Katarzyna Boguszewska-Byczkiewicz[1], Dariusz Jarych[2], Izabela Drożdż[3], Hamed Al Huwaidi[4],
Izabela Zawlik[5], Agnieszka Kołacińska[6]

[1]Department of Surgical Oncology, Copernicus Provincial Multidisciplinary Centre of Oncology and Traumatology, Lodz, Poland [2]Laboratory of Personalized Medicine and Laboratory of Biotechnology, Bionanopark Ltd Poland, Lodz, Poland

[3]Department of Clinical Genetics, Medical University of Lodz, Lodz, Poland

[4]Medical University of Lodz, Poland

[5]Department of Genetics, Institute of Medical Sciences, Laboratory of Molecular Biology, Centre for Innovative Research in Medical and Natural Sciences, Medical College, University of Rzeszow, Poland

[6]Department of Head and Neck Cancer Surgery, Department of Surgical Oncology, Cancer Center, Medical University of Lodz, Poland

A comparison of four commercial kits used for isolating circulating cell-free DNA: QuickGeneMINI8L (Kurabo), Maxwell RSC cfDNA Plasma Kit (Promega), cfKapture 21 Kit (MagBio), and QIAamp MinElute ccfDNA Kit (Qiagen)

Corresponding author:

Katarzyna Boguszewska-Byczkiewicz,
Department of Surgical Oncology,
Copernicus Provincial Multidisciplinary
Centre of Oncology and Traumatology,
93–513 Lodz, Paderewskiego 4, Poland
e-mail: k.boguszewska@kopernik.lodz.pl

ABSTRACT

A minimally-invasive alternative to surgical biopsies is a liquid biopsy (LB), a technique that has recently revolutionized the management of a number of tumors. One potential target biomarker of LB is cell-free DNA (cfDNA), which can act as a very sensitive indicator for certain tumors. Currently, clinical efforts are focused on increasing the quality of the cfDNA isolated for analysis.

The present study compares the efficiency of isolation by four commercial kits: QuickGeneMINI8L (Kurabo), Maxwell RSC cfDNA Plasma Kit (Promega), cfKapture 21 Kit (MagBio), and QIAamp MinElute ccfDNA Kit (Qiagen). In each case, cfDNA was isolated from three plasma samples and one serum sample.

Available method for the isolation give the ability to enrich optimal diagnostic quantity of cfDNA. cfDNA can be successfully separated using all investigated kits. The greatest efficiency was demonstrated by the QIAamp MinElute ccfDNA Kit (Qiagen) and cfKapture 21 (MagBio). Large amounts of cell-free DNA can be successfully isolated from small volumes of plasma.

Key words: liquid biopsy, breast cancer, cell-free DNA

Introduction

Liquid biopsy (LB) is a minimally-invasive diagnostic method based on the isolation of biomarkers dissolved in body fluids such as blood, cerebrospinal fluid, pleura effusion, sputum, saliva or seminal fluid [1]. By analyzing various circulating molecular biomarkers, such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), exosomes, proteins and microRNA(miRNA), it is possible to achieve early diagnoses of a number of diseases.

One of the most promising of these biomarkers is cell-free DNA (cfDNA), first described in 1948 [2]. The cfDNA profile corresponds with the metastatic model and disease progression; it can also reflect co-morbidity and resistance mechanisms, as well as the complete cancer gene expression profile and tumor staging [3–9]. cfDNA also includes circulating tumor DNA (ctDNA) referred to as the tumor code in the blood [10]; however, the ctDNA is mixed with a wide range of cfDNA from various sources and is hence difficult to detect clearly. In line with the recent surge of interest in the development

of innovative technologies to detect cfDNA in LB, the present study evaluates four commercially-available cfDNA extraction kits as candidates for improved cfDNA isolation techniques.

In 2016, cfDNA testing was given FDA approval for clinical usage. Unfortunately, the current high cost of LB technology, and the associated technical challenges in cfDNA isolation, limit its clinical adoption. However, LB offers great potential for daily use as it requires little time and resources to perform, and possesses broad diagnostic potential.

Aim

The present study compares the efficiency of four cfDNA isolation kits in patients with breast cancer to determine which offers the most efficient cfDNA isolation; it also determines whether serum or plasma provides the greatest quantities of cfDNA.

Material and methods

Three patients with breast cancer were enrolled in the study; they were selected from patients hospitalized in the Department of Surgical Oncology, Copernicus Memorial Hospital, Lodz, Poland. The medical records of those patients, including the age of breast cancer removal, type of surgery, size of tumor, histological type of cancer, hormone receptor profile, grading, TNM staging and lymph node involvement, were reviewed retrospectively. Patient baseline characteristics are presented in Table 1.

All participants gave written informed consent to take part in the study. Approval for the study was given by the local ethics committee (study number RNN//345/15/KE dated 15.12.2015).

Patient 3

The patient was 84 years old at the time of the investigation, with lobular type breast cancer on the left side, G2, with no ductal carcinoma in situ (DCIS) component, diameter 1.8 cm, classified as T1NO, after mastectomy. Human epidermal growth factor receptor 2 (HER2) status was negative, estrogen receptor (ER) status was positive (100% of expression) and progesterone receptor (PR) status was positive (90% of expression).

Patient 17

The patient was 54 years old at the time of the investigation, with no special type (NST) breast cancer on the right side, G2 with no DCIS component, diameter 1.1 cm, classified as T1NO, after mastectomy with

sentinel lymph node biopsy (SLNB). HER2 status was negative, ER status was negative (0% of expression), PR status was negative (0% of expression).

Patient 19

The patient was 69 years old at the time of the investigation, with NST breast cancer on the left side, G2, with DCIS component, diameter 2 cm, classified as T1N2 after breast conservation therapy; the patient had undergone axillary lymph node dissection.HER2 status was negative, ER status was positive, PR status was positive. The reason why we have chosen those particular 3 patients is because they fulfill the criteria of TNM class when it comes to all dimensions (size, lymph nodes and metastases).

Method

Blood samples were obtained from the peripheral veins of all participants and frozen at –80°C. Each serum sample was numbered according to the participating patient. The following commercial cfDNA isolating kits were analyzed: QuickGene-Mini8L (Kurabo, Japan), cfKapture 21 Kit (200–400 μL) (MagBio Genomics, USA), Maxwell RSC ccfDNA Plasma Kit (Promega, USA), QIAamp MinElute ccfDNA (Qiagen, Germany). Laboratory testing was performed in the Laboratory of Personalized Medicine and Laboratory of Biotechnology, BioNanoPark in Lodz.

A. Isolation of cfDNA from serum sample number 3: cfDNA was isolated from serum sample 3 using two kits: QuickGeneMINI8L from Kurabo and the QIAamp MinElute ccfDNA Kit from Qiagen.

B. Isolation of cfDNA serum sample number 17: cfDNA was isolated from serum sample 17 using three kits: the aMaxwell RSC (Promega) cfDNA Plasma Kit, the cfKapture 21 Kit (MagBio) and the QIAamp MinElute ccfDNA Kit (Qiagen).

C. Isolation of cfDNA serum sample number 19: cfDNA was isolated from serum sample 19 using four kits: QuickGeneMINI8L (Kurabo), Maxwell RSC cfDNA Plasma Kit (Promega), cfKapture 21 Kit (MagBio) and QIAamp MinElute ccfDNA Kit (Qiagen).

D. Comparison of cfDNA isolation from plasma and serum sample from patient number 19:
Isolation was carried out using two kits: the Maxwell RSC cfDNA Plasma Kit (Promega) and cfKapture 21 Kit (MagBio). Plasma and serum samples were both taken from patient number 19.

In all cases, the obtained cfDNA fragments were analyzed by automated gel electrophoresis on a Tape Station 2200(Agilent) using D1000 High Sensitive Screen Tape.

Table 1. Patient profiles

Patient number	Tumor type/ /DCIS component	TNM/G	Tumor size [cm]	side	HER/ /ER/PR	Operation type
3	Lobular/no	T1N0/G2	1.8	left	−++/ /luminal A	mastectomy
17	NST/no	T1N0/G2	1.1	right	−−−/ /triple negative	mastectomy
19	NST/no	T1N2/G2	2.0	left	−++	Breast conservation therapy (BCT)

Table 2. A comparison of the tested kits

The name of a kit	Purification method	Buffer volume [μL]	Starting material volume [mL]
QuickGeneMINI8L (Kurabo)	DNA affinity columns	100.0	3.0
QIAamp MinElute ccfDNA Kit (Qiagen)	magnetic beads with on-column purification	60.0	1.0
Maxwell RSC cfDNA Plasma Kit (Promega)	magnetic beads	60.0	1.0
cfKapture 21 Kit (MagBio)	magnetic beads	50.0	0.28

Summary of characteristics of all kits used for isolation is shown in Table 2.

Results

A. For sample 3, a high number of cfDNA fragments about 180 bp length were observed; however, only for the QIAamp MinElute ccfDNA kit. The isolated cfDNA is presented in Figure 1.

B. For sample number 17, cfDNA was isolated successfully with all of the kits. The largest amount of cfDNA was obtained by the Qiamp MinElute ccfDNA Kit from 1 ml of starting material. However, the highest yield, *i.e.* the greatest amount of cfDNA per given volume of starting material, was obtained by the cfKapture 21 Kit, as this only required 280 μL of starting material. Figure 2 presents the cfDNA isolated from sample number 17 by three cfDNA isolation kits: A1 — cfKaptur 21 Kit; E1 — QIAamp MinElute ccfDNA Kit; F5 — Maxwell RSC cfDNA Plasma Kit. The efficacy of the isolation method is reflected by cfDNA concentration related to cfDNA fragment size. The horizontal axis and vertical axis represent product size and peak height of DNA concentration, respectively.

C. For sample number 19, the greatest amount of cfDNA was obtained using the Qiamp MinElute ccfDNA Kit (i.e. from 1 mL material), while the highest efficiency was obtained by the cfKapture 21 Kit (i.e. from 280 μL material). The isolated cfDNA is presented in Figure 3.

D. A high level of genomic DNA contamination was observed during cfDNA isolation from serum samples, evidenced by the presence of additional products longer than 180 bp. This is confirmed by the isolation results obtained by the two kits used. In the case of cfDNA isolation from plasma, a single peak characteristic of the size of cfDNA was obtained. The isolated cfDNA is presented in Figure 4 and Figure 5.

Discussion

The concept of circulating tumor cells was first proposed by Ashworth in 1869 following the identification of tumor cells in the peripheral blood of a patient with metastatic disease. However, the relationship between cfDNA and malignant disease was only established in 1975 [16]. In recent years, a large number of studies have attempted to eliminate the background interference generated by large amounts of cfDNA from other sources: the concentration of total cfDNA in healthy individuals ranges from 0 to 100 ng/mL, and this value can be as high as 1000 ng/mL in cancer patients [17, 18]. cfDNA is
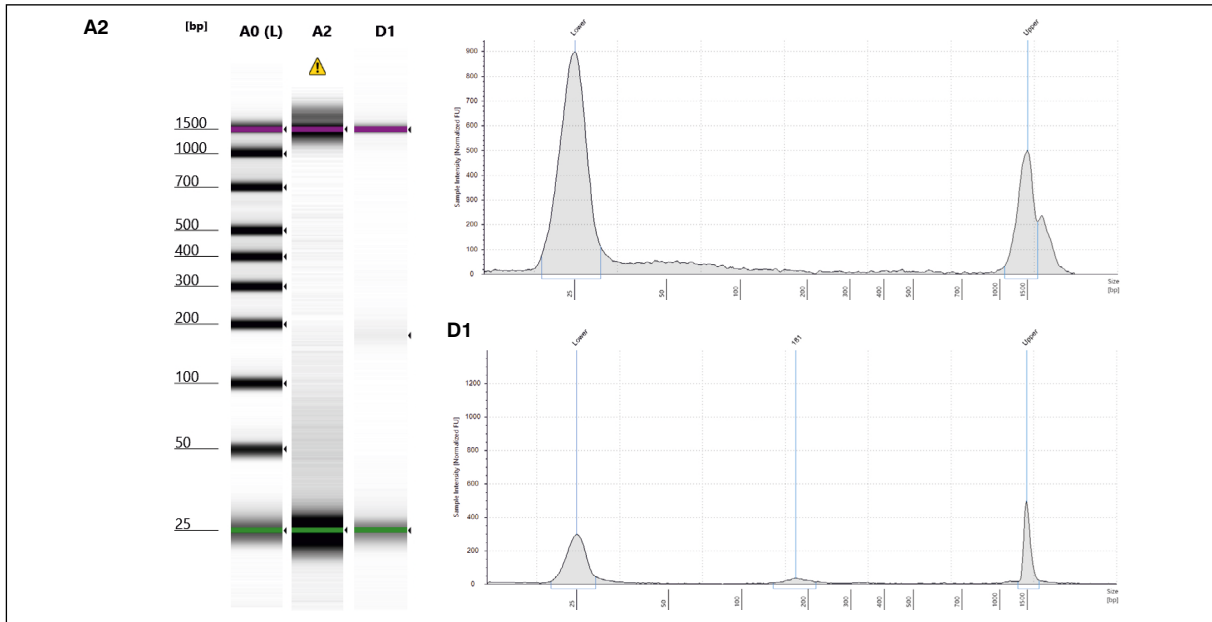
Figure 1. Electrophoresis of cfDNA isolated from sample number 3 by Agilent 2200 TapeStation 2200. A2-QuickGeneMINI8L(Kurabo), D1-QIAamp MinElite ccfDNA Kit(Qiagen). The observed bands are described in more detail in Table 3. and Electropherograms of cfDNA isolation products (A2-Kurabo, D1-Qiagen), obtained with Tape Station 2200 (Agilent)
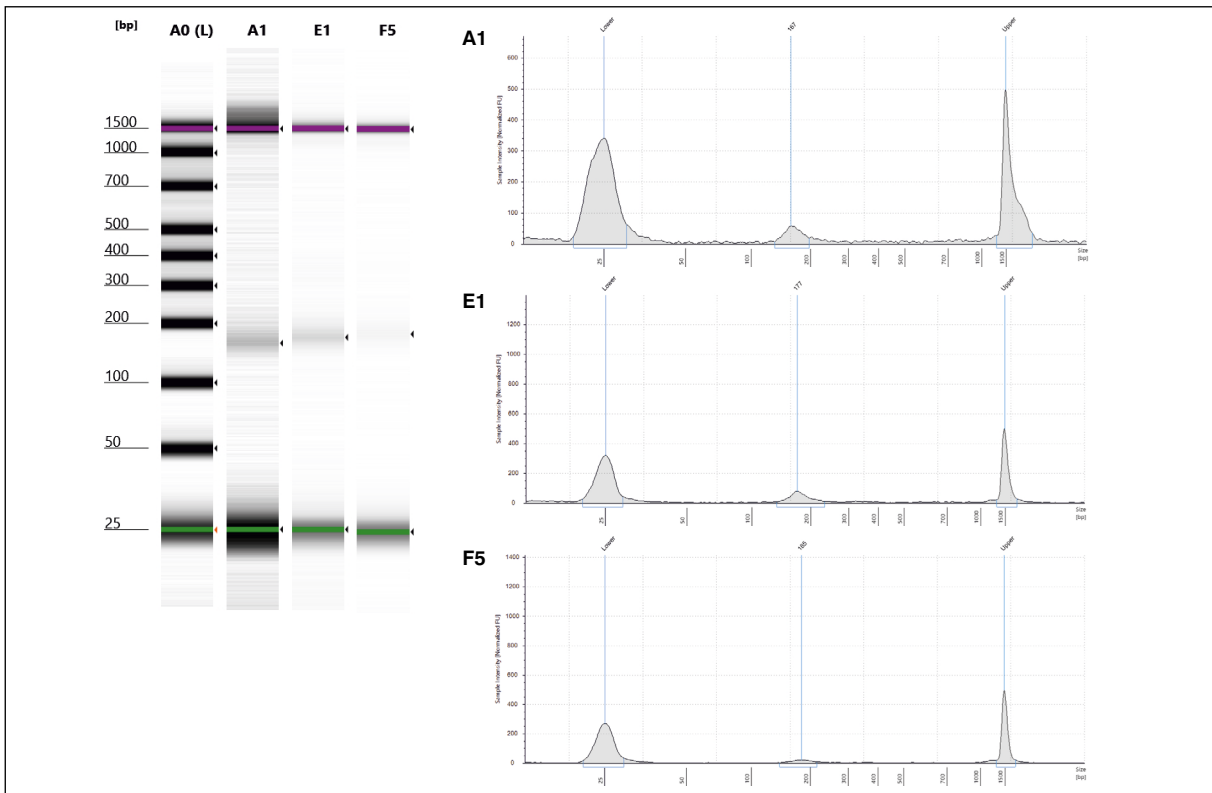

Figure 2. Electrophoresis of cfDNA isolated from sample no. 17 by Agilent 2200 TapeStation 2200. A1 – cfKapture 21 Kit; E1- QIAamp MinElute ccfDNA Kit; F5- Maxwell RSC cfDNA Plasma Kit. The observed bands are described in more detail in Table 3. and Electropherograms for cfDNA isolated fragments (A1 — cfKapture 21 Kit; E1 — QIAamp MinElute ccfDNA Kit; F5 — Maxwell RSC cfDNA Plasma Kit), obtained using an Agilent TapeStation 2200
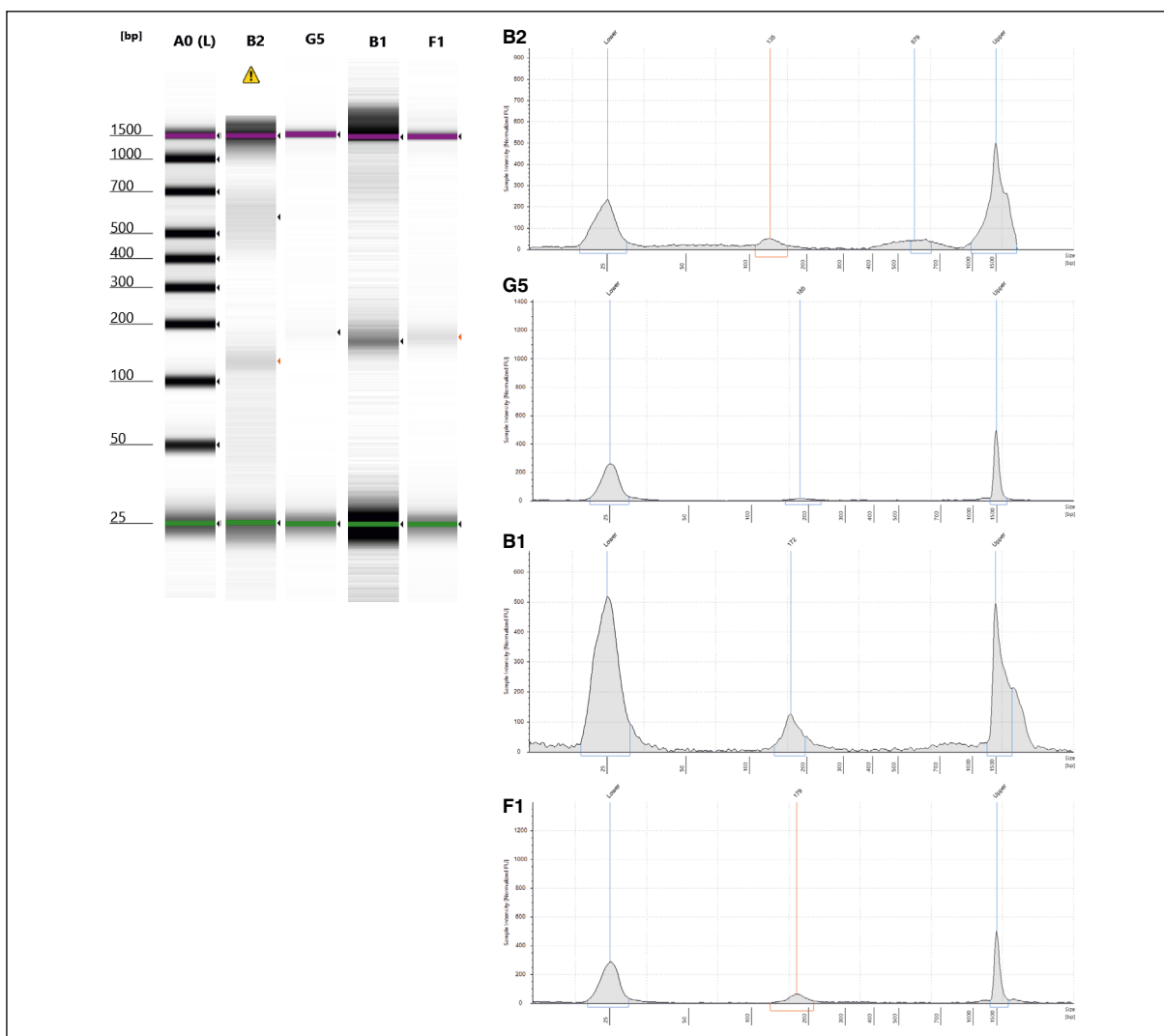
Figure 3. Electrophoresis of cfDNA isolated from sample no. 19 by Agilent 2200 TapeStation 2200: B2-QuickGeneMINI8L; G5- Maxwell RSC cfDNA Plasma Kit; B1 — cfKapture 21 Kit: F1— QIAamp MinElute ccfDNA Kit. The observed bands are described in more detail in Table 3. and Electropherograms of cfDNA isolated from sample no. 19 using the following kits: B2-QuickGeneMINI8L; G5 — Maxwell RSC cfDNA Plasma Kit; B1 — cfKapture 21 Kit: F1 — QIAamp MinElute ccfDNA Kit (TapeStation 2200, Agilent). The efficacy of the isolation method is determined by cfDNA concentration related to cfDNA fragment size. The horizontal axis and vertical axis represent the product size and peak height of DNA concentration, respectively

predominantly of hematopoietic origin, with cfDNA from non-hematopoietic cells being shorter [19, 20].

This preliminary study is intended as a segue to further analyses of isolated ctDNA. Although the approach is expensive, it has established a firm foundation for liquid biopsy (LB) as a minimally-invasive diagnostic tool with strong potential to predict clinically-important changes. Analyses performed on genetic material isolated from neoplastic cells are highly accurate and have a low false-negative rate. The presence of ctDNA can be indicative of even very low levels of potent oncogenic cells.

Our findings may play a significant role in opening this area of study. Previous studies have addressed the standardization of ctDNA measurement and the further improvement of cfDNA extraction kits, and have attempted to simplify longitudinal monitoring. The findings have yielded clinically-significant information regarding the monitoring of ctDNA quantity and accessibility.

In addition to comparing cfDNA isolation kits, the present study also compares the suitability of plasma and serum as bases for ctDNA quantification. While ctDNA is typically quantified by digital PCR (dPCR) specific for the mutations detected in the tumor tissues [9], am-

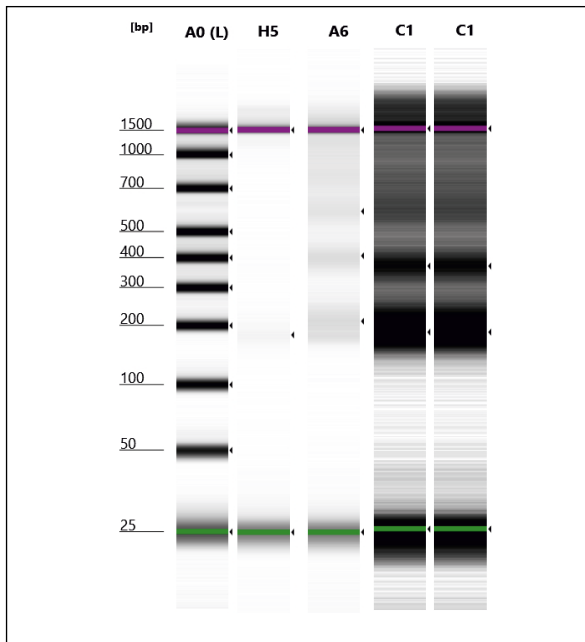plification refractory mutation systems (ARMSs), droplet digital PCR (ddPCR), and next-generation sequencing (NGS) can also be used [11]. In our analysis, cfDNA from the peripheral blood was enriched by size-based separation: a procedure that concentrates the proportion of ctDNA within a sample based on fragment size [15]. By following this approach, a significantly higher proportion of tumor ctDNA is detected by primers that target amplicons shorter than 100 bp and is directly correlated with the increase of ctDNA concentration; in addition, while ctDNA fragments longer than 10,000 bp are likely to originate from necrotic cells, those shorter than 1000 bp, particularly 180 bp or multiples of this size, tend to be from apoptotic cells [12–14].

The amount of cfDNA and the length of ctDNA fragments can be used to predict progression-free survival and overall survival in patients with breast cancer. It has been demonstrated that both cfDNA fragment size and high cfDNA levels pre-treatment are associated with shorter progression-free survival and overall survival; in addition, pre-treatment cfDNA levels could independently predict prognosis for both progression-free survival and overall survival [21, 22]. Studies of metastatic melanoma treated with immune checkpoint inhibitors found lower ctDNA concentration at baseline to be associated with shorter progression-free survival, and for the concentration to increase with tumor burden during treatment [23]. Although the amount of isolated cfDNA depends on the efficiency of the extraction method, a comparable influence is exerted by pre-analytical factors such as type of blood collection tube, centrifugation speed and storage temperature. Pre-analytical variables can also influence the release of non-mutated DNA from leucocytes, resulting in the dilution of the ctDNA fraction [24].

Figure 4. Electrophoresis of cfDNA isolated from sample no. 19 by Agilent 2200 TapeStation 2200. The following cfDNA were assumed to be ctDNA: 184 bp in plasma (H5) and 211bp in serum (A6) for the Maxwell RSC cfDNA Plasma Kit, and 185 bp in serum (C1) for the cfKapture 21 Kit. The observed bands are described in more detail in Table 4

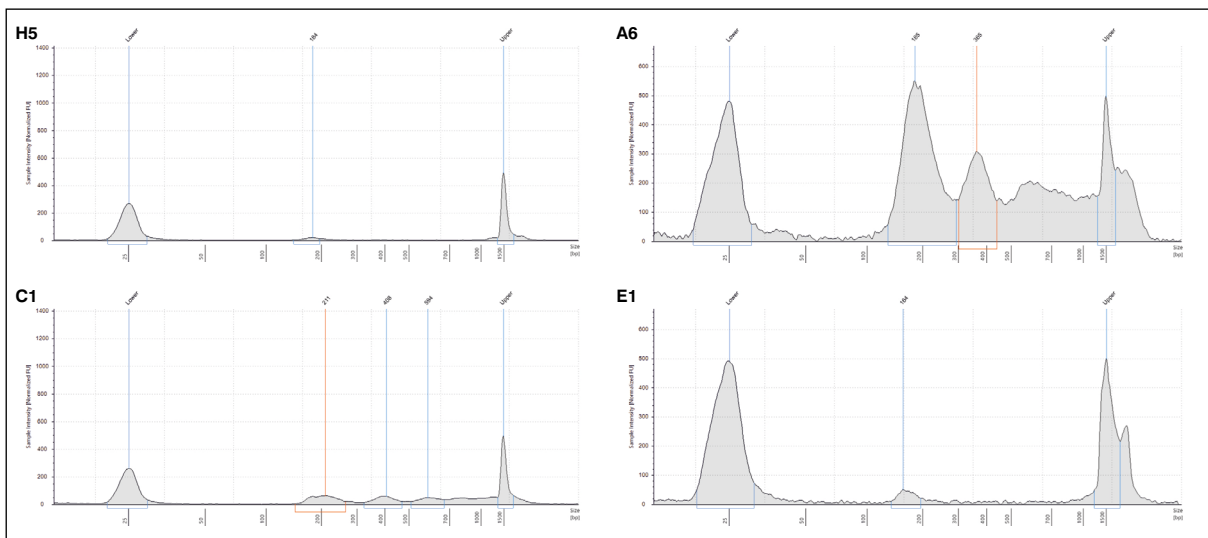**Figure 5.** Electropherograms for cfDNA isolation products using the Maxwell RSC cfDNA Plasma Kit (H5 — Plasma, A6 — Serum), and cfKapture 21 Kit (C1 — Serum, E1 — Plasma) (TapeStation 2200). The efficacy of the isolation method is reflected by cfDNA concentration related to cfDNA fragment size. The horizontal axis and vertical axis represent product size and the peak height of DNA concentration, respectively

Table 3. Sample placement on the gel (Fig. 1, 3, 5) and a comparison of DNA concentration and fragment sizes

Well number	Sample number	The name of the cfDNA isolation kit used	DNA concentration [pg/μL]	Fragment size [bp]
A2	3	QuickGeneMINI8L (Kurabo)	26.8	N/A
D1	3	QIAamp MinElute ccfDNA Kit (Qiagen)	59.8	181
A1	17	CfKapture 21 Kit (MagBio)	47.7	167
E1	17	QIAamp MinElute ccfDNA Kit (Qiagen)	107.0	177
F5	17	Maxwell RSC cfDNA Plasma Kit (Promega)	32.8	185
B2	19	QuickGeneMINI8L (Kurabo)	29.9	135
G5	19	Maxwell RSC cfDNA Plasma Kit (Promega)	24.7	185
B1	19	CfKapture 21 Kit (MagBio)	85.9	172
F1	19	QIAamp MinElute ccfDNA Kit (Qiagen)	93.9	179

Table 4. Sample placement on the gel (Fig. 7) and a comparison of DNA concentration and fragment sizes

Well number	Sample type	The name of the cfDNA isolation kit used	DNA Concentration [pg/μL]	Fragment size [bp]
H5	Plasma	Maxwell® RSC cfDNA Plasma Kit	25.4	184
A6	Serum		105.0 104.0 89.6	211 408 594
C1	Serum	cfKapture™ 21 Kit	439.0 378.0	185 365
E1	Plasma		30.7	164

The kits used in the present study differ with regard to the amount of starting material required. cfDNA has a short half-life of less than two hours and is cleared through the liver and kidneys; hence, failure of these organs can affect cfDNA clearance in cancer patients [25, 26]. Changes in the numbers of chromosomal regions have been detected in plasma tumor-specific cfDNA as copy number aberrations, and these can be used to compute a genomic copy number instability score [27]. The use of cfDNA offers greater clarity regarding tumor information than circulating (CTC) tumor cell analysis [28, 29]. It is important to note that the mass of the detected tumors would typically range from 0.1 g to 1 g, small enough to be present in asymptomatic individuals [30].

The quantity of cfDNA that can be extracted from plasma can vary depending on the efficiency of the chosen extraction method [30]. In the present study, cfDNA levels were established in treatment-naïve samples. Recently, cfDNA analysis has allowed a non-invasive method for the identification of resistance mutations selective for treatment method. cfDNA mutation may well serve as an early predictor of response to standard chemotherapy, and the presence of immune checkpoint inhibitors (immunotherapy) such as PD-1, PDL-1, LAG3, TIMI-3.

Conclusion

Malignant neoplasms release significant amounts of ctDNA into the circulatory system as a result of necrosis and apoptosis. Most of these fragments are in the range of 160–180 bp. These ctDNA constitutes over 90% of total circulating cfDNA. However, the amount of isolated cfDNA ranges from 10 to 100 ng/mL. It is therefore important to develop an efficient isolation method. The present study compares the ability of selected kits to isolate total cfDNA, with the aim of eventually developing a method for detecting ctDNA in patients at an early stage of cancer.

The study group included a small number of patients differing in terms of age distribution, histological type of breast cancer, estrogen receptor (ER) and progesterone receptor (PR) expression status, as well as axillary lymph node metastasis and type of surgery performed: i.e. BCT vs. mastectomy. Nevertheless, the group was homogenous with respect to histological malignancy grade, this being G2, T1 in the TNM-staging system: tumor diameter 1–2 cm, no distant metastases(M0), HER2(–) negative.

Our study confirms that that cfDNA can be successfully isolated using the following kits: QuickGene-Mini8L

(Kurabo, Japan), Catch-cfDNASerum/Plasma Kit (CatchGene, China), cfKapture 21 Kit (200–400 μL) (MagBio Genomics, USA), Maxwell RSC ccfDNA Plasma Kit (Promega, USA) and QIAamp MinElute ccfDNA (Qiagen, Germany). Of these, the greatest efficiency was demonstrated by the QIAamp MinElute ccfDNA Kit (Qiagen) for samples 3 and 17, and cfKapture 21 for sample 19.

The efficiency of cfDNA isolation from plasma and serum samples were compared by the use of the Maxwell RSC cfDNA Plasma Kit and the cfKapture 21 Kit. A high level of genomic DNA contamination was observed in the serum samples, as evidenced by the presence of additional isolation fragments with more than 180 bp, while a single peak characteristic of cfDNA was observed in the plasma.

Conflict of interest

The authors declared that they have no conflict of interest.

Research involving Human Participants. Informed Consent was obtained.

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