J Chin Med Assoc

ORIGINAL ARTICLE

Epidermal growth factor receptor mutations in non–small cell lung cancer undetected by high-sensitivity allele-specific real-time polymerase chain reaction–based assays

Chia-I Shen[a], Hsiang-Ling Ho[b,c], Yi-Chen Yeh[b,d], Chao-Hua Chiu[a,d,*], Teh-Ying Chou[b,d]

[a]Department of Chest Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [b]Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [c]School of Biomedical Science and Engineering, National Yang-Ming University, Taipei, Taiwan, ROC; [d]School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC

Abstract

Background: Identifying epidermal growth factor receptor (EGFR) mutation status is critical for planning lung cancer treatment. Sanger sequencing detects both known and novel mutations but shows poor sensitivity. High-sensitivity allele-specific real-time polymerase chain reaction (ASRP)-based assays offer quick and reliable results, but may overlook uncommon mutations. We aimed to define the rate at which high-sensitivity ASRP-based assays missed uncommon EGFR mutations.

Methods: Non–small cell lung cancer specimens that were diagnosed as EGFR wild-type (EGFR-WT) by high-sensitivity ASRP-based assays and had residual DNA samples were sent for Sanger sequencing. Patient characteristics and clinical features were evaluated by chart review, and outcomes of EGFR-tyrosine kinase inhibitor (EGFR-TKI) therapy were studied.

Results: Hundred DNA specimens diagnosed by high-sensitivity ASRP-based assays as EGFR-WT were rechecked by Sanger sequencing. Two samples which were re-biopsy specimens from patients with EGFR mutations were excluded from the analysis. Sanger sequencing was failed in 24 samples. Among the remaining 74 samples, 6 (8.1%) had EGFR mutations—one exhibited exon 19 deletion (delT751_I759insS), two exhibited substitution mutations (S768I+V769L and L861Q), and three exhibited exon 20 insertions (N771_P772insN, P772_H773insHP, and H773_V774insAH). Only the patient with the exon 19 deletion had received EGFR-TKI therapy. Although the best tumor response was only stable disease, this was maintained for >10 months.

Conclusion: High-sensitivity ASRP-based assays can overlook uncommon mutations. This detection failure rate is worth noting, especially when treating patients from regions known to have a high prevalence of EGFR mutation. Patients carrying uncommon mutations may still benefit from EGFR-TKI therapy.

Keywords: Epidermal growth factor receptor mutation; Non–small cell lung cancer; Sequence analysis

1. INTRODUCTION

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase overexpressed in many malignancies. The frequency of EGFR mutations in non–small cell lung cancer (NSCLC) is higher in Asians, females, and nonsmokers.[1] According to pivotal studies published in 2004, EGFR mutation status is an important factor for EGFR-tyrosine kinase inhibitor (EGFR-TKI) treatment.[2,3] In treatment-naïve, EGFR-mutant advanced NSCLC patients, EGFR-TKI has been proven to provide better outcomes compared with chemotherapy.[4–7] Thus, detection of EGFR mutations is the key step in the management of advanced NSCLC patients. In-frame deletions in exon 19 and substitution mutation L858R in exon 21 account for over 80% of EGFR mutations.[8–10] Other subsets are considered uncommon mutations, and the efficacy of EGFR-TKI in these mutations remains uncertain.[11]

Classical Sanger sequencing detects both known and novel genomic mutations, but it has suboptimal sensitivity and requires the DNA sample to contain roughly 25% of a given mutation to enable its detection.[12] Our previous study revealed Sanger sequencing to have a false-negative rate of up to 21.5% in clinical samples collected from an area where EGFR mutations are prevalent.[13] To meet clinical needs, high-sensitivity allele-specific real-time polymerase chain reaction (ASRP)-based assays using specially designed primers and probes to lower the detection threshold requirement to 1% mutant DNA in the sample. However, it can only detect the predefined mutations to which the primers and probes included in the assay kit have been customized; therefore, uncommon mutations will inevitably be overlooked.

It has been suggested that uncommon EGFR mutations may be less susceptible to EGFR-TKI treatment than common

*Address correspondence. Dr. Chao-Hua Chiu, Department of Chest Medicine, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC. E-mail address: jhchiou@vghtpe.gov.tw (C.-H. Chiu).

Conflicts of Interest Statement: Chao-Hua Chiu had received honoraria from AstraZeneca, Boehringer Ingelheim, Novartis, and Roche. Teh-Ying Chou had received honorarium from AstraZeneca, Merck Sharp & Dohme, Novartis, and Roche. The other authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2020) 83: 345-349.

Received July 5, 2019; accepted October 8, 2019.

doi: 10.1097/JCMA.0000000000000277.

<zdoi: 10.1097/JCMA.0000000000000277>

mutations.[14–16] However, uncommon mutations are a heterogeneous group, and they vary in clinical significance.[17–20] Thus, clinical outcomes for overlooked or uncommon mutations are still inconclusive. In this study, we collected the residues of the same batch of DNA from clinical samples that were shown to be negative for EGFR mutations by high-sensitivity ASRP-based assays, and we reevaluated them using Sanger sequencing. We aimed to define the rate at which high-sensitivity ASRP-based assays fail to detect uncommon EGFR mutations.

2. METHODS

2.1. Patients and samples

We used the database of the Department of Pathology and Laboratory Medicine in Taipei Veterans General Hospital. Patients who were diagnosed as having advanced NSCLC and had had their tumors tested for EGFR mutation by ASRP-based assays between 2009 and 2014 were included in the study. For tumors in which EGFR mutations were not detected by the ASRP method, we rechecked the residual amount of extracted tumor genomic DNA remaining after the mutation testing; if the amount of residual DNA was adequate, it was then sent for further EGFR mutation detection using Sanger sequencing. Patient characteristics and clinical features were collected by chart review. The protocol was approved by the Institution Review Board at Taipei Veterans General Hospital.

2.2. ASRP-based EGFR mutation assay

A high-sensitivity ASRP EGFR mutation assay consisting of a commercial diagnostic kit (cobas EGFR Mutation Test v1; Roche Molecular Diagnostics, Pleasanton, CA, USA) was used as a clinical tool for EGFR mutation detection at Taipei Veterans General Hospital during the years of 2009 through 2014. This kit is designed to detect the most common 41 EGFR mutations occurring throughout exons 18 and 21, including T790M, S768I, two L858R mutations, three G719 missense mutations, 29 exon 19 deletions, and five exon 20 insertions. The EGFR mutation assay was done after the control assay to assess the total DNA in a sample. All procedures were performed according to the manufacturer's instructions. In brief, 1 μL of genomic DNA was used for detection of exon 2 of the EGFR gene using on a Rotor-gene platform to determine the DNA quality. The cycle threshold should fall within the range between 23.0 and 30.69 to be considered acceptable for further EGFR mutation testing. Then, 10 μL of genomic DNA was used for EGFR mutation analysis using the cobas 4800 analyzer for automated amplification and detection.

2.3. EGFR mutation detection by Sanger sequencing

Exons 18, 19, 20, and 21 of the EGFR gene were amplified with minor modification; namely, nested polymerase chain reaction (PCR) was only performed on specimens when their first PCR products could not be visualized on 2% agarose gel electrophoresis. The first round of PCR was performed in a total volume of 25 μL containing 2 μL of DNA, 1× Taq Master Mix Red (Ampliqon III, Odense, Denmark), and 0.5 μM of each primer. This PCR program consisted of 35 cycles of (95°C for 40 s, 56°C for 40 s, and 72°C for 40 s), followed by a 5-minute extension stage at 72°C. For the nested PCR protocol, DNA amplification was performed using the same PCR program as described above, using 2 μL of the first PCR products as a template, 1× Taq Master Mix Red, and 0.5 μM of each primer. Sanger sequencing was performed with forward or reverse primers, and sequence analyses were performed using Mutation Surveyor software (SoftGenetics, State College, PA, USA).

2.4. Statistical methods

The differences in characteristics and discordance rate were compared using chi-square tests, and the associated p values were two-sided. Fisher's exact test was used for data with an expected frequency <5. The Mann-Whitney U test was applied for analyses of cancer cell percentages and Sanger sequencing success rates. Progression-free survival was defined as the duration from dosing of EGFR-TKI to tumor progression as documented by clinical physicians. Kaplan-Meier survival curves were plotted for progression-free-survival and overall survival. These analyses were conducted using the SPSS version 21.0 software application (IBM Corp., Armonk, NY, USA).

3. RESULTS

3.1. Patient characteristics

After reviewing the EGFR mutation testing results and the residual DNA adequacy in the database, 100 tumor genomic DNA samples fulfilled the inclusion criteria of this study were enrolled. All of these samples had been tested for EGFR mutations using a highly sensitive ASRP-based assay, and no variations were detected. However, two samples were re-biopsy specimens and were excluded for further analysis because EGFR mutations had been found in these patients' previous specimens and they had received EGFR-TKI treatment before. Among the remaining 98 samples, most (76.5%) were extracted from tumors that were biopsied or resected in 2014. The samples came from 98 patients consisting of 69 males and 29 females, and the median age was 72 (with an age range of 41-90). Sixty-one patients had a history of tobacco-smoking. Most of the samples were from adenocarcinomas (80.6%), were from patients in stage IV of disease (78.6%), and had good performance status (ECOG 0-1, 76.5%). As of June 30, 2017, only 23 patients had received EGFR-TKI treatment and most of them used it as the second-line or beyond therapy (three used as the first-line therapy because of their poor performance status). The details are summarized in Table 1.

3.2. Performance of Sanger sequencing

Among 98 samples, 24 failed to produce DNA products by nested PCR. For samples obtained from tumors acquired in 2014, the failure rate was 21.3%. Although not amounting to statistically significant differences, the failure rates were numerically higher for samples collected before 2014 (34.8% vs 21.3%, $p = 0.266$). Interestingly, the failure of Sanger sequencing was significantly associated with the percentage of cancer cells in the samples ($p = 0.002$, Mann-Whitney U test): samples that yielded sufficient amounts of PCR product for Sanger sequencing were obtained from slides where over 25% of cells present were cancer cells (88.9% vs 59.1%, $p = 0.001$). Other factors were not associated with the Sanger sequencing failure rate (Table 2).

3.3. Discordance between ASRP and Sanger sequencing

Among 74 samples that yielded sufficient PCR product to proceed to the next step, 6 (8.1%) were found to have EGFR missense mutations, at exons 18 to 21, by Sanger sequencing. The concordance rate between different factors showed no significant differences (Table 3). Higher cancer cell percentages did not yield higher concordance rates (92.1% vs 91.7%, $p = 1.000$). The six overlooked mutations included one short in-frame deletion mutation at exon 19 (delT751_I759insS), one complex substitution mutation (S768I+V769L) and three short insertion mutations (N771_P772insN, P772_H773insHP, H773_V774insAH) at exon 20, and one substitution mutation (L861Q) at exon 21.

Table 1

Patient and tumor characteristics

Characteristics	n (%)
Gender	
Male	69 (70.4%)
Female	29 (29.6%)
Smoking status	
Nonsmoker	37 (37.8%)
Ex-smoker	38 (38.8%)
Current smoker	23 (23.5%)
Histology	
Adenocarcinoma	79 (80.6%)
Nonadenocarcinoma	19 (19.4%)
Disease stage	
Stage I-III	21 (21.4%)
Stage IV	77 (78.6%)
Year of sample collection	
2014	75 (76.5%)
Before 2014	23 (23.5%)
EGFR-TKI treatment	
EGFR-TKI used	23 (23.5%)
EGFR-TKI not used	75 (76.5%)
ECOG	
0–1	75 (76.5%)
≥2	23 (23.5%)

EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor.

3.4. Clinical response to EGFR-TKI

Among the 23 patients who had received EGFR-TKI during the study period, 2 demonstrated objective tumor responses (objective response rate = 8.7%), 6 maintained stable disease for some period of time, 14 were refractory to treatment, and the treatment response for 1 patient could not be evaluated. The tumors obtained from the eight patients who had disease controlled by EGFR-TKI therapy had the following genetic characteristics: five were EGFR-WT, one was the uncommon exon 19 deletion mutation (delT751_I759insS), and the remaining two were unknown because they failed to produce PCR products. The median progression-free survival for patients who had been treated with EGFR-TKI was 56 days (95% confidence interval, 46-66 days).

Among the six patients whose tumors had been found to have EGFR mutations, only one (with the uncommon exon 19 deletion mutation, delT751_I759insS) had received EGFR-TKI treatment. The patient had maintained stable disease up to the last follow-up date and had been treated for over 325 days.

Table 2

Comparison of samples that succeeded or failed Sanger sequencing

	Sanger sequencing		
	Failed (%)	Succeeded (%)	*p*
Gender			0.797
Male	27.6	72.4	
Female	23.2	76.8	
Age			0.239
≤75	19.6	80.4	
>75	31.0	69.0	
Smoking			0.809
Nonsmoker	27	73	
Ever-smoker	23	77	
Disease stage			0.390
Stage I-III	33.3	66.7	
Stage IV	22.1	77.9	
Year of sample collection			0.266
2014	21.3	78.7	
Before 2014	34.8	65.2	
Histology			0.776
Adenocarcinoma	25.3	74.7	
Nonadenocarcinoma	21.1	78.9	
Cancer cell percentage			0.001
<25%	40.9	59.1	
≥25%	11.1	88.9	

Table 3

Discordance between allele-specific real-time PCR and Sanger sequencing

	Sanger sequencing and allele-specific real-time PCR (n = 74)		
	Discordance (%)	Concordance (%)	*p*
Gender			1.000
Male	7.5	92.5	
Female	9.5	90.5	
Age			1.000
≤75	8.9	91.1	
>75	6.9	93.1	
Smoking			0.662
Nonsmoker	11.1	88.9	
Ever-smoker	6.4	93.6	
Disease stage			0.317
Stage I-III	14.3	85.7	
Stage IV	6.7	93.3	
Year of sample collection			1.000
2014	8.5	91.5	
Before 2014	6.7	93.3	
Histology			0.337
Adenocarcinoma	10.2	89.8	
Nonadenocarcinoma	0	100	
Cancer cell percentage			1.000
<25%	7.7	92.3	
≥25%	8.3	91.7	

PCR = polymerase chain reaction.

4. DISCUSSION

Accurate EGFR mutation detection has become one of the most critical steps in the management of advanced NSCLC patients in this era of targeted therapy.[2,3] Sanger sequencing was previously the gold standard of detecting mutation. However, it showed unsatisfied sensitivity and time-consuming. Targeted methods, such as cobas and Amplification Refractory Mutation System (ARMS), focus on specific known mutations by designed primers. These ASRP-based assays reduced turnaround times and elevated the sensitivity for common mutations.[21] In our study, we used cobas for initial EGFR mutation test. Literature review showed positive percentage agreement between cobas and Sanger sequencing was 98.8% and negative percentage agreement was 79.3%.[22] Although high-sensitivity ASRP-based assays have become the standard method for EGFR mutation detection in clinical practice, they may miss some uncommon but clinically meaningful mutations that would respond to EGFR-TKI treatment.[23,24] It is reasonable to expect that some EGFR mutations are not being detected clinically, but the magnitude of this problem is not clear—and for these patients whose EGFR mutations are missed, the chances of having successful outcomes may be significantly compromised. In this study, we examined the

prevalence of failure to detect uncommon EGFR mutations during testing in real-world practice. Using Sanger sequencing, we found that EGFR mutations were observed in about 8.1% of the samples that had been deemed negative for EGFR mutations by high-sensitivity ASRP-based testing. Because the sensitivity of Sanger sequencing is limited, the rate of detection failure in ASRP testing is expected to be underestimated.[12]

Previous studies indicated that patients with rare or complex EGFR mutations have worse survival and inferior responses to EGFR-TKI treatment than those with common EGFR mutations.[25,26] However, the molecular heterogeneity of different uncommon mutations makes it difficult to reach general conclusions. For example, afatinib is reported to be effective in treating selected types of uncommon EGFR mutations, but it has low response rates in exon 20 insertion mutations.[20,27]

In our study, the most common EGFR mutations missed by high-sensitivity ASRP-based EGFR mutation testing were exon 20 insertions. This was an expected result—because exon 20 insertions are among the largest groups of uncommon EGFR mutations, and the commercial kit used in this study is designed to detect only five of the known exon 20 insertion variants.[13,28] Although most tumors bearing EGFR exon 20 insertion mutations do not respond to classical EGFR-TKIs, there have still been studies showing remarkable responses.[27,29,30] Lin et al[19] reviewed the literature and reported that exon 20 insertion A763_Y764insFQEA was an EGFR-TKI sensitive mutation, with a response rate of 73% and disease control rate of 91%. In addition, several novel EGFR-TKIs are under developed and have been shown to have activity against tumors containing exon 20 insertions.[31,32] Some clinical trials (e.g., NCT02716116 and NCT03066206) are trying to evaluate outcomes for patients with exon 20 insertions treated with new generation EGFR-TKIs. This further emphasizes the importance of developing a more comprehensive detection method for EGFR exon 20 insertion mutations.

The failure of the ASRP-based assay to detect EGFR L861Q mutation was also predictable, because this EGFR mutation detection kit used in our study period did not include the primers and probe for L861Q mutation. This problem had already been fixed in the updated version of this FDA-approved kit. Our ASRP-based assay kit dose contain specific primers and probe for S768I mutation; however, the complex EGFR mutation S768I+V769L was not detected. It is probable that a nearby V769L mutation may affect the affinity of the S768I-specific probe and cause a false-negative result.[33] The detection failure of the uncommon exon 19 in-frame deletion mutation, delT751_I759insS, is also anticipated because of the lack of appropriate primers and probe. This patient received erlotinib when disease progression on the first-line chemotherapy, pemetrexed, and carboplatin. She had the best tumor response of stable disease, and she remained disease-controlled for over 10.8 months. In the literature, we found another patient had this same exon 19 deletion mutation and had achieved progression-free survival of 13.3 months under EGFR-TKI treatment.[24]

In addition, our study yielded a few important findings of clinical relevance. Only 23 of our 98 patients (23.5%) had been treated with EGFR-TKI during their entire treatment course. This strongly suggests that, in the real world, clinicians trusted the negative results they had obtained from the high-sensitivity ASRP-based EGFR mutation testing; they apparently had low expectations for EGFR-TKI treatment responses in these patients with "EGFR mutation-negative" NSCLC in the context of second-line therapies or beyond. However, 2 of these 23 patients did benefit from EGFR-TKI treatment, yielding a response rate of 8.7%, which was comparable with the treatment efficacy of second-line chemotherapies such as docetaxel and pemetrexed.[34] A randomized controlled trial, TAILOR study, had clearly shown superior outcomes for chemotherapy over EGFR-TKI in the second-line treatment setting.[35] In this study, the response rate in the EGFR-TKI treatment group was only 3%. Nevertheless, it should be noted that merely 1% of the participants were Asian. Our study results were similar to those of other studies conducted in the same geographic region, suggesting that the background prevalence of EGFR mutations in a given population should be taken into consideration when choosing second-line treatment options in "EGFR-negative" NSCLC patients.[36,37]

Our study had several limitations. First, being a retrospective study, we had no way of knowing whether clinical data and patient conditions had been accurately documented. Second, Sanger sequencing has low sensitivity and therefore requires higher mutant DNA quantities in the specimen to be effective; usually the sample must consist of at least 25% mutant alleles if the mutations are to be detected by Sanger sequencing.[12] The rate of failure to detect uncommon mutations may be even higher if a more sensitive method, such as next generation sequencing, is employed. Third, the low incidence of different uncommon mutations makes it difficult to reach conclusions on their clinical significance. Liang et al[38] reported similar findings with three uncommon EGFR mutations detected by Sanger sequencing, out of 100 ARMS-PCR EGFR-mutant negative stage I to III adenocarcinoma samples. Both studies supported the finding that primer-designed high-sensitivity assays may overlook uncommon EGFR mutations.

There are several ways to improve the detection of uncommon mutations. For example, we may add specific primers of uncommon mutation in current EGFR mutation kit. Another method is by using a sequential evaluation test, that is, screening common mutations first and a targeted survey of uncommon mutations as following. Furthermore, broad-based genomic sequencing methods such as next generation sequencing may offer more comprehensive and sensitive study of gene profile.

In conclusion, ASRP-based EGFR mutation assays, although highly sensitive, will unavoidably miss some uncommon mutations. The detection failure rate, determined by rechecking via Sanger sequencing, was up to 8.1% in an area with high prevalence of EGFR mutations. Patients with these uncommon mutations may still benefit from EGFR-TKI treatment.

ACKNOWLEDGMENTS

This work was supported by Taipei Veterans General Hospital, Taiwan (V105C-181, V107C-106, and V109A-003); the Ministry of Science and Technology, Taiwan (MOST106-2314-B-075-031-MY3-2), and the Ministry of Health and Welfare, Taiwan (MOHW107-TDU-B-211-114019). The authors thank Yu-Ting Huang and Han-Jhih Chang for technical support and Christopher at Editage for English editing.

REFERENCES

1. Sequist LV, Heist RS, Shaw AT, Fidias P, Rosovsky R, Temel JS, et al. Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice. *Ann Oncol* 2011;**22**:2616–24.
2. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;**350**:2129–39.
3. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;**304**:1497–500.
4. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al.; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations

of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;**11**:121–8.
5. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al.; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;**362**:2380–8.
6. Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2011;**12**:735–42.
7. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al.; Spanish Lung Cancer Group in collaboration with Groupe Français de Pneumo-Cancérologie and Associazione Italiana Oncologia Toracica. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 2012;**13**:239–46.
8. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2005;**97**:339–46.
9. Costa DB. Kinase inhibitor-responsive genotypes in EGFR mutated lung adenocarcinomas: moving past common point mutations or indels into uncommon kinase domain duplications and rearrangements. *Transl Lung Cancer Res* 2016;**5**:331–7.
10. Hsu WH, Yang JC, Mok TS, Loong HH. Overview of current systemic management of EGFR-mutant NSCLC. *Ann Oncol* 2018;**29**(suppl_1) :i3–9.
11. Noronha V, Choughule A, Patil VM, Joshi A, Kumar R, Susan Joy Philip D, et al. Epidermal growth factor receptor exon 20 mutation in lung cancer: types, incidence, clinical features and impact on treatment. *Onco Targets Ther* 2017;**10**:2903–8.
12. Pao W, Ladanyi M. Epidermal growth factor receptor mutation testing in lung cancer: searching for the ideal method. *Clin Cancer Res* 2007;**13**:4954–5.
13. Chiu CH, Ho HL, Chiang CL, Lin SF, Ma HH, Chuang YT, et al. Clinical characteristics and treatment outcomes of lung adenocarcinomas with discrepant EGFR mutation testing results derived from PCR-direct sequencing and real-time PCR-based assays. *J Thorac Oncol* 2014;**9**:91–6.
14. Karachaliou N, Molina-Vila MA, Rosell R. The impact of rare EGFR mutations on the treatment response of patients with non-small cell lung cancer. *Expert Rev Respir Med* 2015;**9**:241–4.
15. Watanabe S, Minegishi Y, Yoshizawa H, Maemondo M, Inoue A, Sugawara S, et al. Effectiveness of gefitinib against non-small-cell lung cancer with the uncommon EGFR mutations G719X and L861Q. *J Thorac Oncol* 2014;**9**:189–94.
16. Chiu CH, Yang CT, Shih JY, Huang MS, Su WC, Lai RS, et al. Epidermal growth factor receptor tyrosine kinase inhibitor treatment response in advanced lung adenocarcinomas with G719X/L861Q/S768I mutations. *J Thorac Oncol* 2015;**10**:793–9.
17. Tu HY, Ke EE, Yang JJ, Sun YL, Yan HH, Zheng MY, et al. A comprehensive review of uncommon EGFR mutations in patients with non-small cell lung cancer. *Lung Cancer* 2017;**114**:96–102.
18. Yasuda H, Park E, Yun CH, Sng NJ, Lucena-Araujo AR, Yeo WL, et al. Structural, biochemical, and clinical characterization of epidermal growth factor receptor (EGFR) exon 20 insertion mutations in lung cancer. *Sci Transl Med* 2013;**5**:216ra177.
19. Lin YT, Liu YN, Wu SG, Yang JC, Shih JY. Epidermal growth factor receptor tyrosine kinase inhibitor-sensitive exon 19 insertion and exon 20 insertion in patients with advanced non-small-cell lung cancer. *Clin Lung Cancer* 2017;**18**:324–332.e1.
20. Yang JC, Sequist LV, Geater SL, Tsai CM, Mok TS, Schuler M, et al. Clinical activity of afatinib in patients with advanced non-small-cell lung cancer harbouring uncommon EGFR mutations: a combined post-hoc analysis of LUX-lung 2, LUX-lung 3, and LUX-lung 6. *Lancet Oncol* 2015;**16**:830–8.
21. Ellison G, Zhu G, Moulis A, Dearden S, Speake G, McCormack R. EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. *J Clin Pathol* 2013;**66**:79–89.
22. Lopez-Rios F, Angulo B, Gomez B, Mair D, Martinez R, Conde E, et al. Comparison of molecular testing methods for the detection of EGFR mutations in formalin-fixed paraffin-embedded tissue specimens of non-small cell lung cancer. *J Clin Pathol* 2013;**66**:381–5.
23. Wu JY, Yu CJ, Chang YC, Yang CH, Shih JY, Yang PC. Effectiveness of tyrosine kinase inhibitors on "uncommon" epidermal growth factor receptor mutations of unknown clinical significance in non-small cell lung cancer. *Clin Cancer Res* 2011;**17**:3812–21.
24. Lee VH, Tin VP, Choy TS, Lam KO, Choi CW, Chung LP, et al. Association of exon 19 and 21 EGFR mutation patterns with treatment outcome after first-line tyrosine kinase inhibitor in metastatic non-small-cell lung cancer. *J Thorac Oncol* 2013;**8**:1148–55.
25. Baek JH, Sun JM, Min YJ, Cho EK, Cho BC, Kim JH, et al. Efficacy of EGFR tyrosine kinase inhibitors in patients with EGFR-mutated non-small cell lung cancer except both exon 19 deletion and exon 21 L858R: a retrospective analysis in Korea. *Lung Cancer* 2015;**87**:148–54.
26. Frega S, Lorenzi M, Fassan M, Indraccolo S, Calabrese F, Favaretto A, et al. Clinical features and treatment outcome of non-small cell lung cancer (NSCLC) patients with uncommon or complex epidermal growth factor receptor (EGFR) mutations. *Oncotarget* 2017;**8**:32626–38.
27. Heigener DF, Schumann C, Sebastian M, Sadjadian P, Stehle I, Märten A, et al.; Afatinib Compassionate Use Consortium (ACUC). Afatinib in non-small cell lung cancer harboring uncommon EGFR mutations pretreated with reversible EGFR inhibitors. *Oncologist* 2015;**20**:1167–74.
28. Arcila ME, Nafa K, Chaft JE, Rekhtman N, Lau C, Reva BA, et al. EGFR exon 20 insertion mutations in lung adenocarcinomas: prevalence, molecular heterogeneity, and clinicopathologic characteristics. *Mol Cancer Ther* 2013;**12**:220–9.
29. Kuiper JL, Hashemi SM, Thunnissen E, Snijders PJ, Grünberg K, Bloemena E, et al. Non-classic EGFR mutations in a cohort of Dutch EGFR-mutated NSCLC patients and outcomes following EGFR-TKI treatment. *Br J Cancer* 2016;**115**:1504–12.
30. Voon PJ, Tsui DW, Rosenfeld N, Chin TM. EGFR exon 20 insertion A763_Y764insFQEA and response to erlotinib–letter. *Mol Cancer Ther* 2013;**12**:2614–5.
31. Hirano T, Yasuda H, Hamamoto J, Nukaga S, Masuzawa K, Kawada I, et al. Pharmacological and structural characterizations of naquotinib, a novel third-generation EGFR tyrosine kinase inhibitor, in EGFR-mutated non-small cell lung cancer. *Mol Cancer Ther* 2018;**17**:740–50.
32. Hasako S, Terasaka M, Abe N, Uno T, Ohsawa H, Hashimoto A, et al. TAS6417, a novel EGFR inhibitor targeting exon 20 insertion mutations. *Mol Cancer Ther* 2018;**17**:1648–58.
33. Martínez-Carretero C, Pascual FI, Rus A, Bernardo I. Detection of EGFR mutations in patients with non-small cell lung cancer by high resolution melting. Comparison with other methods. *Clin Chem Lab Med* 2017;**55**:1970–8.
34. Cohen MH, Johnson JR, Wang YC, Sridhara R, Pazdur R. FDA drug approval summary: pemetrexed for injection (ALIMTA) for the treatment of non-small cell lung cancer. *Oncologist* 2005;**10**:363–8.
35. Garassino MC, Martelli O, Broggini M, Farina G, Veronese S, Rulli E, et al.; TAILOR Trialists. Erlotinib versus docetaxel as second-line treatment of patients with advanced non-small-cell lung cancer and wild-type EGFR tumours (TAILOR): a randomised controlled trial. *Lancet Oncol* 2013;**14**:981–8.
36. Kobayashi T, Koizumi T, Agatsuma T, Yasuo M, Tsushima K, Kubo K, et al. A phase II trial of erlotinib in patients with EGFR wild-type advanced non-small-cell lung cancer. *Cancer Chemother Pharmacol* 2012;**69**:1241–6.
37. Tseng JS, Wang CL, Huang MS, Chen CY, Chang CY, Yang TY, et al. Impact of EGFR mutation detection methods on the efficacy of erlotinib in patients with advanced EGFR-wild type lung adenocarcinoma. *PLoS One* 2014;**9**:e107160.
38. Liang C, Wu Z, Gan X, Liu Y, You Y, Liu C, et al. Detection of rare mutations in EGFR-ARMS-PCR-negative lung adenocarcinoma by sanger sequencing. *Yonsei Med J* 2018;**59**:13–9.