Original Article

Circulating Cell-Free DNA Extraction from Liquid Biopsy for Cancer Research

(biomarker / cancer / cell-free DNA / circulating tumour DNA / liquid biopsy / oncology / precision)

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Abstract. As the number of cancer patients globally increases, a need for reliable biomarkers including circulating tumour DNA from liquid biopsy for diagnosis, prognosis and monitoring of the disease is rising. Currently, mainly tissue samples from biopsy are used, but there are certain limitations: firstly, it is an invasive technique, and secondly, in some cases it is almost impossible to obtain an acceptable tissue sample. This could be changed by using circulating cell-free DNA from liquid biopsy, which also gives the possibility of repeated examination. Here, we focus on the options of isolating circulating cell-free DNA from plasma samples using two isolation techniques: precision manual QIAamp Circulating Nucleic Acid Kit and automatic MagNA Pure Compact (MPC) using Nucleic Acid Isolation Kit I. Manual extraction gave significantly better yields of circulating tumour DNA ($P < 0.05$). This DNA also had less contaminants (organic compounds or proteins). DNA obtained by both tested methods of isolation is suitable for subsequent molecular genetic methods.

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Abbreviations: cfDNA – cell-free DNA, ctDNA – circulating tumour DNA, TMB – tumour mutation burden.

Introduction

Liquid biopsy is a relatively novel technique that offers new possibilities, specifically in cancer diagnostics and therapy. It can be used for detecting diagnostically and prognostically significant markers, such as exosomes, microRNAs, circulating tumour cells and circulating cell-free DNA from various body fluids, including blood, serum, plasma, saline, urine and cerebrospinal fluid (Kustanovich et al., 2019; Kilgour et al., 2020; Michela, 2021). Compared to traditional tissue biopsy, it has obvious advantages: it is a minimally invasive technique and easily repeatable; it requires much less sample preparation and retrieval time; it enables real-time monitoring of changes at the molecular level in patients; it allows examination in cases where the traditional tissue biopsy is not possible or is too risky, as well as in cases where there is just a small amount of material obtained by classical biopsy not enabling all suitable examinations.

One of the most promising biomarkers for liquid biopsy in cancer diagnosis, prognosis and monitoring is circulating cell-free DNA (cfDNA) in the blood. cfDNA is single- or double-stranded extracellular DNA released through a combination of apoptosis, necrosis and secretion (Johann et al., 2018). In healthy individuals, cfDNA levels are usually low, ranging from 0 to 100 ng/ml. Higher levels can indicate pathological conditions, such as inflammation, autoimmune diseases or cellular stress. In patients with malignancies, the concentration of cfDNA varies significantly, but increased levels are detected at advanced stages. Depending on the type of cancer and stage of the disease, the cfDNA level can be 1- to 10-fold higher (Gedvilaitė et al., 2017; Kilgour et al., 2020).

Part of the cfDNA in cancer patients consists of cancer-derived circulating tumour DNA (ctDNA) contain-

ing tumour-related genetic and epigenetic changes. Such genetic changes could be used to identify targeted cancer treatment (Pessoa et al., 2020). Since ctDNA is released from multiple tumour regions, cfDNA analysis can provide a broader picture of the patient's disease and prognosis. However, the potential usage of cfDNA is affected by the tumour type, disease development, and choice and volume of biological material (Lee et al., 2020).

Recent studies have shown the possible use of cfDNA in determination of the tumour mutation burden (TMB) (Yeo and Lim, 2018; Addeo and Weiss., 2019; Ghosh et al., 2019). TMB, the approximate amount of somatic gene mutations in tumour cells, is considered to be a promising predictive biomarker for immunotherapy. These studies (Yeo and Lim, 2018; Addeo and Weiss, 2019; Ghosh et al., 2019) were strictly based on the use of plasma and standard manual isolation of cfDNA. However, the possibilities of automation as the key to enabling wider use of the method were not considered.

Here, we compare the isolation yields of cfDNA obtained from low-volume samples of plasma from cancer patients by using two isolation methods: precision manual QIAamp Circulating Nucleic Acid Kit and automatic MagNA Pure Compact (MPC) Nucleic Acid Isolation Kit I.

Material and Methods

Study design

Two methods, manual isolation using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) with a QIAvac Vacuum System (Qiagen, Hilden, Germany) and a MagNA Pure automatic system (Roche Diagnostics, Mannheim, Germany) using a Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany), were compared, and the impact of differing isolation on the quality and quantity of extracted ctDNA was tested.

Samples

The study was performed with the approval of the Ethics Committee of General University Hospital, Prague (No. 1042/19 S-IV, 1.8.2019) and was conducted in accordance with the Declaration of Helsinki. All enrolled patients have signed written informed consent with participation.

Anonymized blood samples were collected from each of 20 patients with oncological diagnosis (breast cancer (6 patients), pancreas cancer (1 patient), lung cancer (3 patients), colorectal cancer (9 patients, 3 of them had liver metastases) and gastric cancer (1 patient)); 11 men, mean age 66 ± 10 years, and 9 women, mean age 59 ± 11 years, into tubes containing ethylenediaminetetraacetic acid (EDTA).

ctDNA isolation

To obtain plasma, each blood sample was centrifuged (900 × *g*) for 15 minutes at laboratory temperature and almost all the plasma was moved into a new tube. These samples of plasma were promptly stored at −80 °C until the second centrifugation and ctDNA extraction were performed. The plasma samples underwent the second centrifugation (16 000 × *g* for 15 minutes at 4 °C) just before the ctDNA isolation.

Both kits, QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) with a QIAvac 24 plus system (Qiagen, Hilden, Germany) and MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume (Roche Diagnostics, Mannheim, Germany) with a MagNA Pure Compact Instrument (Roche Diagnostics, Mannheim, Germany), were used according to manufacturer's instructions.

To allow comparison of both methods, for each sample, 1 ml of plasma was used and 50 µl of extracted genomic DNA product was obtained.

Measurement of DNA quantity and quality

The extracted DNA was evaluated by measuring its concentration and purity spectrophotometrically using a NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). The ratio of absorbance at 260 nm and 280 nm was used to define the DNA purity, and the value in the range 1.8–2.0 was considered to represent pure DNA as it demonstrates good deproteinization. The ratio of absorbance at 260 nm and 230 nm was used as an auxiliary parameter of DNA purity. The second concentration measurement (fluorometrically) was performed using a dsDNA HS Assay Kit with a Qubit fluorometer (Invitrogen, Waltham, MA).

The quality analysis and ctDNA concentration measurement was performed using High Sensitivity D1000 Screen Tape and Reagents in bioanalyser TapeStation 4200 (Agilent, Santa Clara, CA) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the open-source R statistical software v 4.1.10 distributed under the GNU General Public License (Copyright 2007 Free Software Foundation, Inc., http://www.gnu.org/licenses/gpl.html) and the statistical program STATISTICA 12 (Statsoft CR s.r.o., Prague, Czech Republic). Descriptive statistics of numerical variables are presented in the form of mean ± SD (standard deviation). Data comparison was performed using Wilcoxon matched-pairs signed-ranks test and Kruskal-Wallis test. Correlation analyses were performed using Pearson correlation coefficient. $P < 0.05$ was considered as significant.

Results

Firstly, the data from all samples were analysed together. The 260/280 spectrophotometric absorbance ra-

Table 1. Comparison of ctDNA isolation methods – manual and automated
Manual isolation was performed using a QIAamp Circulating Nucleic Acid Kit with a QIAvac Vacuum System and automated isolation using a MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume with a MagNA Pure Compact Instrument.

	Manual isolation	**Automated isolation**
260/280 nm ratio spectrophotometric measurement	0.37 ± 0.07	0.33 ± 0.02
260/230 nm ratio* spectrophotometric measurement	2.30 ± 0.30	1.22 ± 0.05
Mean yield (ng per 1 ml)* spectrophotometric measurement	21 800 ± 3 548.2	24 900 ± 5 665.2
Range of yield (ng per 1 ml) spectrophotometric measurement	17 000–30 000	15 000–41 000
Mean yield (ng per 1 ml)* fluorometric measurement	675.00 ± 372.80	484.80 ± 504.2
Range of yield (ng per 1 ml) fluorometric measurement	268–1750	95–1740
Mean yield (ng per 1 ml)* bioanalyser measurement	107.87 ± 115.70	49.75 ± 36.27
Range of yield (ng per 1 ml) bioanalyser measurement	9.67–454.00	7.40–153.00
Mean length of fragments (bp)	146 ± 26	144 ± 11
Range of lengths of fragments (bp)	115–200	127–166

*P < 0.05. Mean data were analysed using Wilcoxon matched-pairs signed-ranks test and Kruskal-Wallis test. Ranges of data are provided for further information. Bioanalyser measurement data from two samples (breast cancer and lung cancer) were excluded from final statistical analyses due to the technical problems with minimal concentrations.

tios did not differ significantly between automatic and manual isolation (P = 0.08), in contrast to the 260/230 ratios (P < 0.0001). Both spectrophotometric and fluorometric measurements of DNA concentration showed significant differences: spectrophotometric in favour of the automatic method, (P = 0.04) and fluorometric in favour of the manual method (P = 0.03). Data also showed a significant correlation (r = 0.51, P = 0.02) between the automatic and manual method in fluorometric concentration measurements. A significant difference was also found in the ctDNA concentrations measured by the bioanalyser between the compared isolation methods (P = 0.048). The average yield of obtained ctDNA was higher for the manual method than for the automatic method. However, the average lengths of the obtained ctDNA fragments were comparable in both methods (P = 0.98). For overall results see Table 1 and Fig. 1. Bioanalyser measurement data from two samples (breast cancer and lung cancer) were excluded from final statistical analyses due to the technical problems with minimal concentrations.

Subsequently, we were interested in whether there was any difference in the amount and quality of ctDNA among diagnoses. Due to the number of samples, we

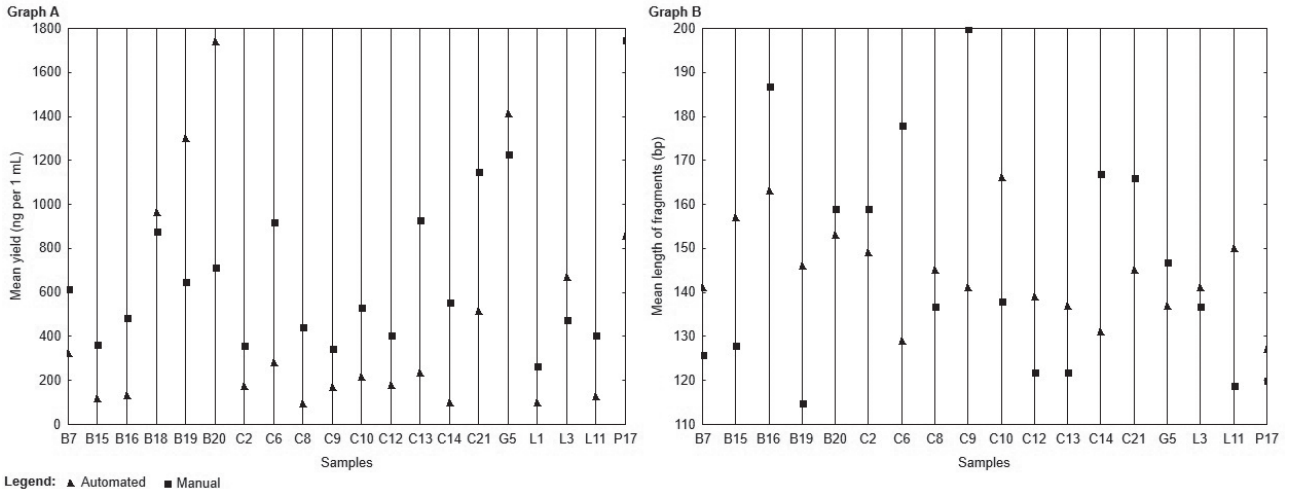

Fig. 1. Mean yield of obtained DNA and mean length of fragments
Graph **A** shows the mean yield of obtained DNA (ng per 1 ml) using fluorometric measurement. Graph **B** shows the mean length of fragments (bp) using bioanalyser measurements. Samples L1 (lung cancer) and B18 (breast cancer) in Graph **B** were excluded due to the technical problems with minimal concentrations. (B = breast cancer, C = colorectal cancer, G = gastric cancer, L = lung cancer, P = pancreas cancer, number = sample ID)

were able to compare only samples from patients with colorectal and breast cancer. Colorectal cancer samples differed significantly ($P < 0.05$) in parameters of fluorometric and spectrophotometric concentrations and both ratios 260/280 nm and 260/230 nm. Data also showed a significant correlation ($r = 0.81$, $P = 0.007$) between the automatic and manual method in colorectal cancer fluorometric concentration measurements. Breast cancer showed statistical significance ($P < 0.05$) in comparison of two parameters: 260/230 nm ratio and ctDNA measurement using the bioanalyser. Bioanalyser measurement data from one sample (breast cancer) were excluded from final statistical analyses due to the technical problems with minimal concentrations.

Finally, the comparison of colorectal and breast cancer showed similar results in the analysed parameters, i.e., the yields, purity ratios and ctDNA length.

Discussion

Liquid biopsy as a source of cfDNA seems to be a promising technique in the care of oncological patients. Many methods for ctDNA isolation exist, e.g., commercial kits for both manual and automated extraction and in-house procedures. All these extraction methods are still being improved in terms of obtained ctDNA quality, quantity and purity as well as in the sense of reducing the input volume of the sample. Thus, there is a lack of consensus on the optimal ctDNA isolation method (Normanno et al., 2017). Generally, serum contains a higher amount of ctDNA than plasma, but this ctDNA tends to be more contaminated with gDNA. Therefore, no clear consensus exists as to whether the serum or plasma is preferable for ctDNA extraction (Taback et al., 2004; Umetani et al., 2006; Pittella-Silva et al., 2020). Here, two isolation methods – manual and automated – were compared in ctDNA extraction from the plasma.

The data showed that manual isolation is more effective – higher yield of extracted DNA ($P < 0.05$) measured fluorometrically using a Qubit fluorometer with a dsDNA HS Assay Kit, which is a more appropriate method than the spectrophotometric method. In contrast, the spectrophotometric measurement showed significantly better yields in favour of automatic isolation; however, this measuring method is less accurate in measuring lower concentrations. Spectrophotometric concentration data can also be affected by different contaminants, e.g., organic compounds or proteins that absorb at 230 nm and/or 280 nm. The 260/280 nm ratio was slightly in favour of manual isolation. The 260/230 nm ratio results were significantly higher and again in favour of manual extraction. Similar results as fluorometric measurement were shown by analysis of ctDNA performed in bioanalyser TapeStation 4200 using High Sensitivity D1000 Screen Tape and Reagents. The mean lengths of ctDNA fragments were almost identical in both techniques; however, larger variability of the fragment length was observed for manual isolation. High deviations in mean values (such as yield), see Table 1, were probably caused by an inhomogeneous group of patient samples.

The results also indicated differences in the analysed parameters between diagnoses and compared methods; here, the colorectal cancer group differed in fluorometric and spectrophotometric concentrations and both ratios (260/280 nm and 260/230 nm). However, due to the small number of samples, this trend cannot be reliably statistically confirmed among the diagnoses included in this study.

The comparison of colorectal and breast cancer in the analysed parameters (yields, purity ratios and ctDNA length) proved similar results.

Conclusion

Although manual isolation gives a higher yield with acceptable purity of DNA, we have shown that automatic isolation is sufficient for the extraction of cfDNA from plasma, even when a small sample volume (1 ml and smaller) is used. Our results clearly indicate that both manual and automated method are suitable for cfDNA isolation from liquid biopsy and its subsequent use for further molecular genetic analyses.

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Contribution of authors

L. P. and M. S. contributed equally to this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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