Effects of Different Centrifugation Protocols on the Detection of *EGFR* Mutations in Plasma Cell-Free DNA

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ABSTRACT

Objectives: Various preanalytical factors, including the collection tube, storage conditions, and centrifugation, affect the detection results of plasma cell-free DNA (cfDNA). We compared the effect of different centrifugation protocols on the detection of *EGFR* mutations in cfDNA.

Methods: We analyzed 117 plasma specimens from 110 patients with non–small cell lung cancer using the cobas *EGFR* Mutation Test v2 (Roche Diagnostics). We compared the identified *EGFR* mutations and semiquantitative index values from the 1- and 2-step centrifugation groups and confirmed the clinical impact of differences in the results after further high-speed centrifugation.

Results: We detected *EGFR* mutations in 44 (37.6%) and 47 (40.2%) samples that were centrifuged once and twice, respectively; the 2 groups showed an 89.7% (105/117) concordance and a strong correlation in their semiquantitative index values ($r = 0.929$). Among the 12 inconsistent result pairs, 9 samples of 2-step centrifugation (75%) were consistent with the results of a recent tissue biopsy.

Conclusions: Additional high-speed centrifugation has been shown to increase the sensitivity of *EGFR* mutation detection in a commercial in vitro diagnostic real-time polymerase chain reaction device and is an optimal preanalytical factor for detecting low-allele frequency gene mutations using low concentrations of cfDNA.

INTRODUCTION

The discovery of frequent molecular alterations in non–small cell lung cancer (NSCLC) has led to the development of treatment approaches focused on targeted therapeutics.[1] For example, erlotinib and gefitinib, which are reversible tyrosine kinase inhibitors (TKIs) of EGFR, and afatinib, an irreversible inhibitor of the ErbB family, have been approved to treat patients with advanced *EGFR* mutation–positive NSCLC. Thus, identifying patients who may benefit from these therapeutic agents is crucial for successfully treating NSCLC.[2]

KEY POINTS

- We detected *EGFR* mutations in 44 (37.6%) and 47 (40.2%) samples that were centrifuged once and twice, respectively, with an 89.7% (105/117) concordance between the sample groups.
- The semiquantitative index values in the 2 groups correlated strongly ($r = 0.929$), with 9 samples of 2-step centrifugation (75%) consistent with the results of a recent tissue biopsy.
- Additional high-speed centrifugation increases the sensitivity of *EGFR* mutation detection in a commercial companion diagnostic real-time polymerase chain reaction test of cell-free DNA.

KEY WORDS

Liquid biopsy; Epidermal growth factor receptor mutations; Cell-free DNA; Non-small cell lung cancer; Preanalytical factors; Centrifugation

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Mutations in *EGFR* (exon 19 deletion and L858R) are associated with the clinical response of patients with NSCLC to first- and second-generation EGFR TKIs.[3,4] The most common mutation is T790M, which is responsible for the development of resistance against EGFR TKIs. This mutation is detected in 49% to 60% of patients with NSCLC who show acquired EGFR TKI resistance.[5-10] Thus, third-generation EGFR TKIs that target the *EGFR* T790M mutant and EGFR TKI–sensitive mutants have been developed. Additionally, in 2021, the US Food and Drug Administration approved amivantamab and mobocertinib to treat adult patients with NSCLC with *EGFR* exon 20 insertion mutations.

The cobas EGFR Mutation Test (Roche Diagnostics) is used as a companion diagnostic test before treatment with erlotinib, which exhibits a higher binding affinity for *EGFR* mutants harboring exon 19 deletion or L858R mutation in exon 21 than wild-type *EGFR*. This test is also used as a companion diagnostic test for osimertinib, an irreversible inhibitor of EGFR TKI–sensitive and EGFR-resistant (T790M mutation) mutants, in patients with advanced NSCLC.

Detection of *EGFR* mutations in circulating cell-free DNA (cfDNA) isolated from plasma is feasible and beneficial, particularly for cases in which tumor biopsy is not possible because of insufficient tumor cells, poor DNA quality, or tissue necrosis or when patients cannot undergo invasive biopsy. Gene mutations in cfDNA reflect genetic variations in the tumor.[11]

The variability in the detection rates and correlation of *EGFR* mutations in plasma with specific patient characteristics or clinical outcomes remain unclear, however.[12] In particular, preanalytical factors, including blood collection, preservation, storage and transport conditions, time elapsed between specimen collection and plasma generation, plasma storage or transport conditions, and cfDNA isolation and storage methods, can affect the detection of *EGFR* mutations in cfDNA.[13]

We conducted this study to verify the effect of a centrifugation protocol that could lead to different results on the detection of an *EGFR* mutation in plasma cfDNA.

MATERIALS AND METHODS

Study Population

This study was reviewed and approved for the deliberation waiver by the institutional review of Pusan National University Yangsan Hospital (05-2018-005). A total of 110 patients with advanced NSCLC who were admitted to our hospital between November 2017 and February 2019 were enrolled in this study: 43 men and 67 women (median [range] age, 67 [35-82] years). We analyzed 117 remnant plasma samples from these 110 patients.

Sample Preparation and DNA Extraction

Venous blood samples were collected from patients using 21G needles into one 10-mL Cell-Free DNA BCT tube (Streck) per patient. For the 1-step centrifugation group, within 4 hours after room-temperature blood collection, blood samples were centrifuged at 1,600*g* for 10 minutes, dispensed at 2 mL each into Eppendorf tubes, and stored at −70°C until analysis FIGURE 1. For the 2-step centrifugation group, a second centrifugation step was performed at 13,200*g* for 10 minutes in a benchtop microcentrifuge just before analysis FIGURE 1. Second centrifugation was performed at 1 week to 1.5 years after first centrifugation. All plasma specimens were analyzed after only 1 freeze-thaw cycle. The plasma cfDNA was extracted from a 2-mL starting volume and eluted in 100 µL of elution buffer provided with the cobas cfDNA sample preparation kit (Roche Diagnostics) according to the manufacturer's instructions.

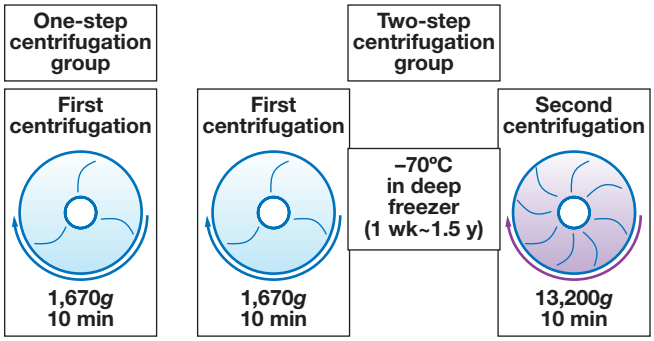

FIGURE 1 Centrifugation protocols for samples in the 2 groups.

EGFR Gene Mutation Test

EGFR mutations were identified using the cobas EGFR Mutation Test v2 on a cobas z 480 analyzer (Roche Diagnostics) according to the manufacturer's instructions. This assay was designed to detect insertions and deletions in *EGFR*, such as deletions in exon 19 and insertions in exon 20, as well as nucleic acid substitutions in *EGFR*, such as G719X, S768I, T790M, L858R, and L861Q. The semiquantitative index (SQI) was determined to reflect trends in the proportion of the mutated copy numbers compared to wild-type copies of *EGFR*. The SQI was derived from a dilution series containing known copy numbers of mutated *EGFR* and a fixed amount of wild-type *EGFR*, with the wild-type DNA serving as an internal control during real-time polymerase chain reaction (PCR). The software assigns an SQI to the detected mutation based on the observed threshold cycle. A higher SQI corresponds to a higher concentration of mutated *EGFR*.[14] We compared identified *EGFR* mutations and SQI values from the results obtained following 1 vs 2 centrifugation steps. We confirmed the clinical impact of the altered results after further centrifugation and compared it with tissue *EGFR* mutations obtained at the nearest time point.

Clinical Confirmation of *EGFR* Mutation Result

Because the results between the 2 centrifugation groups were inconsistent in some cases, the results related to *EGFR* mutation in cfDNA were compared with those obtained following a recently performed tissue biopsy or from pleural fluid. Paraffin-embedded samples from tissue biopsy were placed onto slides to prepare 4-µm-thick sections. One slide of the block was stained with H&E and reexamined for the presence of cancer cells. The enriched area was marked by a pathologist to validate tumor purity of 25% or more. These cancer cell–enriched areas were microdissected, and DNA was extracted. *EGFR* mutations were analyzed using a PNAClamp EGFR Mutation Detection Kit or PANAMutyper (PANAGENE) on a Bio-Rad CFX96 system in the pathology laboratory.

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Statistical Analyses

Statistical analyses were performed using MedCalc statistical software, version 17.7.2 (MedCalc Software). Cohen κ and Pearson χ^2 test were used to analyze qualitative data. Spearman correlation coefficient (ρ) was used to analyze quantitative data. The significance of differences in group parameters was evaluated using the Kruskal-Wallis test and paired *t* test. $P < .05$ was considered statistically significant.

RESULTS

Detected *EGFR* Mutations Were Consistent Between 1- and 2-Step Centrifugation Samples

We detected *EGFR* mutations in 37.6% (44/117) and 40.2% (47/117) of samples in the 1- and 2-step centrifugation groups, respectively. The κ coefficient for the qualitative results of these 2 groups was 0.91. Additionally, the T790M mutation was detected in 10.3% (12/117) of samples in both groups. We observed an 89.7% (105/117) concordance between the 1- and 2-step centrifugation groups, with a 96.6% concordance for the T790M mutation TABLE 1. We detected additional mutations in 5 and 7 samples belonging to the 1- and 2-step centrifugation groups, respectively.

Correlation of SQI Value of Concordant *EGFR* Mutations Between the 1- and 2-Step Centrifugation Groups

The SQI values for 54 mutations in 43 samples were compared for the concordant mutations detected in the 1- and 2-step centrifugation groups. The SQI values based on the mutation type did not differ between the 1- and 2-step centrifugation groups ($P = .288$) TABLE 2. We also observed a strong correlation between the SQI values for the 2 groups (SQI of 1-step centrifugation group = 1.012 × (SQI of 2-step centrifugation group) – 0.0742; Spearman coefficient $r = 0.929$; $P < .0001$) FIGURE 2.

The median SQI differed according to the mutation type in the 1- and 2-step centrifugation groups ($P = .002$ and $P < .001$ in 1- and 2-step centrifugation groups, respectively).

Index Cases With Inconsistent Results Between the 1- and 2-Step Centrifugation Groups

Two patient plasma samples exhibited exon 19 deletions (ex19del; SQI, 6.0 and 8.99), 2 samples showed the T790M mutation (SQI, 4.98 and 8.93), and 1 sample had the L858R mutation (SQI, 4.98) only in the 1-step centrifugation group. In contrast, we identified 2 samples with Ex19del (SQI, 9.09 and 11.82), 2 samples with the T790M mutation (SQI, 3.99 and 4.00), 2 samples with the L858R mutation (SQI, 6.78 and 7.01), and 1 sample with the L861Q mutation (SQI, 1.00) only in the 2-step centrifugation group.

Among the inconsistent mutations observed between the 1- and 2-step centrifugation groups, except for the 2 Ex19del (SQI, 9.09 and 11.82) mutations, 10 mutations showed low SQI values near the limit of detection (LOD).

Index Cases With Inconsistent Results Between the 1- and 2-Step Centrifugation Groups—Comparison With *EGFR* Mutation Results From Recent Tissue Biopsy

Among 12 cases with inconsistent results between the 1- and 2-step centrifugation groups, the 9 samples in the 2-step centrifugation group showed consistent results with those obtained in the *EGFR* mutation test in a recent tissue biopsy (median 1 day after, max

TABLE 1 Concordant *EGFR* Mutations Between Samples in the 1- and 2-Step Centrifugation Groups

	1-Step Centrifugation	2-Step Centrifugation	No.	Comparison With Recent Tissue Biopsy	
				EGFR Mutation	Origin of Tissue
Concordant mutations	Wild-type	Wild-type	69		
	Ex19del	Ex19del	17		
	L858R	L858R	9		
	T790M/Ex19del	T790M/Ex19del	2		
	T790M/L858R	T790M/L858R	6		
	S768I/L858R	S768I/L858R	2		
Discordant mutations	Wild-type	Ex19del[a]	1	Ex19del, L858R	PCNA
	Wild-type	L858R	2	L858R	PCNA, EBUS-TBNA
	Wild-type	L861Q	1	L861Q	Parietal pleura biopsy
	Ex19del	Wild-type[a]	1	Ex19del	TBLB
	T790M/L858R	L858R	1	L858R	EBUS-TBNA
	T790M/Ex19del/L858R	T790M/Ex19del	1	T790M/Ex19del	Metastatic liver biopsy
	T790M	Ex19del/T790M	1	Ex19del/T790M	TBLB
	L858R/T790M	L858R	1	L858R	PCNA, metastatic lumbar biopsy
	L858R	L858R/T790M[a]	1	L858R	PCNA
	Ex19del/L858R	L858R	1	L858R	Metastatic lymph node biopsy
	Ex19del	T790M/Ex19del	1	T790M/Ex19del	PCNA

EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; PCNA, percutaneous fine-needle lung aspiration; TBLB, transbronchial lung biopsy.
[a]Two-step sample inconsistent with tissue biopsy.

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TABLE 2 Median SQI and Difference SQI of Concordant *EGFR* Mutations in the 2 Groups

Mutation Type	No.	SQI in 1-Step Centrifugation Group, Median (min-max)	SQI in 2-Step Centrifugation Group, Median (min-max)	Difference SQI,[a] Median (min-max)
Ex19del	22	14.28 (7.97-20.53)	14.4 (11.4-20.71)	–0.09 (–1.86 to 4.08)
L858R	20	11.28 (4.99-16.55)	10.89 (4.98-20.36)	–0.005 (–2.54 to 3.81)
S768I	1	8.06	7.04	–1.02
T790M	11	5.23 (3.00-12.84)	8.64 (4.98-14.56)	–1.28 (–3.55 to 3.32)

SQI, semiquantitative index.
[a]Difference SQI = SQI in 2-step centrifugation group – SQI in 1-step centrifugation group.

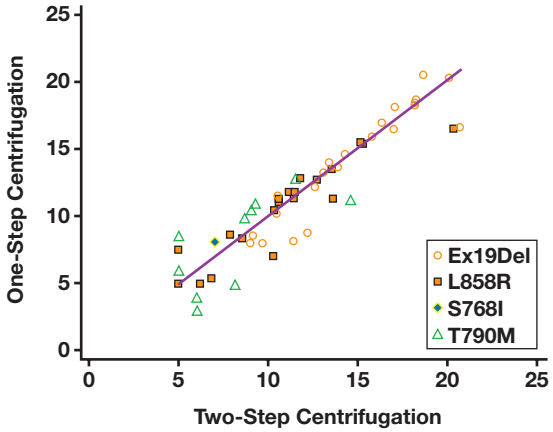

FIGURE 2 Scatter plot of the semiquantitative index values of each *EGFR* mutation between 1- and 2-step centrifugation groups (n = 54).

73 days prior). The 2 samples in the 2-step centrifugation group showed inconsistent results with those obtained in the *EGFR* mutation test in a recent tissue biopsy.

In the first case, Ex19del (SQI, 8.99) was observed only in 1-step centrifuged plasma, which is consistent with the results obtained using lung tissue biopsy samples. In the second case, Ex19del was detected in 1-step centrifuged plasma and transbronchial lung biopsy and not detected only in 2-step centrifuged plasma. Six months later (last line of TABLE 1), Ex19del and T790M mutations were detected in 2-step centrifuged plasma and percutaneous fine-needle lung aspiration samples.

In the last case, L858R was detected in 1-step centrifuged plasma samples and metastatic lymph node biopsy, and L858R and additional T790M were detected only in 2-step centrifuged plasma samples. Five months before this result, only L858R mutation was detected in the tissue and in both 1- and 2-step centrifuged plasma samples. Six months after this result, L858R and T790M mutations were revealed in 1- and 2-step centrifuged plasma samples.

DISCUSSION

The 2-step centrifugation protocol, performed in a Cell-Free DNA BCT container, consisted of an initial low-speed centrifugation step to separate the plasma from the buffy layer and to avoid cell lysis. This step was followed by a high-speed centrifugation step to eliminate any remaining cellular material, including genomic DNA. This protocol has been reported to increase plasma volume and quality.[15-17] We observed an 89.7% concordance between samples in the 1- and 2-step centrifugation groups, with a strong correlation between the SQI values in these groups. Among 12 discordant result pairs, most detection results for *EGFR* mutations using the 2-step centrifugation protocol were consistent with those observed in recently biopsied EGFR-expressing tissues.

LOD may vary depending on the mutation type being detected. For example, in this study, the T790M and L858R mutations were detected at lower SQIs compared with Ex19del FIGURE 2. According to the manufacturer's information, the LOD score for the cobas assay is less than 0.1% (75 copies/mL for exon 19 deletion, 25 copies/mL for exon 20 insertion, and 100 copies/mL for p.L858R and p.T790M mutations). Kim et al[18] found that the LOD scores of the cobas EGFR Mutation assay were 5 to 27 copies/mL for exon 19 deletion (0.1%-0.5% allele frequency), 35 to 70 copies/mL for L858R mutation (0.4%-0.8% allele frequency), 18 to 36 copies/mL for T790M mutation (0.4%-0.8% allele frequency), and 15 to 31 copies/mL for exon 20 insertion (0.3%-0.7% allele frequency). In contrast, Han et al[19] detected Ex19del, T790M, and L858R mutations with SQI values of 7.0 to 9.42, 3.98 to 4.99, and 6.01 to 6.70, respectively, using the cobas EGFR Mutation assay. This difference in performance, based on detection of the target mutation, may be related to the assay design and target gene characteristics.[20,21] Thus, analyzing the sensitivity of an assay based on the SQI is important for detecting *EGFR* mutations, even those at low frequencies. Tumor-derived cfDNA often accounts for a small percentage of the total cfDNA because of tumor heterogeneity and can be present at allele frequencies as low as 0.01%,[22] but previous studies of the correlation between the SQI and variant allele frequency (VAF) or *EGFR* mutated copies/mL showed contradictory results. For example, Marchetti et al[14] found a significant correlation between the SQI and VAF value and between the SQI and mutated copies/mL using droplet digital PCR (ddPCR), but no significant correlations have been detected between the SQI and VAF or *EGFR* mutated copies/mL for different *EGFR* mutations.[23-25]

Ten mutations in the 12 sample pairs among all samples showing inconsistent mutations between the 1- and 2-step centrifugation groups (barring 2) exhibited SQI values near the LOD. Kim et al[18] observed a good correlation between the SQI and VAF for *EGFR* Ex19del but reported low reproducibility for the SQI when the VAF was less than 1%. The cobas assay showed good reproducibility, with a coefficient of variation of 1.29% to 7.35% for target mutations,

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but for the T790M mutation and exon 20 insertion, the coefficient of variation for a sample with an expected allele frequency of 0.05% to 0.8% (13.1%-30.98%) for these mutations was poorer than that for samples harboring other mutations.[18]

EGFR mutation–positive NSCLC tumors are genetically heterogenous and undergo clonal evolution. Activating *EGFR* mutations are generally truncal mutations, and clonal mutations are present in all tumor cells and regions in approximately 90% of samples. T790M is more frequently restricted to branch mutations, which later become subclonal events that occur in only a small proportion of tumor cells and may emerge in subpopulations within discrete tumor locations (~30% of samples).[26,27] Thus, T790M often shows a lower mutant allele frequency compared with the truncal mutation (exon 19 deletion, L858R) and may be undetected in the plasma.[28] It is thought that the detection of T790M differs between the 1- and 2-step centrifugation procedures, as observed in this study. In many countries, T790M is a relatively unimportant clinical issue because osimertinib is used as a front-line treatment.

Compound *EGFR* mutation is defined as double or multiple mutations in the EGFR tyrosine kinase domain. Most compound mutations are combinations of the atypical mutation and typical mutations (exon19 deletion, L858R or G719X substitutions, or exon 20 insertion).[29] In this study, however, Ex19del/L858R compound mutations were detected in 2 cases in the 1-step centrifugation group. In a previous study,[30] among 3,925 patients with *EGFR* mutation, 5 (0.12%) possessed Ex19del/L858R according to sequencing analysis. Another study[31] revealed co-occurring Ex19del/L858R mutations in 10 patients (3.14%) among 318 patients with *EGFR* mutation. Three hypotheses may explain why both variants were identified. In subcloning analysis, Yokoyama et al[32] and Zhang et al[33] showed that complex mutations, including both Del-19 and L858R, were on the same allele. Sakurada et al[34] detected intratumoral tissue heterogeneity of *EGFR* mutations in lung adenocarcinoma. Two or more cells may have different *EGFR* mutation sites. Additionally, the possibility of experimental artifacts in small, paraffin-embedded samples has been reported,[35] but experimental artifacts can be excluded in the current study because we used cfDNA from whole blood.

One limitation of this study is that the 2 protocols were not performed simultaneously; thus, variability in reagent lots or storage conditions cannot be excluded. The high agreement between the results for 2-step centrifugation samples and those for the tissue, however, indicates that the dilution effect can be reduced by double-centrifugation. In addition, samples with differing *EGFR* mutations, as detected using the 2 protocols, could not be verified using next-generation sequencing or ddPCR, which are highly sensitive methods. We overcame other potential limitations, however, by collecting plasma from clinical patients in tubes containing preservatives, and the accuracy of the study results was validated using the plasma test results and tissue results from experiments conducted at specific time intervals.

Detecting *EGFR* mutations, even those at low frequencies, in the cfDNA can significantly affect the treatment approach for patients with lung cancer. Because *EGFR* mutations with low allele frequencies in cfDNA may be undetected in low-sensitivity tests, it is important to establish an optimal test process by analyzing preanalytical factors. Two-step, high-speed centrifugation is an optimization process that increases the sensitivity of the protocol required for detecting *EGFR* mutations in cfDNA.

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REFERENCES

1. Novello S, Barlesi F, Califano R, et al. Metastatic non-small-cell lung cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* 2016;27(suppl 5):v1-v27.
2. Olsen D, Jørgensen JT. Companion diagnostics for targeted cancer drugs—clinical and regulatory aspects. *Front Oncol.* 2014;4:105.
3. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science.* 2004;304:1497-1500.
4. Lynch TJ, Bell DW, Sordella R, 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;350:2129-2139.
5. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med.* 2005;2:e73.
6. Sequist LV, Waltman BA, Dias-Santagata D, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med.* 2011;3:75ra26.
7. Yu HA, Arcila ME, Rekhtman N, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clin Cancer Res.* 2013;19:2240-2247.
8. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* 2007;316:1039-1043.
9. Kuiper JL, Heideman DAM, Thunnissen E, et al. Incidence of T790M mutation in (sequential) rebiopsies in EGFR-mutated NSCLC-patients. *Lung Cancer.* 2014;85:19-24.
10. Arcila ME, Oxnard GR, Nafa K, et al. Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. *Clin Cancer Res.* 2011;17:1169-1180.
11. Molina-Vila MA, Mayo-de-Las-Casas C, Giménez-Capitán A, et al. Liquid biopsy in non-small cell lung cancer. *Front Med (Lausanne).* 2016;3:69.
12. Wu YL, Sequist LV, Hu CP, et al. EGFR mutation detection in circulating cell-free DNA of lung adenocarcinoma patients: analysis of LUX-Lung 3 and 6. *Br J Cancer.* 2017;116:175-185.
13. Grölz D, Hauch S, Schlumpberger M, et al. Liquid biopsy preservation solutions for standardized pre-analytical workflows—venous whole blood and plasma. *Curr Pathobiol Rep.* 2018;6:275-286.
14. 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;10:1437-1443.
15. Sherwood JL, Corcoran C, Brown H, et al. Optimised pre-analytical methods improve KRAS mutation detection in circulating tumour DNA (ctDNA) from patients with non-small cell lung cancer (NSCLC). *PLoS One.* 2016;11:e0150197.
16. Sorber L, Zwaenepoel K, Jacobs J, et al. Circulating cell-free DNA and RNA analysis as liquid biopsy: optimal centrifugation protocol. *Cancers (Basel).* 2019;11:458.

HTTPS://DOI.ORG/10.1093/AJCP/AQAC024

17. van Ginkel JH, van den Broek DA, van Kuik J, et al. Preanalytical blood sample workup for cell-free DNA analysis using droplet digital PCR for future molecular cancer diagnostics. *Cancer Med*. 2017;6:2297-2307.
18. Kim Y, Shin S, Lee KA. A comparative study for detection of EGFR mutations in plasma cell-free DNA in Korean clinical diagnostic laboratories. *Biomed Res Int*. 2018;2018:7392419.
19. Han AL, Kim HR, Choi KH, et al. Comparison of cobas EGFR mutation test v2 and PANAMutyper-R-EGFR for detection and semi-quantification of epidermal growth factor receptor mutations in plasma and pleural effusion supernatant. *Ann Lab Med*. 2019;39:478-487.
20. O'Donnell P, Ferguson J, Shyu J, et al. Analytic performance studies and clinical reproducibility of a real-time PCR assay for the detection of epidermal growth factor receptor gene mutations in formalin-fixed paraffin-embedded tissue specimens of non-small cell lung cancer. *BMC Cancer*. 2013;13:210.
21. Xu T, Kang X, You X, et al. Cross-platform comparison of four leading technologies for detecting EGFR mutations in circulating tumor DNA from non-small cell lung carcinoma patient plasma. *Theranostics*. 2017;7:1437-1446.
22. Volik S, Alcaide M, Morin RD, et al. Cell-free DNA (cfDNA): clinical significance and utility in cancer shaped by emerging technologies. *Mol Cancer Res*. 2016;14:898-908.
23. Macías M, Alegre E, Alkorta-Aranburu G, et al. The dynamic use of EGFR mutation analysis in cell-free DNA as a follow-up biomarker during different treatment lines in non-small-cell lung cancer patients. *Dis Markers*. 2019;2019:7954921.
24. Esteva-Socias M, Enver-Sumaya M, Gómez-Bellvert C, et al. Detection of the *EGFR* G719S mutation in non-small cell lung cancer using droplet digital PCR. *Front Med (Lausanne)*. 2020;7:594900.
25. So MK, Park JH, Kim JW, et al. Analytical validation of a pan-cancer panel for cell-free assay for the detection of *EGFR* mutations. *Diagnostics (Basel)*. 2021;11:1022.
26. Blakely CM, Watkins TBK, Wu W, et al. Evolution and clinical impact of co-occurring genetic alterations in advanced-stage EGFR-mutant lung cancers. *Nat Genet*. 2019;49:1693-1704.
27. Burrell RA, Swanton C. Re-evaluating clonal dominance in cancer evolution. *Trends Cancer*. 2016;2:263-276.
28. Kohsaka S, Mark P, Flavio S, et al. Tumor clonality and resistance mechanisms in EGFR mutation-positive non-small-cell lung cancer: implications for therapeutic sequencing. *Future Oncol*. 2019;15:637-652.
29. Kim EY, Cho EN, Park HS, et al. Compound EGFR mutation is frequently detected with co-mutations of actionable genes and associated with poor clinical outcome in lung adenocarcinoma. *Cancer Biol Ther*. 2016;17:237-245.
30. Yu X, Zhang X, Zhang Z, et al. First-generation EGFR tyrosine kinase inhibitor therapy in 106 patients with compound EGFR-mutated lung cancer: a single institution's clinical practice experience. *Cancer Commun (Lond)*. 2018;38:51.
31. Hata A, Yoshioka H, Fujita S, et al. Complex mutations in the epidermal growth factor receptor gene in non-small cell lung cancer. *J Thoracic Oncol*. 2010;5:1524-1528.
32. Yokoyama T, Kondo M, Goto Y, et al. EGFR point mutation in non-small cell lung cancer is occasionally accompanied by a second mutation or amplification. *Cancer Sci*. 2006;97:753-759.
33. Zhang GC, Lin JY, Wang Z, et al. Epidermal growth factor receptor double activating mutations involving both exons 19 and 21 exist in Chinese non-small cell lung cancer patients. *Clin Oncol (R Coll Radiol)*. 2007;19:499-506.
34. Sakurada A, Lara-Guerra H, Liu N, et al. Tissue heterogeneity of EGFR mutation in lung adenocarcinoma. *J Thorac Oncol*. 2008;3:527-529.
35. Marchetti A, Felicioni L, Buttitta F. Assessing EGFR mutations. *N Engl J Med*. 2006;354:526-528; author reply 526-528.

HTTPS://DOI.ORG/10.1093/AJCP/AQAC024