**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 companion diagnostic real-time polymerase chain reaction test of cell-free DNA.

**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. 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. 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.

The variability in the detection rates and correlation of EGFR mutations in plasma with specific patient characteristics or clinical outcomes remain unclear, however. 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.

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 1,600g for 10 minutes, dispensed at 2 mL each into Eppendorf tubes, and stored at –70°C until analysis. For the 2-step centrifugation group, a second centrifugation step was performed at 13,200g for 10 minutes in a benchtop microcentrifuge just before analysis.

FIGURE 1 Centrifugation protocols for samples in the 2 groups.
Statistical Analyses
Statistical analyses were performed using MedCalc statistical software, version 17.7.2 (MedCalc Software). Cohen κ and Pearson χ² 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.

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).

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...
months later (last line of biopsy and not detected only in 2-step centrifuged plasma. Six detected in 1-step centrifuged plasma and transbronchial lung using lung tissue biopsy samples. In the second case, Ex19del was centrifuged plasma, which is consistent with the results obtained

tion test in a recent tissue biopsy.

One-Step Centrifugation

Two-Step Centrifugation

Difference SQI = SQI in 2-step centrifugation group ‒ SQI in 1-step centrifugation group.

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

In the first case, Ext19del (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, Ext19del 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 biopsy), Ext19del 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.\(^\text{14-17}\) 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 Ext19del mutation (0.3%-0.7% allele frequency). In contrast, Han et al\(^\text{19}\) detected Ext19del, T790M, and L858R mutations with SQI values of 7.0 to 9.42, 3.98 to 4.99, and 6.0 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.\(^\text{20,21}\) 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%,\(^\text{22}\) 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\(^\text{14}\) 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.\(^\text{23-25}\)

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\(^\text{18}\) observed a good correlation between the SQI and VAF for *EGFR* Ext19del 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.
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 heterogeneous 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, Ex9del/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 Ex9del/L858R according to sequencing analysis. Another study31 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 al32 and Zhang et al33 showed that complex mutations, including both Del19 and L858R, were on the same allele. Sakurada et al34 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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