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Evaluation of PCR-HRM, RFLP, and direct sequencing as simple and cost-effective methods to detect common *EGFR* mutations in plasma cell–free DNA of non–small cell lung cancer patients

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Abstract

Background: Lung cancer patients with mutations in epidermal growth factor receptor (*EGFR*) gene are treated with tyrosine kinase inhibitor (TKI).

Aims: We aimed to evaluate polymerase chain reaction (PCR)–high-resolution melting (HRM), restriction fragment length polymorphism (RFLP), and direct sequencing (DS) to detect *EGFR* mutations in cell-free DNA (cfDNA) before and after TKI treatment in real-world settings of a developing country.

Methods: Paired cytology and plasma samples were collected from 116 treatment-naïve lung cancer patients. DNA from both plasma and cytology specimens was isolated and analyzed using PCR-HRM (to detect exon 19 insertion/deletion), RFLP (to genotypes L858R and L861Q), and DS (to detect uncommon mutations G719A, G719C, or G719S [G719Xaa] in exon 18 and T790M and insertion mutations in exon 20).

Results: *EGFR* genotypes were obtained in all 116 (100%) cfDNA and 110/116 (94.82%) of cytological specimens of treatment-naïve patient (baseline samples). *EGFR*-activating mutations were detected in 46/110 (40.6%) plasma samples, and 69/110 (63.2%) mutations were found in routine cytology samples. Using cytological *EGFR* genotypes as reference, we found that sensitivity and specificity of baseline plasma *EGFR* testing varied from 9.1% to 39.39% and 83.12% to 96.55%, respectively. In particular, the sensitivity and specificity of this assay in detecting baseline T790M mutations in exon 20 were 30% and 89.58%, respectively. Three months after TKI treatment, plasma T790M and insertion exon 20 mutations appeared in 5.4% and 2.7% patients, respectively.

Conclusions: Despite low sensitivity, combined DS, RFLP, and PCR-HRM was able to detect *EGFR* mutations in plasma cfDNA with high specificity. Moreover, TKI resistance exon 20 insertions mutation was detected as early as 3 months post TKI treatment.

KEYWORDS

EGFR, liquid biopsy, lung cancer, PCR-HRM, Sanger sequencing, T790M

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1 | INTRODUCTION

Non–small cell lung cancer (NSCLC) is the most common histological type of lung cancer, which has a poor prognosis that has not improved over the recent 20 years in terms of overall survival (OS) before the invention of the present epidermal growth factor receptor (*EGFR*) in NSCLC patients in 2004.[1] *EGFR* mutation is the actionable target that has been widely studied as the subject for targeted therapy in NSCLC treatment.

First-generation EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib are widely used in the treatment of NSCLC and significantly improved the progression-free survival of the patients.[2] Two most common genetic alterations in *EGFR* gene are exon 19 deletion (del746-750) and L858R substitution mutations. In addition, there are uncommon *EGFR* mutations such as G719Xaa (G719A, G719C, and G719S) in exon 18, insertion mutation and T790M substitution in exon 20, and L861Q in exon 2.[3] These common and uncommon mutations are TKI-sensitive mutations, except for T790M, being a TKI-resistant mutation.

In 2015, the World Health Organization has proposed using small biopsies and cytological samples as alternatives to surgical or tissue samples.[4] However, those samples may have issues with limited quantity and poor quality.[5] Recently, cell-free DNA (cfDNA) has been widely studied as an alternative DNA source that can be used to detect *EGFR* mutation.[6] cfDNA may be useful to genotype *EGFR* gene in newly diagnosed lung cancer patients with poor performance status of whom repeat biopsy is not possible.[7] Moreover, cfDNA may also track impending appearances of *EGFR* T790M–resistant mutations that affect efficacy of first-generation TKI.[8] Recently, osimertinib treatment shows promising results based on appearance of *EGFR* T790M mutations in cfDNA of patients previously treated with first-generation TKI.[9] According to recent review,[10] there are several methods that have been used to detect *EGFR* T790M mutations using cfDNA such as Droplet Digital polymerase chain reaction (ddPCR), next-generation sequencing (NGS), and amplification-refractory mutation system (ARMS). These methods generally require special equipment, complex procedures, and expensive reagents that are rarely available and affordable in developing countries.

PCR high-resolution melting (HRM), restriction fragment length polymorphism (RFLP), and direct sequencing (DS) are relatively simple and affordable and used routine equipment to detect *EGFR* mutations in real-life clinical setting. PCR-HRM and RFLP could detect genetic alterations in the samples containing as low as 1% of mutant alleles.[11,12]

In this study, we aimed to determine the ability of PCR-HRM, RFLP, and DS to detect *EGFR* mutations from paired cytology and plasma cfDNA and cytology samples before and after TKI treatment in lung cancer patients. We also aimed to explore some genetic alterations that correlates with clinical outcomes.

Key Points

- Combined PCR-HRM, RFLP, and direct sequencing (DS) methods were able to detect *EGFR* common mutations in plasma of lung cancer patients.
- Resistance marker was found in plasma as early as 3 months after targeted therapy.
- This combined method was simple and cost-effective and may be useful to track plasma *EGFR* mutations in developing countries

2 | MATERIALS AND METHODS

2.1 | Patients

Both cytology and plasma samples were obtained from 116 lung cancer patients with the inclusion criteria: newly diagnosed lung cancer patients, treatment naïve, and provided written informed consent to participate in the study. The patients was recruited nonconsecutively from a tertiary hospital, Persahabatan Hospital, Jakarta, Indonesia (hereinafter referred to as baseline samples). Informed consent was obtained from all individual participants included in the study. First, baseline *EGFR* mutation in both cytology and plasma was tested, then patients shown to be *EGFR* mutation positive in cytological samples were then treated with EGFR TKI, and subsequently, blood samples were taken every 3 months for a period of 9 months. Six of 116 patients have to be excluded from this study because of insufficient samples or low content of tumor cells. As many as 45 patients were followed up until their 9 months or the last period of surveillance in this study, while the rest were not followed up because they were lost to follow-up or wild-type (WT) alleles were detected on the basis of their cytology result analysis. The Ethical Committee of the Faculty of Medicine, Universitas Indonesia (396/UN2.F1/ETIK/2016), Jakarta, had approved this study, which was performed in accordance with the 1964 Helsinki Declaration and its later amendments.

2.2 | DNA isolation from cytology samples

Tumor cells were marked by experienced independent pathologists, scraped from the slides, and transferred into microtubes containing preloaded proteinase K and tissue lysis buffer. The pathologists rejected specimens containing less than 100 tumor cells. DNA was isolated using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to described protocol and stored at −20°C before use. The concentration of DNA was determined by using Nanodrop UV-vis Spectrophotometer.

2.3 | DNA isolation from plasma samples

Whole blood specimens from patients were collected in EDTA tube and then centrifuged at 1600 rpm for 10 minutes. Plasma was obtained by collecting the supernatants as results of another round of centrifugation at 14 000 rpm for 10 minutes. The final supernatants or plasma fraction were transferred into fresh microtubes and stored at −80°C before use. DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden Germany) according to described protocol and stored at −20°C before use. DNA concentration was determined using Nanodrop UV-vis Spectrophotometer.

2.4 | Analysis of *EGFR* mutations in exons 18, 19, and 21 on baseline samples only

DNA was assayed using Rotor Gene Q system (Qiagen, Germany). PCR mix included 10 mM of PCR buffer; 1.5 mM of $MgCl_2$; 0.4 μM each of two primers for the amplification of exons 18, 19, and 21 of *EGFR* gene; 200 μM each of deoxynucleotide triphosphate; 0.5 U of Hotstart Taq Polymerase (Qiagen, Germany); and 0.1 mM of SYTO9. Primer sequences to amplify *EGFR* mutations in exons 18, 19, and 21 were adopted from Do et al.[13]

The temperature cycling protocol included the predenaturation step at 95°C for 15 minutes and then followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 65°C for 10 seconds, and extension at 72°C for 30 seconds. After the amplification process, the samples were heated in the Rotor Gene Q at 97°C for 15 seconds and then cooled at 45°C for 15 seconds. HRM analysis was performed by heating the PCR product from 79°C to 90°C at 0.1°C/s. HRM data and melting curve data were analyzed using Rotor-Gene Q Series Software (Qiagen, Germany). PCR products from HRM analysis were further analyzed by using direct sequencing method (exon 18), gel electrophoresis (exon 19), and restriction fragment length polymophism (RFLP) method (genotyping L858R and L861Q mutations in exon 21 as described[12]).

2.5 | Direct sequencing to detect *EGFR* mutations in exon 20 on both baseline and follow-up samples

EGFR exon 20 mutations from both cytology and plasma samples were amplified in Veriti Thermal Cycler (Applied Biosystem). PCR amplification mixture contained 10 mM of PCR buffer, 1.5 mM of $MgCl_2$, 0.4 μM each of two primers for the amplification of exon 20, 200 μM each of deoxynucleotide triphosphate, and 0.5 U of Hotstart Taq Polymerase (Qiagen, Germany). PCR products were purified by Exosap-IT (Affymetrix) and then followed by cycle sequencing reaction with Big Dye Terminator v3.1 (Applied Biosystems). Direct sequencing process was performed in 3500 Genetic Analyzer (Applied Biosystems). Primer sequences to amplify *EGFR* mutation in exon 20 were adopted from Amann et al.[14]

2.6 | Statistical analysis

Graphpad Prism 7 was used for the statistical analysis. The detection sensitivity and specificity of plasma *EGFR* mutations using PCR-HRM, RFLP, and direct sequencing, compared with the cytology, were analyzed using Fisher exact test (χ^2). Survival data were available from 38 patients. The time from the date of starting the therapy to death or last follow-up was used to compare OS between the different groups. At the time of statistical analysis (September 2017), the cohort had a median follow-up of 7.16 months (2.83-12 months). Survival curves were estimated by Kaplan-Meier method using SPSS 20.

3 | RESULTS

3.1 | Patient characteristics

Clinical information is shown in Table 1. Median age was 55 years (range: 29-84 years). Seventy-five patients were males, and 35 patients were females. Of all patients, 59.09% were smokers. The

TABLE 1 Patients demography (N = 110)

Variable	Number n (%)	*EGFR*[a]		*P* Value[b]
		Mutation n (%)	WTn (%)	
Gender				
Male	75 (68.18)	43 (55.84)	32 (44.16)	0.087
Female	35 (31.82)	26 (78.79)	9 (21.21)	
Age				
≤55	55 (50.00)	34 (61.82)	21 (38.18)	0.843
>55	54 (49.09)	35 (64.81)	19 (35.19)	
Unknown	1 (0.91)			
Smoking history				
Ever smoker	65 (59.09)	36 (55.38)	29 (44.62)	0.078
Never smoker	34 (30.91)	25 (73.53)	9 (26.47)	
Unknown	11 (10.00)			
Histological type				
Adenocarcinoma	108 (98.18)	67 (62.04)	41 (37.96)	0.593
Nonadenocarcinoma	2 (1.82)	2 (100)	0	
TNM staging				
II + IIIA	9 (8.49)	6 (66.67)	3 (33.33)	0.801
IIIB + IV	45 (42.45)	28 (62.22)	17 (37.78)	
Unknown	52 (49.06)			

[a]*EGFR* mutation results were obtained from cytology samples; WT (*EGFR* wild type or normal).

[b]*P* value was calculated between each subvariable using Fisher exact test (two-sided). Associations between variables were considered statistically significant; *P* values were less than 0.05.

most common histological type in this study was adenocarcinoma (108 patients, 98.18%), and the majority of patients (45 patients, 42.45%) had stage IIIB to IV diseases. *EGFR* mutations were detected more frequently in female (78.79% vs 55.84% in male) and never smoker (73.53% vs 55.38% in smokers) patients.

3.2 | *EGFR* mutations in cytology and plasma samples in treatment-naïve patients

In total, both cytology and plasma samples were collected from 116 TKI-naïve lung cancer patients. These samples were analyzed using combination of PCR-HRM with RFLP to detect *EGFR* common mutations (exon 19 ins/dels, L858R) and direct sequencing methods to genotype *EGFR* uncommon mutations in exons 18 (G719X) and 20 (T790M and insertion mutations). *EGFR* testing was unsuccessful for the six patients mainly because of insufficient samples; therefore, 110 out of 116 patients had paired cytology and plasma samples undergoing *EGFR* mutations testing using identical methods. *EGFR* mutations were detected in 69 patients (62.7%) and 46 patients (41.8%) in cytology and plasma samples, respectively. Common mutations (exon 19 ins/dels and L858R) contributed major proportion of *EGFR* mutations in both cytological and plasma samples. Notably, rates of detectable T790M mutations were 14% (10 of 69) in cytological samples and 28% (13 of 46) in plasma samples (Table 2).

3.3 | The specificity and sensitivity of combined PCR-HRM, RFLP, and DS to detect *EGFR* mutations in cfDNA

As shown in Table 3, 10 and 22 patients had exon 19 insertion/deletion (ins/dels) mutations in plasma and cytology samples, respectively. Seven patients had exon 19 ins/dels mutants in both cytology and plasma samples. Eighty patient samples showed no mutations in both cytology and plasma samples, yielding sensitivity and specificity of 30.43% (95% CI, 15.60-50.87) and 96.43% (95% CI, 90.35-99.06), respectively.

Another mutation, L858R, was shown in half of 26 patients in both plasma and cytology samples. The presence of WT alleles was found in 62 patients. From those results, the sensitivity and specificity of this assay to detect L858R in the plasma sample were 39.39% (95% CI, 24.68-56.32) and 83.12% (95% CI, 73.23-89.86), respectively.

While common mutations were mainly detected using HRM and RFLP, uncommon mutations were detected using direct sequencing. Eleven and six patients had TKI-sensitive G719X mutations in cytology and plasma sample, respectively. Among G719X positive patients, only one patient had matched *EGFR* mutations status with their paired cytology sample. The remaining 90 patients had WT alleles in exon 18. The sensitivity and specificity to detect G719X mutations were 9.1% (95% CI, 0.4-37.74) and 93.94% (95% CI, 87.40-97.19), respectively. Another TKI-sensitive uncommon L861Q mutation was also detected in three and 11 plasma and cytology patients, respectively. The sensitivity and specificity of L861Q detection were 9.1% (95% CI, 0.4-37.74) and 97.98% (95% CI, 92.93-99.64), respectively.

TABLE 2 *EGFR* mutation types found in cytology and plasma samples

Sources of EGFR testing	N (%)
Cytology samples	
Common mutation	
19 ins/dels	17 (15.45)
L858R	24 (21.81)
Uncommon mutation	
G719X	2 (1.82)
L861Q	6 (5.45)
T790M	2 (1.82)
Mix mutation	
G719X, 19 ins/dels	3 (2.73)
G719X, T790M	1 (0.91)
G719X, L858R	3 (2.73)
G719X, L861Q	1 (0.91)
19 ins/dels, T790M	2 (1.82)
19 ins/dels, L861Q	1 (0.91)
T790M, L858R	3 (2.73)
T790M, L861Q	1 (0.91)
L858R, L861Q	2 (1.82)
T790M, G719S, L858R	1 (0.91)
Wild type/normal	41 (37.27)
Plasma samples	
Common mutations	
Exon 19 ins/dels	6 (5.45)
L858R	15 (13.64)
Uncommon mutations	
G719X	4 (3.64)
L861Q	2 (1.82)
T790M	6 (5.45)
G719X, L858R	3 (2.73)
19 ins/dels, L858R	2 (1.82)
T790M, 19 ins/dels	1 (0.91)
T790M, L858R	5 (4.54)
L858R, L861Q	1 (0.91)
T790M, 19 ins/dels, L858R	1 (0.91)
WT	64 (58.18)

Lastly, plasma samples of 12 patients were positive for uncommon EGFR TKI–resistant T790M mutations in exon 20 of *EGFR* gene. However, only three patients had matched T790M mutations in their cytology samples. Seven patients had shown T790M mutation in the cytology samples, but not in their plasma samples. The remaining 86 patients had WT alleles in both plasma and cytological samples. Thus, sensitivity and specificity of this assay in detecting T790M mutation were 30% (95% CI, 10.78-60.32) and 90% (95% CI, 82.56-94.48), respectively.

Furthermore, categorizing *EGFR* mutations into common (exon 19 in/del and L858R) and uncommon mutations (G719X, T790M, L861Q, and mix) yields similar specificity (82.86%; 95% CI, 67.32-91.90). However, these routine methods had higher sensitivity trend to detect common *EGFR* mutations than uncommon *EGFR* mutations (43.33%; 95% CI, 27.38-60.80 vs 30.77%; 95% CI, 16.50-49.99, respectively).

TABLE 3 Comparison of individual *EGFR* mutations detected in plasma versus cytology samples

	Cytology		
Plasma	**Mutant**	**Wild Type**	**Total**
Common mutations			
EGFR 19 Ins/dels			
Mutant	7	3	10
Wild type	16	84	10
Total	23	87	110
EGFR L858R			
Mutant	13	13	26
Wild type	20	64	84
Total	33	77	110
Uncommon mutations			
EGFR G719X			
Mutant	1	6	7
Wild type	10	93	103
Total	11	99	110
EGFR L861Q			
Mutant	1	2	3
Wild type	10	97	107
Total	11	99	110
EGFR T790M			
Mutant	3	10	13
Wild type	7	90	97
Total	10	100	110

3.4 | Detection of *EGFR* exon 20 mutations in TKI-treated patients

Out of 110 patients, 45 patients with baseline *EGFR* mutations in their cytology samples were followed up until 9 months or the last period of the surveillance. Their blood plasma was then taken every 3 months to detect the presence of TKI resistance *EGFR* mutations in EGFR exon 20 such as insertion and T790M substitution mutations. Eight out of 45 patients were deceased in less than the first 3-month follow-up. Two patients had T790M, and one patient had V774_C775insHV insertion in their 3-month plasma as shown in Table 4 and Figure 1. The rate of detection of 3-month plasma *EGFR* mutations T790M and insertion exon 20 mutations was 5.4% and 2.7% patients (N = 37), respectively. In this study, we found exon 20 *EGFR* mutations in four patients during their TKI treatment. Of those four patients, all three patients with T790M mutations were still alive until the last period of the monitoring. One patient with exon 20 insertion mutation was deceased 1 month after the mutation was detected.

4 | DISCUSSION

Plasma cfDNA may be used as alternative to tissue biopsy for detect *EGFR* mutations. However, most studies of *EGFR* mutations detection in plasma cfDNA have described the utility of sophisticated methods such as ARMS, ddPCR, or NGS.[15-21] These methods have shown high sensitivity to detect *EGFR* mutations in samples with low tumor contents such as cytology and cfDNA. Despite high sensitivity, those methods are neither accessible nor affordable in developing countries with limited resources because of high investment and requirements for advance operating skills.

Therefore, there are unmet needs for economical, routine, and simple methods such as PCR-HRM[22] or RFLP[23-25] to detect and track plasma EGFR mutations. However, the utility of routine, widely used, and relatively inexpensive methods in real-life clinical *EGFR* testing, namely, DS, RFLP, and PCR-HRM have not been evaluated in detail. In this study, we addressed and described two major findings. First, we described that the overall sensitivity and specificity of these routine methods were similar to IGNITE study in detecting both common and uncommon *EGFR* mutations in cfDNA of the treatment-naïve patients. Secondly, the routine methods were able to detect acquired TKIs resistance mutation T790M as well as exon 20 insertion mutation as early as 3 months after TKIs treatment.

4.1 | Sensitivity and specificity

The inability to detect mutation at level of less than 20% of total DNA, direct or Sanger sequencing has been dismissed as unsuitable method to detect plasma *EGFR* mutations.[26] Indeed, a previous study using sequencing shows overall sensitivity of 18% to detect *EGFR* common mutations (exon 19 in/dels and L858R),[27] which is lower than that of our study showing 30% to 40% sensitivity. The lower sensitivity in previous study than ours may be due to mixed population of both treated and nontreated patients. It is known that the level of *EGFR* mutations decreases after TKI treatment[28] and may contribute to low sensitivity of sequencing method in TKI-treated patients enrolled in previous study.[27]

TABLE 4 A comparison of *EGFR* second-site mutations between baseline and treated samples

			Plasma Follow-up[a]		
Samples	**Cytology**	**Plasma Baseline**	**Month 3**	**Month 6**	**Month 9**
Case 1	L858R	WT	WT	WT	T790M
Case 2	In/del 19	WT	T790M	WT	WT
Case 3	G719S, L861Q	WT	Ins20 V774_C775insHV	Deceased	N/D
Case 4	In/del 19	T790M	T790M	WT	WT

Abbreviations: N/D, not determined; WT, wild-type EGFR.

[a]Plasma DNA samples isolated from post-TKI–treated patients were genotyped for mutations in exon 20 of *EGFR* gene using direct sequencing method.

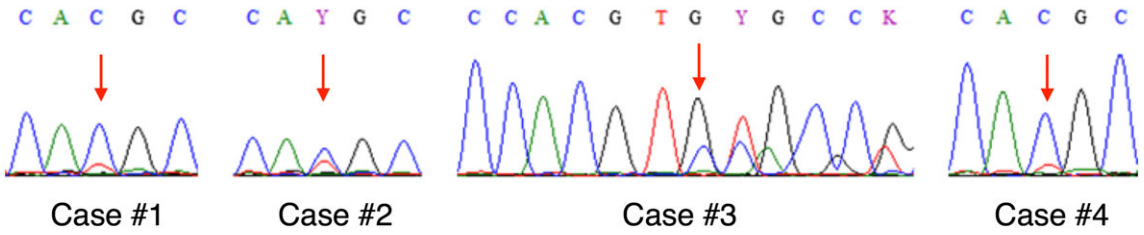

FIGURE 1 The result of direct sequencing to detect exon 20 tyrosine kinase inhibitor epidermal growth factor receptor–resistant mutations. Red arrows point to mutations

The sensitivity to detect pre–TKI treatment or baseline common *EGFR* mutations in plasma cfDNA in our study (30%-40%) with high specificity (83%-96%) was surprising given a well-known low sensitivity of combined PCR-HRM and Sanger sequencing. In fact improvement of this routine method maybe possible, which has been shown by incorporating two rounds of PCR amplification of cfDNA yielding high sensitivity[29] up to 60%. Interestingly, the use of ARMS PCR in real-life testing of paired tumor and plasma samples as shown in IGNITE[30] (n = 2581 patients) and ASSESS[31] (n = 1162) studies has reported 46% sensitivity, which is similar to our findings. However, in clinical trial setting involving 1060 subjects, the use of ARMS PCR can yield up to 65% sensitivity.[32] According to the Couraud et al[15] and Zhu et al[21] studies, NGS and ddPCR are more sensitive methods ranging from 80% to 100% than our methods.

Moreover, the use of PCR-HRM to detect *EGFR* mutations in plasma[33] or serum[34] has been described demonstrating high sensitivity 60% and 90%, respectively. We also used PCR-HRM to screen mutations mainly in exons 18, 19, and 21. However, we did not use HRM alone to detect clinically relevant mutations such G719X (in exon 18) and L858R and L861Q (both in exon 21). The combined PCR-HRM and sequencing (to genotype G719X) and RFLP (to genotype L858R and L861Q) did not yield sensitivity higher than 40%. The difference with previous study may lie in primer designs and/or PCR equipment being used for HRM screening. In principle, HRM alone will not be able to genotype clinically relevant variants,[35] and careful designs of HRM primers must avoid polymorphic *EGFR* variants in exons 20 and 21 that do not impact clinical outcome such as Q787Q and R836R.[30,36]

In spite of low sensitivity of our methods to detect *EGFR* mutations in plasma cfDNA, showed in our study findings, these methods are more feasible, economical, and relatively simple than other more complex methods requiring specialized training to detect *EGFR* mutations in plasma cfDNA. According to IASLC's recommendation of genotyping cfDNA, the feasibility of platforms to be used to genotyping cfDNA should be a prime concern of each clinical setting in its region. Each clinical setting should have to evaluate and balance between cost, availability, and assay sensitivity.[37] Thus, the combined method of DS, RFLP, and PCR-HRM might be useful in resource-limited settings even with such a low sensitivity. Based on our previous study performed in our population, the overall rate of *EGFR* mutations was varied ranging[38] from 35% to 44%, which is higher than the average rates of *EGFR* mutations in Caucasian patients (7%).[39] Therefore, our low-sensitivity test may be compensated by high rates of *EGFR* mutations in Asian patients.

4.2 | Detection of exon 20 *EGFR* mutations in treated-patients cohort

Previously, we have described the presence of exon 20 insertions and T790M substitution mutations in 8.4% of treatment-naïve lung cancer patients.[38] In this current study, we recruited independent patients' cohort and assessed the feasibility of direct sequencing to detect acquired TKI resistance in plasma at regular 3-month time interval after TKI treatment. Out of 37 patients, we found three patients with acquired T790M and one patient with Ins20. To our knowledge, this is the first description of exon 20 insertion mutations in post–TKI treatment patient. Moreover, one of those three samples that were positively detected to have acquired T790M mutation (case 1 in Table 4) was also positively detected using NGS with 14% mutant allele frequency (MAF) of acquired T790M mutation according to our preliminary study of plasma cfDNA (Figure S1). The sensitivity of sequencing to detect plasma T790M in this study (5%) was similar to previous study detecting plasma T790M mutations in 6% of gefitinib-resistant patients.[40]

The patient with exon 20 insertion mutation V774_C775insHV was deceased 1 month after the mutation was detected. On the other hand, all three patients having acquired T790M mutations were still alive until the last period of surveillance. Arcila et al[41] had analyzed the effect of amino acid alterations in V774_C775insHV to the three-dimensional structure of EGFR kinase domain. This type of mutation affects a loop region of a drug-binding pocket resulting into a significant reduction of the drug-binding affinity, which may explain resistance to first-generation TKIs.

Detection of T790M mutation has been reported at variable times. Some studies have reported as early as 3 weeks in two of 27 patients[42] (7.4%). While others using cobas system[43] and ddPCR show 6 months (three of nine patients 33%)[44] and 8 months.[28] However, the clinical utility of early detection of T790M before showing clinical progressive disease was not clear.[28] There has been no specific therapeutic recommendation based on T790M appearance alone without evidence of radiological progression. Recent study demonstrates that any positivity of *EGFR* T790M mutation shows good response to osimertinib independently of MAF.[45] Therefore, patients with positive *EGFR* mutations detected using the combined methods of DS, PCR-HRM, and RFLP may also benefit from osimertinib.

The utility of T790M appearance during TKI therapy to predict progressive disease may be hampered by the dynamics of T790M burden. Quantitative monitoring studies using highly sensitive and sophisticated methods such as NGS[44-46] and matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)[47] have shown that T790M mutation burdens may not necessarily remain elevated up to presentation of frank radiological progression. Indeed, some patients demonstrate only transient elevation of T790M mutations during early phase of TKI treatment, and the mutations eventually disappear near baseline level at later time points prior to disease progression. Using direct sequencing, we also noticed in two of our patients (cases 2 and 4) that the initial presence of T790M in month 3 was not sustained at later follow-ups in months 6 and 9. While the ups and downs of T790M mutation burden may reflect the natural course of the disease, we speculated that the low analytical sensitivity of direct sequencing method may also contribute to the disappearance of T790M.

In conclusion, our analysis showed that combination of routine inexpensive methods (PCR-HRM, RFLP, and DS) could be used to detect plasma *EGFR* mutations in both pre– and post–TKI treatments with high specificity, albeit with low sensitivity. Patients residing in Asian countries with limited resources may benefit from using the combined method. While patients with mutations may be recommended for treatments, patients with negative results should opt for rebiopsy when possible. Interestingly, the combined method also demonstrated the appearance of exon 20 insertion mutations for the first time in the plasma of post–TKI treatment.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHORS' CONTRIBUTION

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, J.Z., S.L.A., A.H., A.R.U.; *Methodology*, E.S., M.Y., N.M., R.F., A.R.; *Investigation*, J.Z., E.S., M.Y., S.L.A., A.H., N.M., A.R.,H.H.; *Formal Analysis*, J.Z., M.Y., S.L.A., A.H.; *Resources*, E.S., A.H.,H.H.; *Writing - Original Draft*, M.Y., A.R.U.; *Writing - Review & Editing*, J.Z., E.S., M.Y., S. L.A., A.H., N.M., R.Y., A.R., H.H., F.N., S.S., A.R.U.; *Visualization*, M.Y., N. M., A.R.; *Supervision*, A.H., S.S., A.R.U.; *Funding Acquisition*, A.H., A.R.U.

COMPLIANCE WITH ETHICAL STANDARDS

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ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7-30. https://doi.org/10.3322/caac.21332
2. Miller VA. EGFR mutations and EGFR tyrosine kinase inhibition in non–small cell lung cancer. *Semin Oncol Nurs*. 2008;24(1):27-33. https://doi.org/10.1016/j.soncn.2007.11.009
3. Siegelin MD, Borczuk AC. Epidermal growth factor receptor mutations in lung adenocarcinoma. *Lab Invest*. 2014;94(2):129-137. https://doi.org/10.1038/labinvest.2013.147
4. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol*. 2015;10(9):1243-1260. https://doi.org/10.1097/JTO.0000000000000630
5. Fenizia F, De Luca A, Pasquale R, et al. EGFR mutations in lung cancer: from tissue testing to liquid biopsy. *Future Oncol*. 2015;11(11):1611-1623. https://doi.org/10.2217/fon.15.23
6. Seki Y, Fujiwara Y, Kohno T, et al. Circulating cell-free plasma tumour DNA shows a higher incidence of EGFR mutations in patients with extrathoracic disease progression. *ESMO Open*. 2018;3(2):e000292. https://doi.org/10.1136/esmoopen-2017-000292
7. Kwapisz D. The first liquid biopsy test approved. Is it a new era of mutation testing for non–small cell lung cancer? *Ann Transl Med*. 2017;5(3):46-46. https://doi.org/10.21037/atm.2017.01.32
8. Riediger AL, Dietz S, Schirmer U, et al. Mutation analysis of circulating plasma DNA to determine response to EGFR tyrosine kinase inhibitor therapy of lung adenocarcinoma patients. *Sci Rep*. 2016;6(1):33505. https://doi.org/10.1038/srep33505
9. Remon J, Caramella C, Jovelet C, et al. Osimertinib benefit in EGFR-mutant NSCLC patients with T790M-mutation detected by circulating tumour DNA. *Ann Oncol*. January 2017:mdx017-mdx017. doi:https://doi.org/10.1093/annonc/mdx017. 28(4): 784-790
10. Liang Z, Cheng Y, Chen Y, et al. EGFR T790M ctDNA testing platforms and their role as companion diagnostics: correlation with clinical outcomes to EGFR-TKIs. *Cancer Lett*. 2017;403:186-194. https://doi.org/10.1016/j.canlet.2017.06.008
11. Diaz LA, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol*. 2014;32(6):579-586. https://doi.org/10.1200/JCO.2012.45.2011
12. Kawada I, Soejima K, Watanabe H, et al. An alternative method for screening EGFR mutation using RFLP in non–small cell lung cancer patients. *J Thorac Oncol*. 2008;3(10):1096-1103. https://doi.org/10.1097/JTO.0b013e318186fadd
13. Do H, Krypuy M, Mitchell PL, Fox SB, Dobrovic A. High resolution melting analysis for rapid and sensitive EGFR and KRAS mutation detection in formalin fixed paraffin embedded biopsies. *BMC Cancer*. 2008;8(1):142. https://doi.org/10.1186/1471-2407-8-142.
14. Amann J, Kalyankrishna S, Massion PP, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res*. 2005;65(1):226-235.
15. Couraud S, Vaca-Paniagua F, Villar S, et al. Noninvasive diagnosis of actionable mutations by deep sequencing of circulating free DNA in lung cancer from never-smokers: a proof-of-concept study from BioCAST/IFCT-1002. *Clin Cancer Res*. 2014;20(17):4613-4624. https://doi.org/10.1158/1078-0432.CCR-13-3063
16. Goto K, Ichinose Y, Ohe Y, et al. Epidermal growth factor receptor mutation status in circulating free DNA in serum: from IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non–small cell lung

cancer. *J Thorac Oncol*. 2012;7(1):115-121. https://doi.org/10.1097/JTO.0b013e3182307f98

17. Kimura H, Suminoe M, Kasahara K, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). *Br J Cancer*. 2007;97(6):778-784. https://doi.org/10.1038/sj.bjc.6603949
18. Kukita Y, Uchida J, Oba S, et al. Quantitative identification of mutant alleles derived from lung cancer in plasma cell-free DNA via anomaly detection using deep sequencing data. *PLoS One*. 2013;8(11):e81468. https://doi.org/10.1371/journal.pone.0081468
19. Wang S, Han X, Hu X, et al. Clinical significance of pretreatment plasma biomarkers in advanced non–small cell lung cancer patients. *Clin Chim Acta*. 2014;430:63-70. https://doi.org/10.1016/j.cca.2013.12.026
20. Zheng D, Ye X, Zhang MZ, et al. Plasma EGFR T790M ctDNA status is associated with clinical outcome in advanced NSCLC patients with acquired EGFR-TKI resistance. *Sci Rep*. 2016;6(1):20913-20919. https://doi.org/10.1038/srep20913
21. Zhu G, Ye X, Dong Z, et al. Highly sensitive droplet digital PCR method for detection of EGFR-activating mutations in plasma cell-free DNA from patients with advanced non–small cell lung cancer. *J Mol Diagn*. 2015;17(3):265-272. https://doi.org/10.1016/j.jmoldx.2015.01.004
22. Abou Tayoun AN, Burchard PR, Malik I, Scherer A, Tsongalis GJ. Democratizing molecular diagnostics for the developing world. *Am J Clin Pathol*. 2014;141(1):17-24. https://doi.org/10.1309/AJCPA1L4KPXBJNPG
23. Kim H-J, Yong T-S, Shin MH, et al. Practical algorisms for PCR-RFLP-based genotyping of *Echinococcus granulosus* sensu lato. *Korean J Parasitol*. 2017;55(6):679-684. https://doi.org/10.3347/kjp.2017.55.6.679
24. Chaâbane-Banaoues R, Oudni-M'rad M, M'rad S, Amani H, Mezhoud H, Babba H. A novel PCR-RFLP assay for molecular characterization of *Echinococcus granulosus* sensu lato and closely related species in developing countries. *Parasitol Res*. 2016;115(10):3817-3824. https://doi.org/10.1007/s00436-016-5143-x
25. Faleel FDM, Zoysa MIMD, Lokuhetti MDS, Gunawardena YINS, Chandrasekharan VN, Dassanayake RS. Modified mismatch polymerase chain reaction-restriction fragment length polymorphism detected mutations in codon 12 and 13 of exon 2 of K-ras gene in colorectal cancer patients and its association with liver metastases: data from a South Asian country. *J Cancer Res Ther*. 2016;12(4):1272-1277. https://doi.org/10.4103/0973-1482.187294
26. Normanno N, Denis MG, Thress KS, Ratcliffe M, Reck M. Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non–small-cell lung cancer. *Oncotarget*. 2017;8(7):12501-12516. https://doi.org/10.18632/oncotarget.13915
27. He C, Liu M, Zhou C, et al. Detection of epidermal growth factor receptor mutations in plasma by mutant-enriched PCR assay for prediction of the response to gefitinib in patients with non–small-cell lung cancer. *Int J Cancer*. 2009;125(10):2393-2399. https://doi.org/10.1002/ijc.24653
28. Lee JY, Qing X, Xiumin W, et al. Longitudinal monitoring of EGFR mutations in plasma predicts outcomes of NSCLC patients treated with EGFR TKIs: Korean Lung Cancer Consortium (KLCC-12-02). *Oncotarget*. 2016;7(6):6984-6993. https://doi.org/10.18632/oncotarget.6874
29. Jiang B, Liu F, Yang L, et al. Serum detection of epidermal growth factor receptor gene mutations using mutant-enriched sequencing in Chinese patients with advanced non–small cell lung cancer. *J Int Med Res*. 2011;39(4):1392-1401. https://doi.org/10.1177/147323001103900425
30. Han B, Tjulandin S, Hagiwara K, et al. EGFR mutation prevalence in Asia-Pacific and Russian patients with advanced NSCLC of adenocarcinoma and non-adenocarcinoma histology: the IGNITE study. *Lung Cancer*. 2017;113:37-44. https://doi.org/10.1016/j.lungcan.2017.08.021
31. Reck M, Hagiwara K, Han B, et al. ctDNA determination of EGFR mutation status in European and Japanese patients with advanced NSCLC: the ASSESS study. *J Thorac Oncol*. 2016;11(10):1682-1689. https://doi.org/10.1016/j.jtho.2016.05.036
32. Douillard J-Y, Ostoros G, Cobo M, et al. Gefitinib treatment in EGFR mutated Caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. *J Thorac Oncol*. 2014;9(9):1345-1353. https://doi.org/10.1097/JTO.0000000000000263
33. Jing C-W, Wang Z, Cao H-X, Ma R, Wu J-Z. High resolution melting analysis for epidermal growth factor receptor mutations in formalin-fixed paraffin-embedded tissue and plasma free DNA from non–small cell lung cancer patients. *Asian Pac J Cancer Prev*. 2013;14(11):6619-6623. https://doi.org/10.7314/APJCP.2013.14.11.6619
34. Hu C, Liu X, Chen Y, et al. Direct serum and tissue assay for EGFR mutation in non–small cell lung cancer by high-resolution melting analysis. *Oncol Rep*. 2012;28(5):1815-1821. https://doi.org/10.3892/or.2012.1987
35. Gonzalez-Bosquet J, Calcei J, Wei JS, et al. Detection of somatic mutations by high-resolution DNA melting (HRM) analysis in multiple cancers. *PLoS One*. 2011;6(1):e14522-e14529. https://doi.org/10.1371/journal.pone.0014522
36. Xu S, Duan Y, Lou L, Tang F, Shou J, Wang G. Exploring the impact of EGFR T790M neighboring SNPs on ARMS-based T790M mutation assay. *Oncol Lett*. 2016;12(5):4238-4244. https://doi.org/10.3892/ol.2016.5184
37. Rolfo C, Mack PC, Scagliotti GV, et al. Liquid biopsy for advanced non–small cell lung cancer (NSCLC): a statement paper from the IASLC. *J Thorac Oncol*. 2018;13(9):1248-1268. https://doi.org/10.1016/j.jtho.2018.05.030
38. Syahruddin E, Wulandari L, Sri Muktiati N, et al. Uncommon EGFR mutations in cytological specimens of 1,874 newly diagnosed Indonesian lung cancer patients. *Lung Cancer (Auckl)*. 2018;9:25-34. https://doi.org/10.2147/LCTT.S154116
39. Zhou W, Christiani DC. East meets west: ethnic differences in epidemiology and clinical behaviors of lung cancer between East Asians and Caucasians. *Chin J Cancer*. 2011;30(5):287-292. https://doi.org/10.5732/cjc.011.10106
40. He C, Zheng L, Xu Y, Liu M, Li Y, Xu J. Highly sensitive and noninvasive detection of epidermal growth factor receptor T790M mutation in non–small cell lung cancer. *Clin Chim Acta*. 2013;425:119-124. https://doi.org/10.1016/j.cca.2013.07.012
41. Arcila ME, Nafa K, Chaft JE, et al. EGFR exon 20 insertion mutations in lung adenocarcinomas: prevalence, molecular heterogeneity, and clinicopathologic characteristics. *Mol Cancer Ther*. 2013;12(2):220-229. https://doi.org/10.1158/1535-7163.MCT-12-0620
42. Marchetti A, Palma JF, Felicioni L, et al. Early prediction of response to tyrosine kinase inhibitors by quantification of EGFR mutations in plasma of NSCLC patients. *J Thorac Oncol*. 2015;10(10):1437-1443. https://doi.org/10.1097/JTO.0000000000000643
43. Sorensen BS, Wu L, Wei W, et al. Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non–small cell lung cancer during treatment with erlotinib. *Cancer*. 2014;120(24):3896-3901. https://doi.org/10.1002/cncr.28964
44. Oxnard GR, Paweletz CP, Kuang Y, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer*

Res. 2014;20(6):1698-1705. https://doi.org/10.1158/1078-0432.CCR-13-2482

45. Aggarwal C, Thompson JC, Black TA, Katz SI, Fan R, Yee SS, Chien AL, Evans TL, Bauml JM, Alley EW, Ciunci CA, Berman AT, Cohen RB, Lieberman DB, Majmundar KS, Savitch SL, Morrissette JJD, Hwang WT, Elenitoba-Johnson KSJ, Langer CJ, Carpenter EL Clinical implications of plasma-based genotyping with the delivery of personalized therapy in metastatic non–small cell lung cancer. *JAMA Oncol* October 2018. https://doi.org/10.1001/jamaoncol.2018.4305.
46. Kato K, Uchida J, Kukita Y, et al. Numerical indices based on circulating tumor DNA for the evaluation of therapeutic response and disease progression in lung cancer patients. *Sci Rep*. 2016;6(1):29093-29099. https://doi.org/10.1038/srep29093
47. Su K-Y, Tseng J-S, Liao K-M, et al. Mutational monitoring of EGFR T790M in cfDNA for clinical outcome prediction in EGFR-mutant lung adenocarcinoma. *PLoS One*. 2018;13(11):e0207001. https://doi.org/10.1371/journal.pone.0207001

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