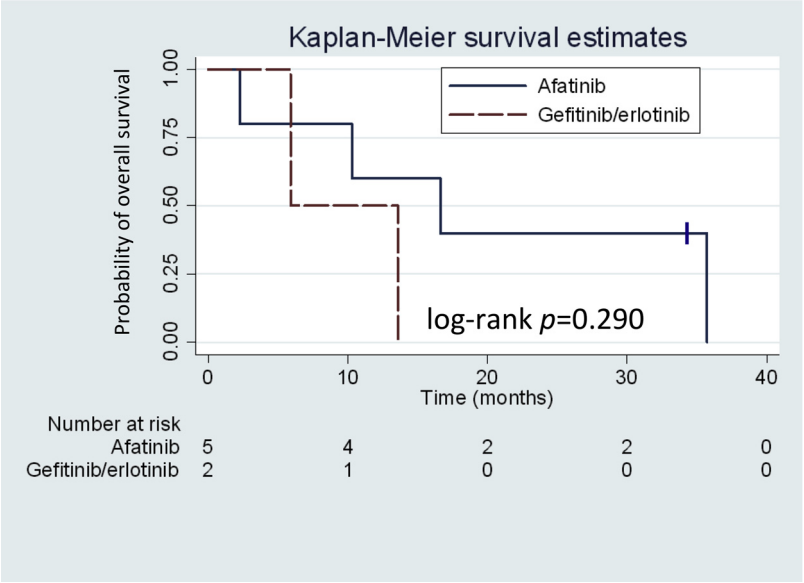

Fig. 2. Kaplan–Meier overall survival (OS) curves according to tyrosine kinase inhibitor use. Patients receiving afatinib or gefitinib/erlotinib are represented by solid blue and dashed red lines, respectively.

p.L747P as a 19DEL mutation may stem from the fact that a 2-bp mutations in exon 19 (c.2239_2240delinsCC) or may result from the mispriming of oligonucleotides used for PCR [12,17]. Remarkably, two patients harboring p.L747 P were both detected as L747-P753 > Q of exon 19 in the *EGFR* gene, which was interpreted as 19DEL, by using method of MassARRAY®. Accordingly, we should carefully check the detailed genetic information of the broad spectrum of 19DEL mutations, and the result of variants on codon 747 of exon 19 in-frame deletions by MassARRAY® particularly need further investigation and identification. In addition, the results of direct DNA and cDNA sequencing of the *EGFR* gene in six patients were compared in the NTUH cohort population. Three of the six (50%) patients were identified as having wild-type *EGFR* by DNA sequencing, rather than having p.L747P or p.L747S mutant *EGFR*. Several methodological studies have emphasized that direct DNA sequencing is highly dependent on DNA quality and the enrichment of tumor cells in specimens [34,35]. With poor DNA quality or a low proportion of tumor cells in specimens, patients could be misdiagnosed as having wild-type *EGFR* rather than a p.L747P mutation.

The above conditions could result in misled clinicians making inappropriate treatment decisions. In the era of personal and precision medicine, the methodological limitations of laboratory methods should be clarified. NGS offers an alternative method for obtaining comprehensive information on the mutational spectrum of NSCLC, revealing rare mutations to better target therapies [36]. For that reason, in patients treated with a first-generation EGFR TKI as a targeted therapy for a 19DEL in *EGFR* based on the results of commercial kits who develop primary resistance to gefitinib or erlotinib, NGS could be used to recheck these specimens, providing additional information for more personalized therapies. With widespread application of NGS in clinical practice, more patients with *EGFR* p.L747P or p.L747S mutations are likely to be identified, and these patients should preferentially be treated with afatinib.

The mutation p.T790M is the main mechanism of acquired resistance after EGFR TKI failure and was shown to be associated with common *EGFR* mutations, especially 19DEL [37,38]. Among our three patients harboring *EGFR* p.L747P or p.L747S mutations and receiving afatinib, none showed the acquisition of the p.T790M mutation after developing resistance. Nevertheless, more data is required to explore the mechanisms of acquired resistance in this specific population.

There are some limitations of our study, including a limited sample size and retrospective design in our NTUH cohort study, as well as potential publication bias and missing data in our review of the literature. While patients harboring p.L747P or p.L747S mutations had favorable responses and improved PFS after afatinib treatment, the lack of a significant improvement in OS may be a result of our limited sample size. Therefore, additional cases are needed to confirm our findings.

5. Conclusions

The uncommon p.L747P and p.L747S *EGFR* mutations can be misclassified as a 19DEL mutation using commercial kits or wild-type *EGFR* using DNA sequencing because of poor DNA quality or a small proportion of tumor cells in specimens. Considering the limitations of above methods, we should cautiously interpret the results of *EGFR* mutations from above methods. In clinical, NGS should be widely used in correctly detecting these mutations and guide physicians making clinical decisions. Moreover, our results suggest that the irreversible inhibitor afatinib may provide improvements in PFS and OS in the treatment of lung adenocarcinoma with *EGFR* p.L747P or p.L747S mutations.

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Conflict of interest

Sheng-Kai Liang has received honoraria from Roche, AstraZeneca, Merck Sharp & Dohme, and Boehringer Ingelheim for speeches. Jen-Chung Ko has received honoraria from Roche, AstraZeneca, Eli Lilly, and Boehringer Ingelheim for speeches. James Chih-Hsin Yang reports personal fees from Boehringer Ingelheim, Eli Lilly, Bayer, Roche/Genentech, Chugai, Astellas, Merck Sharp & Dohme, Merck Serono, Pfizer, Novartis, Clovis Oncology, Celgene, Merrimack, Yuhan Pharmaceuticals, BMS, Ono Pharmaceuticals, Daiichi Sankyo, AstraZeneca, Hansoh Pharmaceutica, and Takeda Pharmaceuticals. Jin-Yuan Shih has received speaking honoraria from AstraZeneca, Roche, Boehringer Ingelheim, Eli Lilly, Pfizer, Novartis, Merck Sharp & Dohme, Ono Pharmaceutical, AbbVie, Chugai, and Bristol-Myers Squibb.

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