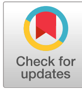
원저
Lab Med Online
Vol. 9, No. 2: 45-56, April 2019
https://doi.org/10.3343/lmo.2019.9.2.45

임상화학 LMO

액체생검을 위한 세포유리 DNA 추출에 검사 전 변동요인이 미치는 영향

Effects of Pre-analytical Variables on Cell-free DNA Extraction for Liquid Biopsy

정태동[1] · 김미화[1] · 박설희[1] · 정혜선[1] · 이진화[2] · 장중현[2] · 허정원[1]
Tae-Dong Jeong, M.D.[1], Mi Hwa Kim, M.T.[1], Sholhui Park, M.D.[1], Hae-Sun Chung, M.D.[1], Jin Wha Lee, M.D.[2], Jung Hyun Chang, M.D.[2], Jungwon Huh, M.D.[1]

이화여자대학교 의과대학 진단검사의학과[1], 호흡기내과[2]
Department of Laboratory Medicine[1], College of Medicine, Ewha Womans University, Seoul; Division of Pulmonary and Critical Care Medicine[2], Department of Internal Medicine, College of Medicine, Ewha Womans University, Seoul, Korea

Background: Extraction of cell-free DNA (cfDNA) is a key step for determining the quality of cfDNA-related molecular diagnostics. We evaluated the effect of sample containers and sample storage conditions on cfDNA extraction.
Methods: The cfDNA extraction using the MagMAX Cell-Free DNA Isolation Kit from five healthy controls and five lung cancer patients was evaluated according to the type of sample container and storage conditions: K2-EDTA container, <1, 6, 24, and 48 hr storage at 4°C after immediate plasma separation; and Cell-Free DNA BCT container, <1, 3, 7, and 14 days stored at room temperature. Mutation analysis of *EGFR* exons 18–21 was performed. To assess the effect of a delay in centrifugation, EDTA whole blood samples from five healthy individuals were stored at 4°C for 6, 12, and 24 hr before plasma separation.
Results: There was no significant difference in the amount and nucleic acid size of cfDNA in both controls and patients with cancer when EDTA plasma was stored at 4°C up to 48 hr. The amount and size of cfDNA in the BCT container were not different up to 7 days; however, the 14-day sample showed an increase in cfDNA concentration due to genomic DNA contamination. *EGFR* mutations were detected on EDTA containers up to 48 hr and with BCT containers up to 14 days. When EDTA whole blood was stored at 4°C and plasma separation was delayed, the cfDNA concentration increased from 24 hr.
Conclusions: The cfDNA extraction was affected by the sample containers and storage conditions.

Key Words: Cell-free DNA, Extraction, Storage, Container, *EGFR*

INTRODUCTION

The concept of liquid biopsy has been introduced not only in research, but also in clinical practice to detect cancer-related gene mutations using circulating cell-free DNA (cfDNA). It is well known that, unlike invasive tissue biopsy, one advantage of liquid biopsies is the evaluation of cancer-related gene mutations through blood sampling, which is a minimally invasive technique [1-4]. In addition, tissue biopsies are difficult to perform repeatedly, and complications related to the procedure may occur depending on the site of the biopsy organ [5-7]. Above all, tumor heterogeneity is a significant challenge when detecting genetic alteration of tumors by tissue biopsy [5, 8, 9]. In contrast, liquid biopsy can be performed easily and repeatedly through blood collection. Since DNA is released into the blood vessels from the tumor tissue, another advantage of liquid biopsies is that it can reflect the real-time genetic characteristics of the tumor [5-9].

The cfDNA is rapidly degraded by nucleases in circulating blood [10, 11], and delayed plasma separation can lead to contam-

Corresponding author: Tae-Dong Jeong, M.D., Ph.D.
https://orcid.org/0000-0002-3873-3117
Department of Laboratory Medicine, College of Medicine, Ewha Womans University, 260 Gonghang-daero, Gangseo-gu, Seoul 07804, Korea
Tel: +82-2-6986-3386, Fax: +82-2-6986-3389, E-mail: tdjeong@ewha.ac.kr

Received: March 23, 2018
Revision received: June 5, 2018
Accepted: June 18, 2018

This article is available from http://www.labmedonline.org
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ination by genomic DNA from cellular components in the specimen [12, 13]. In addition, as the amount of cfDNA present in the blood is very small, a large volume of sample (plasma) is required compared with that required for the extraction of genomic DNA [14]. Subsequent molecular diagnostic techniques after cfDNA extraction can be performed in clinical practice only if sufficient quantity and quality of cfDNA is available. Although quality management of cfDNA extraction is very important to obtain reliable liquid biopsy results, standardization of the cfDNA extraction process has not yet been achieved [15, 16]. Some previous studies have reported results for the evaluation of pre-analytical variables that may affect cfDNA extraction [17-21], but there are still insufficient data on cfDNA extraction methods that can be applied to clinical laboratories as a routine practice.

In this study, we aimed to evaluate the pre-analytical factors affecting cfDNA extraction for liquid biopsy by comparing the type of specimen container and sample storage conditions.

MATERIALS AND METHODS

1. Study design and sample collection

The schematic flow of the study is summarized in Fig. 1. To assess the effect of specimen containers and storage conditions, venous blood from 10 subjects (five healthy volunteers and five lung cancer patients) was collected using two 6 mL K2-EDTA containers (BD Vacutainer, Franklin Lakes, NJ, USA) and one 10 mL Cell-Free DNA BCT container (Streck, La Vista, NE, USA). Blood collection using the BCT container was performed according to the instruction manual [22]. To minimize transportation time of samples, venous blood collection of all study participants was performed in the laboratory. Venous blood in K2-EDTA containers was immediately processed by two steps of centrifugation to separate the plasma, which was aliquoted into four microtubes and stored at 4°C. The cfDNA was extracted from the stored EDTA plasma samples and the performance of cfDNA extraction was compared based on storage time (<1 hr or baseline, and 6, 24, and 48 hr) (Fig. 1A). Venous blood drawn into the BCT container was stored at room temperature for up to 14 days. On days 1, 3, 7, and 14 after the blood draw, approximately 2.5 mL of blood was removed from the BCT container and plasma was separated by two steps of centrifugation. Subsequently, cfDNA was extracted from the plasma and the performance of cfDNA extraction was compared up to 14 days (<1 day or baseline, and 3, 7, and 14) (Fig. 1A).

To assess the effect of delayed centrifugation time for K2-EDTA samples, whole blood was collected in five K2-EDTA containers from five distinct healthy volunteers (Fig. 1B). Blood in two containers was immediately centrifuged in two steps, aliquoted into four microtubes, and stored at 4°C for 48 hr. The remaining three K2-EDTA containers were stored at 4°C for 48 hr. The performance of cfDNA extraction was compared between plasma samples obtained by immediate centrifugation after blood draw and those with delayed centrifugation (6, 24, and 48 hr) of refrigerated whole blood (Fig. 1B).

This study was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (approval number: EUMC 2017-05-031). Written informed consent was obtained from all participants.

2. Plasma separation

Centrifugation for plasma separation was performed in two steps as described in the reagent package insert of the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). First, whole blood samples were centrifuged at 2,000×g for 10 minutes at 4°C. After the first centrifugation, the plasma part of the upper layer was carefully removed, transferred to a new microtube, and centrifuged at 16,000×g for 10 minutes at 4°C. After the second centrifugation, the upper plasma layer was carefully removed and transferred to a new microtube.

3. Extraction of cfDNA

The cfDNA was extracted in an automated manner using a MagMAX Cell-Free DNA Isolation Kit and the KingFisher Duo Prime Purification System (Thermo Fisher Scientific). All procedures were carried out according to the manufacturer's instructions. When blood samples were collected from the BCT specimen, proteinase K treatment was performed after plasma separation. The input plasma volume was 1.0 mL and the cfDNA was eluted in 30 μL of the elution buffer provided by the manufacturer. The extracted cfDNA was transferred to a microtube and stored at lower than -70°C.

4. Measurement of cfDNA concentration and fragment size

The Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and Qubit 3.0 fluorometry (Thermo Fisher Scientific) were used to

https://doi.org/10.3343/lmo.2019.9.2.45

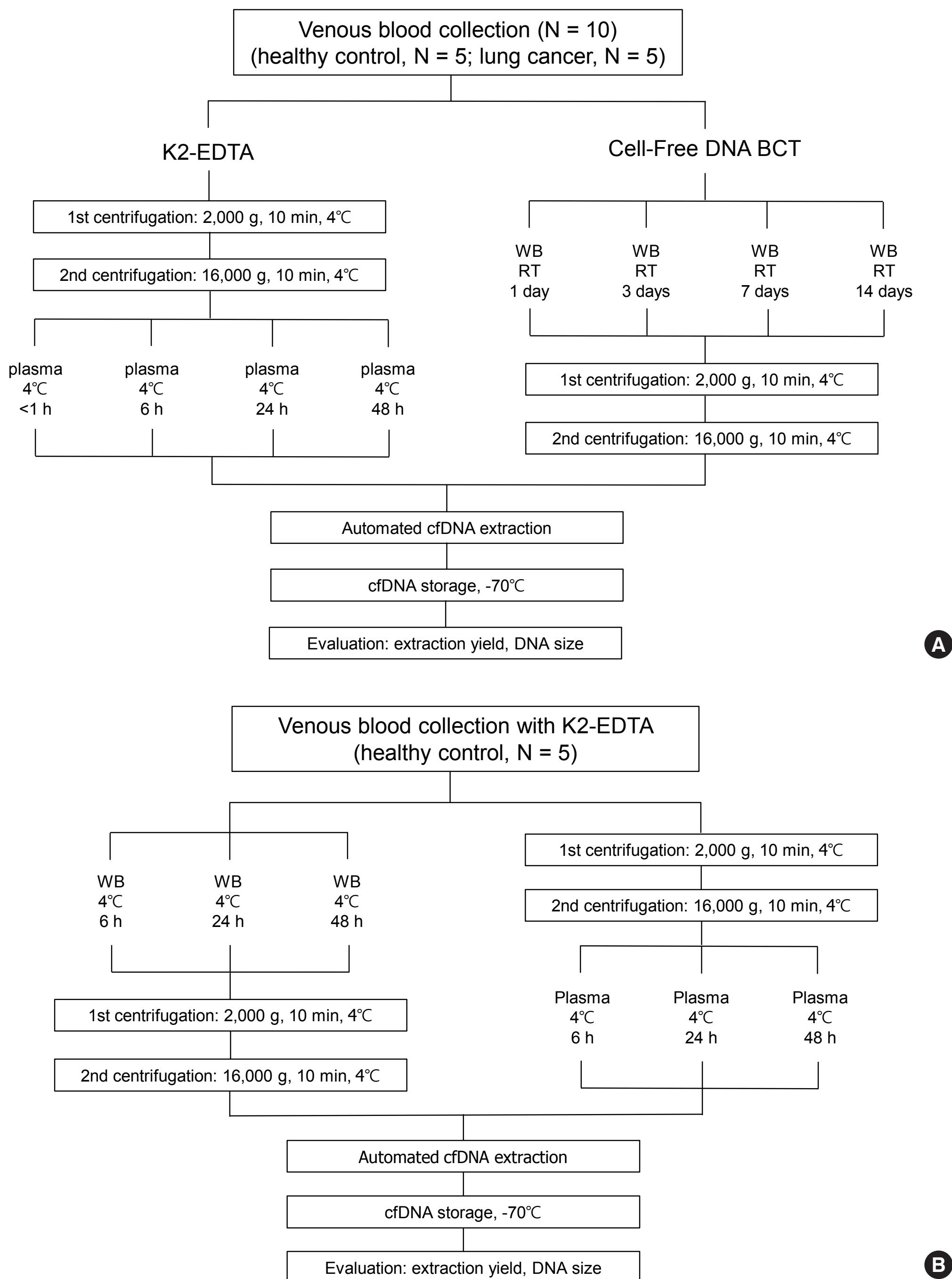

Fig. 1. Schematic flow of the study design. (A) Venous whole blood (WB) was drawn from 10 subjects (healthy controls, N = 5; lung cancer patients, N = 5). The cell-free DNA (cfDNA) was extracted under different specimen storage conditions in K2-EDTA at 4℃ and Cell-Free DNA BCT containers at room temperature (RT). (B) Venous blood was drawn from five healthy subjects in K2-EDTA containers, and the cfDNA was extracted under different centrifugation conditions.

measure the concentration of double-strand DNA in the stored cfDNA samples. The High Sensitivity DNA Analysis Kit and Bioanalyzer 2100 (Agilent Technologies, Inc. Palo Alto, CA, USA) were used to evaluate the fragment size of nucleic acids.

5. *EGFR* mutation analysis

EGFR gene mutation test (cobas EGFR Mutation Test v2 from Roche Diagnostics, Indianapolis, IN, USA) was performed with cfDNA extracted from five patients with lung cancer. The clinical

characteristics of the patients were summarized in Table 1. The cfDNA was extracted from 2 mL of plasma using the cobas cfDNA Sample Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA). The *EGFR* gene mutation test was performed using the cobas z 480 analyzer (Roche Diagnostics) according to the manufacturer's instructions. Patients with *EGFR* mutations underwent additional *EGFR* testing to confirm whether the same type of *EGFR* mutation was detected in cfDNA extracted by the MagMAX Cell-Free DNA Isolation Kit from different sample containers under different storage conditions (K2-EDTA, <1, 6, 24, and 48 hr; BCT, 1, 3, 7, and 14 days).

6. Statistics

The Shapiro-Wilk test was used to evaluate the normality of the continuous variables. Depending on whether the continuous variables were normally distributed, paired *t*-test or Wilcoxon signed-rank test was used to compare the paired samples, and independent sample *t*-test or Mann Whitney *U* test was used to compare the two independent groups. Similarly, one way repeated measures analysis of variance (ANOVA) or Friedman test was used to compare three or more repeated measurements. Statistical analyses were performed using MedCalc software, version 14.12.0 (MedCalc software, Ostend, Belgium). A value of $P<0.05$ was considered statistically significant.

RESULTS

1. Comparison of cfDNA between K2-EDTA and BCT containers

A significantly larger amount of cfDNA was extracted from the K2-EDTA container than the BCT container in the healthy control group (0.173 vs. 0.148 ng/μL; $P=0.0325$ by paired *t*-test), but not in lung cancer patients (0.629 vs. 0.524 ng/μL; $P=0.1173$ by Wilcoxon signed-rank test; Fig. 2). The mean cfDNA yield of baseline K2-EDTA samples was 0.173 ng/μL for healthy controls and 0.629 for patients with lung cancer, which showed a statistically significant difference ($P=0.0143$ by independent sample *t*-test). Similarly, the mean cfDNA yield of baseline BCT samples from healthy controls was significantly lower than that of patients with lung cancer (0.148 vs. 0.524 ng/μL; $P=0.0090$ by Mann Whitney *U* test; Fig. 2). Detailed cfDNA extraction yields according to specimen container and storage conditions in five healthy controls and five lung cancer patients are described in Table 2.

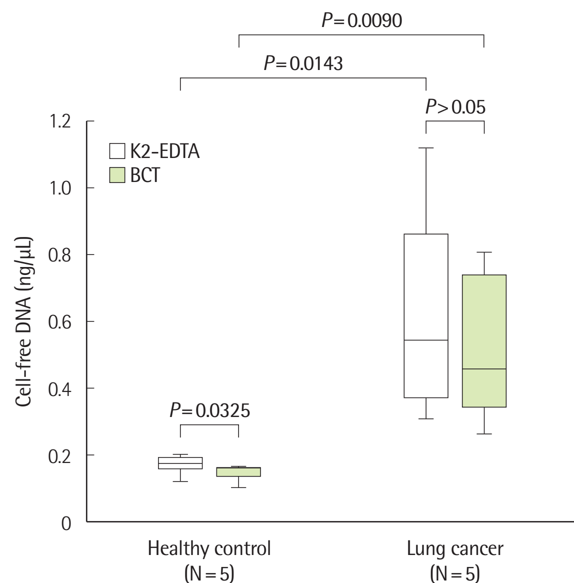

Fig. 2. Comparison of cell-free DNA (cfDNA) concentrations according to specimen container in healthy controls and patients with lung cancer. The cfDNA concentration of patients with lung cancer was significantly higher than that of healthy controls. In the healthy control group, the mean concentration of cfDNA in the K2-EDTA container was significantly higher than that of the Cell-Free DNA BCT containers.

Table 1. Clinical characteristics of five patients with lung cancer

Case No.	Age at diagnosis	Sex	Histology of lung cancer	*EGFR* mutation analysis (date)	
				Tissue biopsy*	Plasma†
LC06	59	F	NSCLC (adenocarcinoma)	L858R (Aug/2016)	L858R (Oct/2017)
LC07	65	F	NSCLC (adenocarcinoma)	Exon 19 deletion (Oct/2014)	No mutation detected (Oct/2017)
LC08	80	F	NSCLC (adenocarcinoma)	Exon 19 deletion (Oct/2017)	Exon 19 deletion (Oct/2017)
LC09	69	F	NSCLC (adenocarcinoma)	Exon 19 deletion, T790M (Mar/2017)	No mutation detected (Oct/2017)
LC10	58	F	NSCLC (adenocarcinoma)	Exon 19 deletion (Sep/2015)	Exon 19 deletion (Oct/2017)

*EGFR mutation was determined using PNAClamp™ EGFR Mutation Detection Kit (PANAGENE Inc., Daejeon, Korea); †EGFR mutation was determined using cobas® EGFR Mutation Test v2 (Roche Diagnostics, Indianapolis, IN, USA).
Abbreviations: F, female; NSCLC, non-small cell lung cancer.

https://doi.org/10.3343/lmo.2019.9.2.45

2. Comparison of cfDNA according to storage conditions in K2-EDTA containers

There was no difference in the amount of cfDNA according to EDTA plasma storage time for both healthy controls (P=0.4696 by Friedman test) and patients with lung cancer (P=0.9168 by one-way repeated ANOVA test) (Fig. 3A). In the evaluation of DNA fragment size, nucleic acids of approximately 150–200 bp were observed, and the size of cfDNA in EDTA plasma stored at 4℃ was not significantly different up to 48 hr for both groups (Fig. 3B and 3C).

3. Comparison of cfDNA according to storage conditions in BCT containers

When BCT containers were stored at room temperature, there was a significant difference in cfDNA concentration over time in both healthy controls (P=0.0097 by Friedman test) and lung cancer patients (P=0.0015 by Friedman test). There was no significant difference in both groups until day 7 compared to baseline. On day 14, however, the amount of cfDNA significantly increased compared to baseline in both healthy controls (P=0.0253 by Friedman test) and patients with lung cancer (P=0.0253 by Friedman test) (Fig. 3D).

In both groups, nucleic acids of about 150–200 bp were mainly observed. The fragment size of cfDNA in BCT samples did not change significantly up to day 7. On day 14, however, an increase in nucleic acid size to greater than 1,000 bp, suggesting genomic DNA, was observed in both healthy controls and patients with lung cancer (Fig. 3E and 3F).

4. Effect of delayed centrifugation in K2-EDTA containers

There was no significant difference in the extraction of cfDNA immediately after plasma separation when cfDNA was extracted after whole blood was refrigerated, and the total amount of cfDNA significantly increased with time (P=0.0256 by one-way repeated ANOVA test). In addition, there was a significant difference in cfDNA concentration from the 24 hr storage sample (P=0.0056 by paired t-test) depending on centrifugation delay (Fig. 4A). The amount of cfDNA according to plasma separation conditions (immediate centrifugation vs. delayed centrifugation) for each of the five healthy subjects is described in Table 3. There was no significant difference in the fragment size of the nucleic acid until 48 hr for stored EDTA plasma (Fig. 4B). However, for

Table 2. Cell-free DNA yield according to the specimen container and storage duration

Case No.	K2-EDTA (ng/μL)				Cell-Free DNA BCT (ng/μL)			
	<1 hr	6 hr	24 hr	48 hr	1 day	3 days	7 days	14 days
Healthy controls (N=5)								
HC01	0.202	0.181	0.193	0.144	0.149	0.143	0.247	1.150
HC02	0.188	0.184	0.225	0.202	0.160	0.141	0.152	0.386
HC03	0.177	0.175	0.194	0.175	0.166	0.161	0.159	0.232
HC04	0.175	0.188	0.181	0.178	0.161	0.142	0.238	0.436
HC05	0.122	0.094	0.102	0.127	0.105	0.108	0.128	0.111
Mean	0.1728	0.1644	0.1790	0.1652	0.1482	0.1390	0.1848	0.4630
SD	0.0304	0.04	0.046	0.03	0.025	0.019	0.054	0.405
Shapiro-Wilk test for normal distribution	W=0.8600 accept normality (P=0.2283)	W=0.6650 reject normality (P=0.0040)	W=0.8508 accept normality (P=0.1970)	W=0.9567 accept normality (P=0.7849)	W=0.7588 reject normality (P=0.0358)	W=0.8651 accept normality (P=0.2470)	W=0.8545 accept normality (P=0.2093)	W=0.8344 accept normality (P=0.1501)
Patients with lung cancer (N=5)								
LC06	0.545	0.438	0.489	0.541	0.460	0.470	0.391	1.080
LC07	0.310	0.286	0.277	0.289	0.265	0.245	0.438	1.040
LC08	0.776	0.618	0.826	0.566	0.716	0.590	0.460	7.690
LC09	1.120	1.320	1.190	1.080	0.806	1.010	0.702	1.350
LC10	0.396	0.480	0.457	0.467	0.372	0.393	0.237	0.411
Mean	0.6294	0.6284	0.6478	0.5886	0.5238	0.5416	0.4456	2.3142
SD	0.3263	0.404	0.362	0.295	0.229	0.29	0.168	3.025
Shapiro-Wilk test for normal distribution	W=0.9324 accept normality (P=0.6125)	W=0.8164 accept normality (P=0.1096)	W=0.9226 accept normality (P=0.5471)	W=0.8661 accept normality (P=0.2509)	W=0.9299 accept normality (P=0.5954)	W=0.9175 accept normality (P=0.5141)	W=0.9430 accept normality (P=0.6872)	W=0.6595 reject normality (P=0.0034)

Abbreviations: BCT, cell-free DNA BCT container; SD, standard deviation.

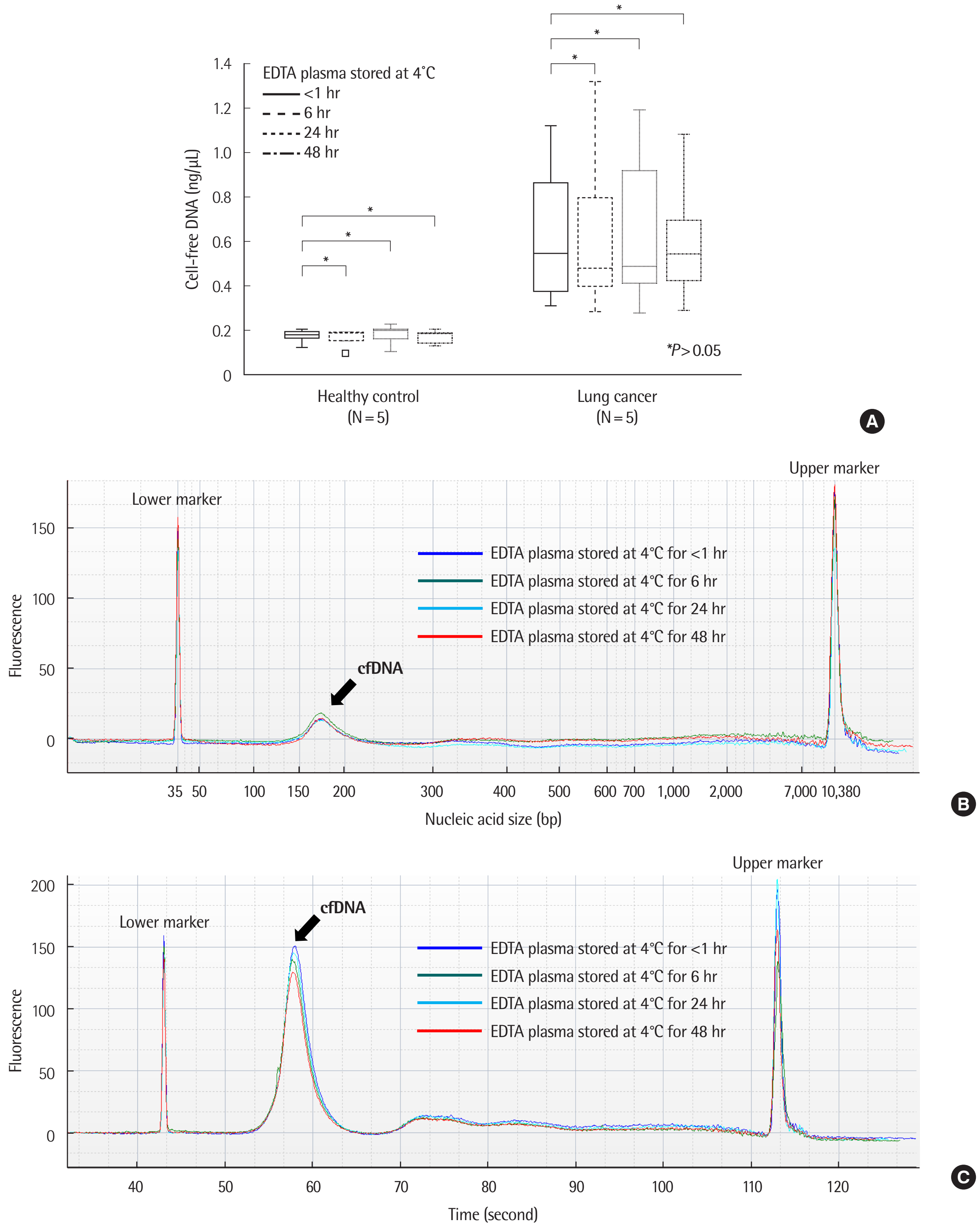

Fig. 3. Comparison of cfDNA concentration and size according to storage conditions in healthy controls and patients with lung cancer. (A) Changes in cfDNA concentration over time in EDTA plasma. There were no significant differences in cfDNA concentration up to 48 hr in EDTA plasma stored at 4˚C. *P>0.05 (one way repeated measures ANOVA or Friedman test, N=15). (B) Example of size distribution of nucleic acids in EDTA plasma in a healthy control (case number: HC04). No significant differences were observed according to time. (C) Example of size distribution of nucleic acids on EDTA plasma in a patient with lung cancer (case number: LC09). No significant differences were observed according to time.

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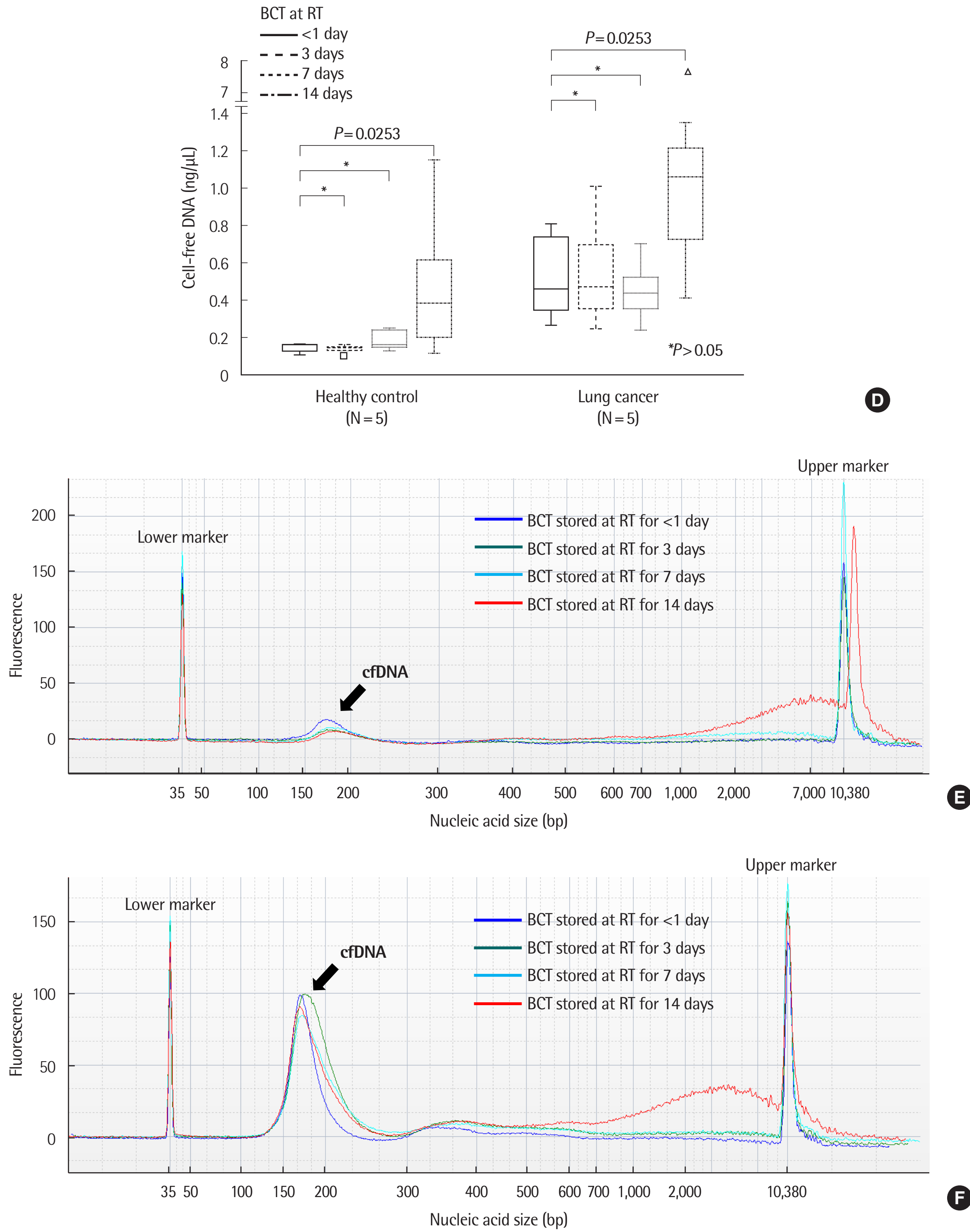

Fig. 3. *Continued.* (D) Changes of cfDNA concentrations over time in BCT containers. There were no statistically significant differences in cfDNA concentration up to 14 days in BCT stored at room temperature (RT). On day 14, an increase in cfDNA concentration was observed. *$P>0.05$ (one way repeated measures ANOVA or Friedman test, N=15). (E) Example of size distribution of nucleic acids in BCT in a healthy control (case number: HC04). (F) Example of size distribution of nucleic acids in BCT in a lung cancer patient (case number: LC09).

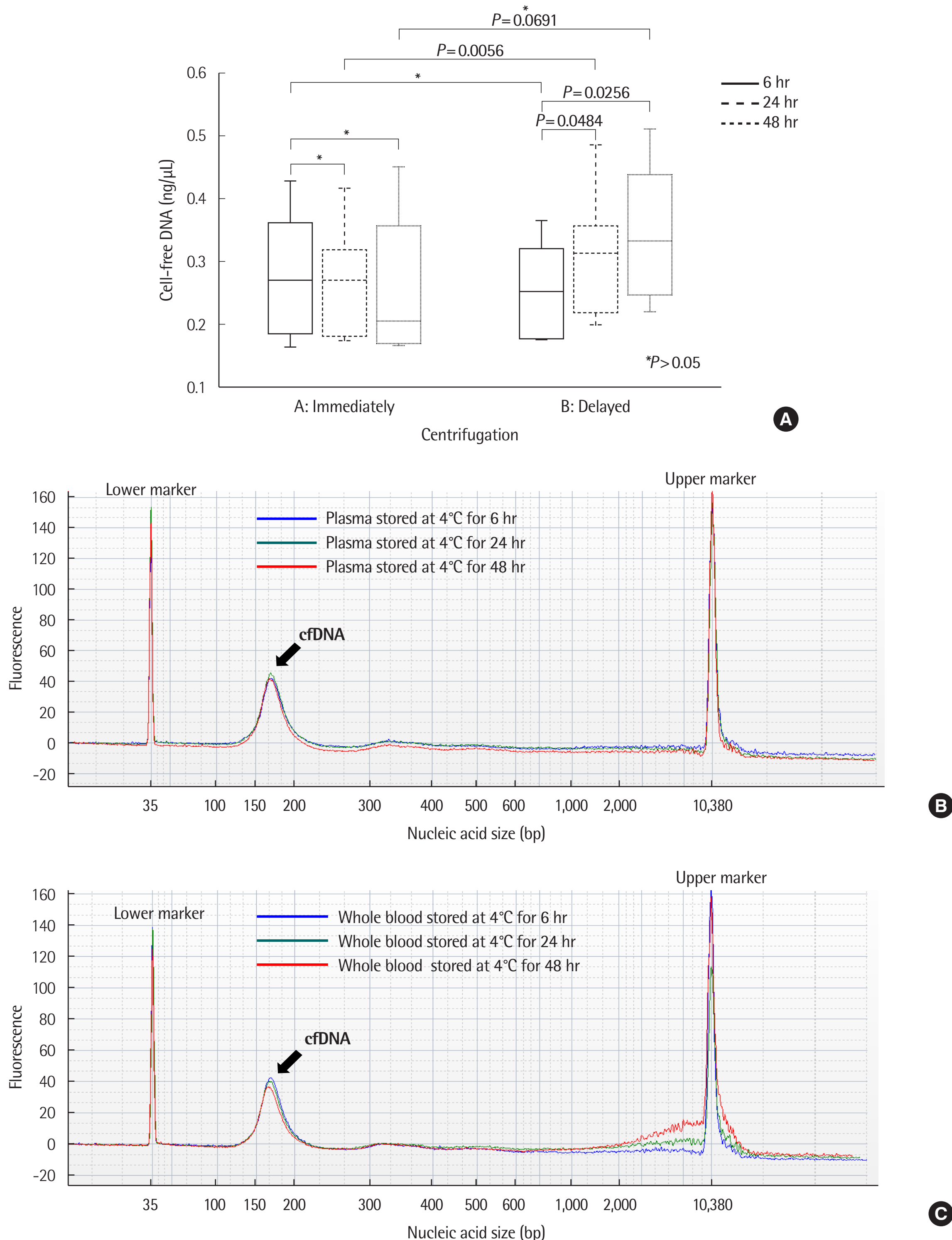

Fig. 4. Comparison of cell-free DNA (cfDNA) in EDTA containers depending on time of centrifugation. X-axis represents centrifugation condition (A, immediate: immediate plasma separation from EDTA whole blood and the plasma stored at 4℃; B, delayed: stored EDTA whole blood at 4℃ and delayed plasma separation). (A) The concentration of cfDNA increased when EDTA whole blood was stored at 4℃ for more than 24 hr. (B) Example of size distribution of nucleic acids in EDTA plasma over time in healthy control (case number: HC13). No significant differences were observed according to storage time. (C) Example of size distribution of nucleic acids in EDTA whole blood over time in healthy control (case number: HC13). Large-sized nucleic acids were observed with increasing storage time.

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Table 3. Cell-free DNA yield according to centrifugation conditions in K2-EDTA containers

Case No.	Immediate plasma separation from EDTA whole blood and stored at 4˚C			EDTA whole blood stored at 4˚C with delayed plasma separation		
	6 hr	24 hr	48 hr	6 hr	24 hr	48 hr
HC11	0.193	0.185	0.172	0.178	0.227	0.222
HC12	0.166	0.176	0.168	0.176	0.201	0.257
HC13	0.270	0.271	0.207	0.253	0.314	0.413
HC14	0.339	0.287	0.325	0.305	0.315	0.333
HC15	0.428	0.417	0.451	0.366	0.485	0.511
Mean	0.2792	0.2672	0.2646	0.2556	0.3084	0.3472
SD	0.1073	0.0974	0.1221	0.0821	0.1112	0.1174
Shapiro-Wilk test for Normal distribution	W=0.9520 accept normality (P=0.7516)	W=0.9006 accept normality (P=0.4130)	W=0.8493 accept normality (P=0.1924)	W=0.9124 accept normality (P=0.4821)	W=0.8923 accept normality (P=0.3686)	W=0.9566 accept normality (P=0.7840)

Abbreviation: SD, standard deviation.

Table 4. Detection of *EGFR* gene mutation in plasma samples from patients with lung cancer

Sample collection and storage conditions	Input plasma volume (mL)	cfDNA extraction kit manufacturer	Case No. LC06 (L858R)	Case No. LC08 (Exon 19 deletion)	Case No. LC10 (Exon 19 deletion)
			EGFR mutation analysis (semi-quantitative index)*		
K2-EDTA, <1 hr	2	Roche	L858R (5.70)	Exon 19 deletion (10.97)	Exon 19 deletion (9.42)
K2-EDTA, <1 hr	1	Thermo Fisher	L858R (5.12)	Exon 19 deletion (8.41)	Exon 19 deletion (8.71)
K2-EDTA, 6 hr	1	Thermo Fisher	L858R (5.79)	Mutation not detected	Exon 19 deletion (8.23)
K2-EDTA, 24 hr	1	Thermo Fisher	L858R (5.03)	Exon 19 deletion (10.17)	Exon 19 deletion (8.97)
K2-EDTA, 48 hr	1	Thermo Fisher	L858R (4.00)	Exon 19 deletion (8.40)	Exon 19 deletion (8.69)
BCT, 1 day	1	Thermo Fisher	L858R (6.04)	Exon 19 deletion (9.32)	Exon 19 deletion (6.98)
BCT, 3 days	1	Thermo Fisher	L858R (6.98)	Exon 19 deletion (9.01)	Exon 19 deletion (8.26)
BCT, 7 days	1	Thermo Fisher	L858R (6.00)	Exon 19 deletion (9.22)	Exon 19 deletion (8.71)
BCT, 14 days	1	Thermo Fisher	L858R (4.92)	Exon 19 deletion (9.12)	Exon 19 deletion (8.66)

**EGFR* mutation was determined using cobas® EGFR Mutation Test v2 (Roche Diagnostics, Indianapolis, IN, USA).
Abbreviations: BCT, cell-free DNA BCT container; SD, standard deviation.

EDTA whole blood stored at 4˚C, an increase in the size of nucleic acids to 2,000 bp or greater was observed after 24 hr (Fig. 4C).

5. *EGFR* gene mutation analysis

The *EGFR* test results with the semi-quantitative index (SQI) of the cfDNA specimens extracted from each patient according to the specimen container type and storage conditions are described in Table 4. *EGFR* mutations were observed in three out of five lung cancer patients (two with exon 19 deletion [Case No. LC08 and LC10], one with L858R [Case No. LC06]). In case No. LC06 with the L858R mutation and case No. LC10 with the exon 19 deletion, the same mutations were observed in all of the extracted cfDNA samples irrespective of the sample container type and storage conditions. SQI values of case No. LC06 and case No. LC10 were 4.00–5.79 and 8.40–10.17, respectively, for K2-EDTA containers and 4.92–6.98 and 9.01–9.32, respectively, for BCT containers. In case No. LC08, no mutation was detected in the samples stored in K2-EDTA for 6 hr at 4˚C after immediate plasma separation, although the exon 19 deletion mutation was detected in all other samples. SQI values of the case No. LC08 were 8.23–8.97 for K2-EDTA containers and 6.98–8.71 for BCT containers.

DISCUSSION

In the era of precision medicine, cfDNA analyses have been implemented in clinical laboratories. The cfDNA testing can be applied to non-invasive prenatal screening as well as diagnosis, real-time monitoring of treatment, and prediction of prognosis in patients with cancer by screening for cancer-related gene mutations and copy number variation [1, 21, 23]. A representative *in vitro* diagnostic test screens for *EGFR* gene mutations in blood samples as a companion diagnostic for the selection of therapeutic drugs in patients with lung cancer [4]. The starting point for cfDNA analyses is the extraction of cfDNA, which is a very im-

portant step in determining the quality of the test results. Several studies have previously reported various pre-analytical factors that can affect cfDNA extraction performance such as sample matrix, collection and processing, storage condition, freeze-thawing, isolation method of DNA, and quantification of DNA [12, 15-21]. Recently, quality control material for cfDNA analysis has been reported [24], and efforts to improve the quality of cfDNA-related tests are expected to continue.

Although there is no standardized operating procedure guideline for cfDNA extraction that can be used in clinical laboratories, it is widely accepted that blood samples are collected in a container containing EDTA anticoagulant, and the plasma can be separated by two steps of centrifugation under different conditions [12, 15-21]. To obtain optimal cfDNA for downstream analysis, plasma specimens are preferred over serum, and the samples are centrifuged twice to remove the cellular components from the specimen and thus minimize the contamination with genomic DNA. Most of the current cfDNA-related tests are based on mutation gene analysis; therefore, if the cfDNA is contaminated by genomic DNA, the percentage of circulating tumor DNA (ctDNA) could be decreased. Since the amount of both ctDNA and cfDNA is very small, minimizing the contamination of genomic DNA during sample processing is one way to improve the quality of tumor-related gene mutation tests using ctDNA.

Although the type of specimen, the anticoagulant, and the centrifugation method for cfDNA extraction were consistent in previous studies [12, 15-21], the safe time to delay centrifugation without genomic DNA contamination was reported to vary from 2 hr [25] to 72 hr [19] at 4°C (EDTA tubes) depending on the researcher. In routine laboratory practice, one of the most important and practical factors is the duration a blood sample can be stored under certain conditions for cfDNA extraction. In our study, no significant changes in cfDNA concentration was observed up to 48 hr for EDTA plasma and up to 6 hr for EDTA whole blood stored at 4°C (Fig. 4A). Large-sized nucleic acids were observed at 24 hr from baseline in the EDTA whole blood, and consequently there was a tendency for the cfDNA concentration to increase after 24 hr (Fig. 4A and 4C). Since the number of specimens evaluated by the authors was small (N=5), we concluded that refrigeration of EDTA whole blood for up to 6 hr did not affect the cfDNA concentration. More samples and time points (e.g., 8 or 12 hr) are needed to confirm whether cfDNA was released from cellular components after 6 hr.

In our results, there was no significant difference in the amount and fragment size of cfDNA up to 48 hr when the plasma was immediately separated from the EDTA whole blood and stored at 4°C (Fig. 3). These findings suggest that even after storage for 48 hr in refrigerated conditions, there was negligible genomic DNA contamination and cfDNA degradation for EDTA plasma. This was observed in both healthy controls and patients with lung cancer, and might be because the early stage two-step centrifugation process minimized genomic DNA contamination by removing as much of the cellular components as possible. In addition, in all three patients with lung cancer with *EGFR* gene mutations, the same mutation present at baseline was detected in EDTA plasma samples stored for 48 hr, and although statistical analysis was not possible due to the small number of sample, the SQI values of *EGFR* mutations were similar up to 48 hr. These results suggest that storage of immediately separated EDTA plasma for 48 hr probably does not affect the results of subsequent real-time PCR-based molecular analyses. In the present study, the exon 19 *EGFR* mutation was not detected in a EDTA plasma sample stored at 4°C for 6 hr, which might be the result of a random error or lack of a duplicate or triplicate test.

Ideally, plasma should be separated as soon as possible from the drawn blood to obtain optimal cfDNA for downstream molecular analysis; however, this may be limited in some clinical laboratories depending on the available labor, facilities, and equipment. Therefore, on a practical level, if the EDTA plasma is immediately separated and stored at 4°C, it may be possible to secure approximately 48 hr of time compared with storing EDTA whole blood at 4°C for cfDNA analysis. However, further evaluation is necessary to determine whether ctDNA from stored EDTA plasma is preserved compared to the baseline. Although the half-life of ctDNA is unclear [26], theoretically if the sample storage time is prolonged, the ctDNA will be gradually degraded and this might affect the final results of the cfDNA analysis (e.g., *EGFR* gene mutation) due to the detection limit of the molecular test.

The BCT containers containing the K3-EDTA anticoagulant and cell preservative, and cfDNA can be stored on BCT at room temperature for up to 14 days [22]. Previous studies have reported the performance of BCT containers for cfDNA extraction, but the samples were usually evaluated after less than 5 days at room temperature [17, 19, 21]. We evaluated the cfDNA extraction per-

formance using BCT stored at room temperature for up to 14 days. Our results showed no significant difference in cfDNA concentration using the BCT containers until day 7 between samples from healthy subjects and patients with cancer. On day 14, the total amount of cfDNA was increased, and larger nucleic acids were observed compared to baseline (Fig. 3). BCT containers stabilize cfDNA and cellular genomic DNA for 14 days, whereas epithelial cells (tumor cells) are stable only up to 7 days [22]. Therefore, after 7 days, nucleic acid released from epithelial cells rather than white blood cells may cause an increase in cfDNA concentration. In this study, the *EGFR* gene mutation was confirmed in samples stored in BCT containers for up to 14 days in all three patients with lung cancer and confirmed mutations. In addition, the SQI values of *EGFR* mutations were similar up to 14 days. These results suggest that the BCT container could be safely used to store cfDNA for 14 days. However, on day 14, a decrease in the relative concentration of ctDNA was expected due to an increase in the total cfDNA concentration. Therefore, storage in the BCT container for up to 14 days may affect *EGFR* gene testing, and further studies with more samples are needed.

There were some limitations in this study. First, the number of specimens evaluated was small, and it is difficult to generalize the results of the authors. Second, only automated cfDNA extraction using the MagMAX Cell-Free DNA Isolation Kit was evaluated, and various other methods were not studied. Third, we confirmed *EGFR* mutations according to specimen containers and storage conditions; however, we did not apply a quantitative approach for *EGFR* mutations in patients with lung cancer.

In summary, cfDNA isolated using the MagMAX Cell-Free DNA Isolation Kit could be stable for 6 hr for EDTA whole blood at 4°C, for 48 hr for immediately centrifuged EDTA plasma at 4°C, and for 7 days in BCT container at room temperature. It was obvious that cfDNA extraction was a crucial step in cfDNA-related analyses, therefore the influence of several pre-analytical factors that can affect the results of downstream assays after cfDNA extraction should be minimized. The results of this study are expected to provide useful and practical data for clinical laboratories working with cfDNA.

요 약

배경: 세포유리 DNA (cell-free DNA, cfDNA) 추출 과정은 cfDNA와 연관된 분자진단 검사의 질을 결정하는 중요한 단계이다. 저자들은 검체 용기 종류와 검체 보관 조건이 cfDNA 추출에 미치는 영향에 대해 평가하였다.

방법: 다섯 명의 건강인과 다섯 명의 폐암 환자를 대상으로 MagMAX Cell-Free DNA 추출 시약을 사용하여 cfDNA를 추출하였다. K2-EDTA 항응고제가 첨가된 검체 용기는 채혈 후 즉시 혈장을 분리하였고, 냉장 상태에서 <1, 6, 24, 48시간 동안 혈장을 보관한 후 cfDNA를 추출하였다. cfDNA 전용 검체 용기(Cell-Free DNA BCT)는 채혈 후 <1, 3, 7, 14일 동안 실온에서 보관한 후 cfDNA를 추출하였다. 폐암 환자에서 *EGFR* 유전자(엑손 18-21) 변이 여부를 검사하였다. 혈장 분리 시간이 cfDNA 추출에 미치는 영향을 평가하기 위해 다섯 명의 건강인을 대상으로 K2-EDTA 전혈을 6, 12, 24시간 동안 냉장 보관한 후 혈장을 분리하여 cfDNA를 추출하였다.

결과: 건강인과 폐암 환자 모두 냉장 보관된 K2-EDTA 혈장 검체는 48시간까지 cfDNA 양과 핵산의 크기에 차이가 없었다. BCT 검체 용기는 건강인과 폐암 환자 모두 7일까지 cfDNA의 양과 핵산의 크기에 차이가 없었으나, 14일째에는 유전체 DNA의 오염으로 인해 cfDNA 양이 증가하였다. *EGFR* 유전자 변이는 냉장 보관된 K2-EDTA 혈장 검체에서 48시간까지, 실온 보관된 BCT 검체 용기는 14일까지 검출되었다. 냉장 보관된 K2-EDTA 전혈 검체에서 24시간 이상 혈장 분리가 지연된 경우 cfDNA의 양이 증가하였다.

결론: cfDNA 추출 성능은 검체 용기 종류와 검체 보관 조건에 영향을 받을 수 있다.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article were reported.

ACKNOWLEDGEMENTS

This study was supported by the Laboratory Medicine Foundation (Korea).

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 https://doi.org/10.3343/lmo.2019.9.2.45