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ORIGINAL ARTICLE

Blood Collection and Cell-Free DNA Isolation Methods Influence the Sensitivity of Liquid Biopsy Analysis for Colorectal Cancer Detection

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Abstract
During colorectal cancer (CRC) development tumor-derived cell-free DNA (cfDNA) can be released into the bloodstream. Many different cfDNA isolation methods and specific blood collection tubes preventing the release of genomic DNA and stabilizing cfDNA with preservative reagents became available. These factors may affect greatly on the further liquid biopsy analyses. Our aim was to test different blood collection tubes and cfDNA isolation methods to determine whether these factors influence the cfDNA amount and the promoter methylation of four previously described hypermethylated biomarkers. Three manual isolation methods (High Pure Viral Nucleic Acid Large Volume Kit; Epi proColon 2.0 Kit; Quick-cfDNA™ Serum & Plasma Kit) and automated sample preparation systems (InviGenius and InviGenius PLUS) were examined. Furthermore, K3EDTA Vacuette tubes and Streck Cell-Free DNA BCT® tubes were compared. After cfDNA isolation and bisulfite conversion of samples, the methylation level of *SFRP1*, *SFRP2*, *SDC2*, and *PRIMA1* were defined with MethyLight assays. We have ascertained that there are differences between the cfDNA amounts depending on the isolation methods. Higher cfDNA yield was observed using InviGenius system than column-based manual isolation method; however, InviGenius PLUS has produced lower cfDNA amounts. No remarkable variance could be found between K3EDTA and Streck tubes; slightly higher cfDNA quantity was detected in 60% of plasma samples using Streck tubes. In point of methylation level and frequency, manual column-based isolation produced more consistent results. Automated cfDNA extraction systems are easy-to-use and high-throughput; however, further improvements in the isolation protocols might lead to the increase of the sensitivity of further methylation analysis.

Keywords Liquid biopsy · Plasma · cfDNA isolation · Blood collection · Colorectal cancer · DNA methylation

Introduction

Colorectal cancer (CRC) is among the leading causes of cancer-related deaths in both men and women worldwide and over 1.3 million new cases were diagnosed in 2012. CRC shows wide geographical variation in incidence across the world and occurs in almost 60% of the cases in developed regions [1]. To reduce mortality, it is necessary to recognize and treat precancerous stages as early as possible. Visual approaches such as colonoscopy, flexible sigmoidoscopy or virtual colonoscopy are the most commonly used methods for the detection of CRC; however, these procedures have high costs, are invasive and uncomfortable for patients. The participation in screening could be increased by using non-invasive methods including identification of blood and stool-based biomarkers [2–4].

CRC develops as a result of the appearance of genetic and epigenetic alterations. During tumor formation, a predictable sequential accumulation of mutations is observed (e.g. in *APC, KRAS,* and *P53* genes). Moreover, epigenetic instability, primarily aberrant DNA methylation also seems to be a common phenomenon in CRC [5]. Cancer-linked DNA hypermethylation has been occurred most often in CG dinucleotides in the promoter regions of target genes influencing gene expression and contributing

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to cancer development [6, 7]. Various methods are available for the analysis of methylation pattern of specific genes. Most of these methods start with bisulfite conversion of genomic DNA, followed by pyrosequencing, PCR combined with high resolution melting or methylation-specific PCR (MSP) [8]. The other group of DNA methylation analysis techniques is based on the selective digestion of DNA by restriction enzymes, coupled with quantitative PCR. The methylation pattern of genes could be examined in different sample types, such as formalin-fixed paraffin-embedded (FFPE) or fresh frozen tissues, but the most easily available sources are body fluids including blood and urine, or stool [9, 10]. Analysis of the methylation status of circulating cell-free DNA (cfDNA) in plasma samples provides a good opportunity for cancer detection. CfDNA can be derived from tumor cells through apoptosis, necrosis, or direct secretion by active manner, and DNA methylation, mutations or microsatellite alterations can be detected in it [11]. Results about the size of cfDNA are quite diverse, but electrophoretic studies indicate that it varies between 180 bp and 10,000 bp [12]. The half-life time of cfDNA is about 16 minutes, and it can be complexed with cellular or non-cellular components, e.g. with glycoprotein increasing the stability and can act as a signaling molecule between different cells and tissues [13, 14]. The quantity of cfDNA in healthy individuals is in a low range (1.8–44 ng/ml), but the concentration is elevated in cancer patients and in the case of other conditions, such as physical activity or during the first trimester of pregnancy [15]. The level of cfDNA is influenced by cancer-dependent factors, e.g. tumor size, stage or location [14]. CfDNA amount in the circulatory system also depends on the activity of DNase enzymes and it has been shown that the increased cfDNA concentration in cancers can be related to decreased DNase I enzyme activity [16–18]. To enhance the stability of cfDNA, new types of blood collection tubes have become available, containing preservative reagents to inhibit the degradation of cfDNA and the release of genomic DNA from the peripheral white blood cell compartment. Moreover, there are significant differences in the exact quantities of cfDNA between studies. The amount of purified cfDNA is greatly influenced by the isolation methods as several diverse DNA extraction methods are commercially available such as magnetic beads or silica-gel membrane technology [19]. In the case of large sample size, the duration of cfDNA isolation can be reduced by the use of automated cfDNA extraction methods. These features suggesting that the quantitative measurement of cfDNA due to the large variability is not the suitable marker for cancer detection; however, the analysis of the qualitative, tumor-specific changes of cfDNA can be an appropriate method for tumor prescreening.

Several CRC-specific mutation and methylation markers have been already reported [20]. In our previously published study, we analyzed the methylation pattern of *SFRP1, SFRP2, SDC2*, and *PRIMA1* genes in 121 plasma and 32 biopsy samples of healthy, adenoma and cancer patients using MethyLight PCR method, and further 45 tissue samples by pyrosequencing [21]. We demonstrated increased methylation levels of the selected markers in adenoma and cancer patients compared to normal controls, and our observations were confirmed on a large independent set of tissue samples with in silico analysis. With MethyLight PCR based on the altered methylation profile of our biomarker panel, we are able to discriminate CRC and also precancerous adenoma samples from normals with high specificity and sensitivity using both plasma and tissue specimens. Recently, new cfDNA isolation techniques and blood collection tubes have become available, which promise more stable and higher yield of cfDNA fraction. However, limited information is presented about the effect of these new procedures on the methylation pattern of cfDNA. Therefore, in the present study, we aimed to compare different sample preparation and cfDNA isolation methods to determine whether these factors influence the amount of cfDNA and the methylation level of the four analyzed markers. Besides three different manual isolation methods, automated sample preparation techniques were also performed. Moreover, two types of blood collection tubes were compared: standard K3EDTA Vacuette tubes (Greiner Bio-One Gmbh) and Cell-Free DNA BCT® (Streck) tubes containing stabilization reagents.

Methods

Patients and Sample Collection

A total of 139 blood samples were collected in the 2nd Department of Internal Medicine, Semmelweis University, Budapest, Hungary after all patients underwent a screening colonoscopy. Before sample collection, written informed consent was obtained from all patients. The study was approved by the local ethics committee and government authorities (Regional and Institutional Committee of Science and Research Ethics; TUKEB Nr: 116/2008). Five-five blood samples were obtained from patients with colorectal adenoma (AD) and cancer (CRC) in Cell-Free DNA BCT® (Streck) collection tubes and blood specimens were stored for 48 hours at room temperature before plasma separation. Cell-Free DNA BCT tube [22] was developed for the preservation of cfDNA stability, which makes the cfDNA to remain stable for up to 14 days before plasma separation at room temperature. This collection tube contains formaldehyde-free preservative reagent that inhibits the cfDNA degradation mediated by nucleases and prevents

the release of cellular genomic DNA. All the other blood samples were drawn in K3EDTA Vacuette tubes and plasma fraction was separated within 4 hours. All plasma separation was performed by double centrifugation at 1350 rcf for 12 min and was stored at −20 °C until use.

Cell-Free DNA Isolation and Bisulfite Conversion Methods

Three different manual and two types of automated cfDNA isolation methods were tested. As the first step, High Pure Viral Nucleic Acid Large Volume Kit (*HP*) (Roche Applied Science) was used for the cfDNA isolation of healthy ($n = 10$), adenoma (n = 10) and CRC (n = 10) plasma samples, and the results were compared to Epi proColon 2.0 (*EpC*) (Epigenomics AG) manual, commercially available cfDNA isolation, and bisulfite conversion kit. Next step was the comparison of High Pure Viral Nucleic Acid Large Volume Kit and InviGenius (*I*) (STRATEC Biomedical AG) system using Epi proColon 2.0 kit on plasma samples of 27 normal, 25 AD and 17 CRC patients. Moreover, we tested Quick-cfDNA™ Serum & Plasma Kit (*QcD*) (Zymo Research) and compared to InviGenius PLUS (*IP*) (STRATEC Biomedical AG) using InviMag Free Circulating DNA Kit (*IM*) (STRATEC Biomedical AG) on 10 normal, 10 AD and 10 CRC plasma specimens. InviGenius and InviGenius PLUS are automated DNA sample preparation systems, with the ability to extract and purify cfDNA from 12 plasma samples in parallel, and bisulfite conversion can also be performed with Epi proColon 2.0. As the final step, we tried out the Cell-Free DNA BCT® (Streck) tubes in comparison to K3EDTA Vacuette tubes using High Pure Viral Nucleic Acid Large Volume Kit on 5–5 adenoma and CRC samples. The proteinase K digestion time was extended to 1 hour at 70 °C in the case of Cell-Free DNA BCT® collected samples according to the latest instructions of the manufacturer (Fig. 1).

The initial plasma volume was 3.5 ml, except for the comparison of Quick-cfDNA™ Serum & Plasma Kit (Zymo Research) and InviMag Free Circulating DNA Kit (STRATEC Biomedical AG), where 4 ml plasma samples were used. Following the cfDNA isolation with Quick-cfDNA™ Serum & Plasma Kit and High Pure Viral Nucleic Acid Large Volume Kit, the bisulfite conversion of plasma DNA was made using EZ DNA Methylation Direct Kit (Zymo Research). CfDNA isolation and bisulfite conversion steps were performed according to the instructions of the manufacturers in all cases. The amount of cfDNA was quantified with Qubit 1.0 fluorometer using Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Bisulfite-converted DNA was eluted in 15 μl elution buffer and was used immediately or stored at −80 °C in aliquots.

Multiplex Preamplification and MethyLight Assay

After bisulfite conversion of all samples, multiplex bisulfite-specific preamplification and MethyLight PCR were carried out to determine the methylation pattern of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* promoters. Using automated isolation methods, the volume of eluted bisulfite-converted DNA was variable; therefore the different quantities were concentrated to 15 μl for multiplex preamplification using Eppendorf Concentrator 5301 (Eppendorf AG). The reaction volume of preamplification was 30 μl containing Multiplex PCR Master Mix (2×) (Qiagen), the mixture of four bisulfite-specific primers (each 10 μM) and the bisulfite-modified DNA. EpiTect Methylated and Unmethylated Controls (Qiagen) were amplified in parallel with the samples to determine the methylation level of plasma samples. The preamplified DNA samples were diluted in 1:10,000 in RNase- and DNase-free water, and were stored at −20 °C until use. For MethyLight assay, 5 μl diluted DNA was utilized, and the reaction contained 10 μl LightCycler® 480 Probes Master (2×) (Roche Applied Science), 1.8 μl of each primer (10 μM) and 0.5 μl MGB TaqMan Probes (ThermoFisher Scientific) in 250 nM final concentration. The detailed protocols of preamplification and MethyLight analysis were previously described by our research group [21].

Statistical Analysis

In order to define the methylation values of plasma samples subjected to all cfDNA isolation methods, linear derivation formula was used from dilution series of each potential biomarker after MethyLight PCR. Pairwise comparisons (AD vs. N, CRC vs. N and CRC vs. AD) were applied using Student's t-test with the significance criterion $p < 0.05$.

Results

Comparison of Two Manual cfDNA Isolation Methods: High Pure Viral Nucleic Acid Large Volume Kit vs. Epi proColon 2.0

The cfDNA concentration and the methylation level of *SFRP1, SFRP2, SDC2*, and *PRIMA1* were analyzed in 10 normal, 10 adenoma and 10 CRC plasma specimens using two types of isolation and bisulfite-conversion kits. The protocol of Epi proColon 2.0 does not contain concentration measurement step, as the DNA isolation directly followed by bisulfite conversion, thus the amount of

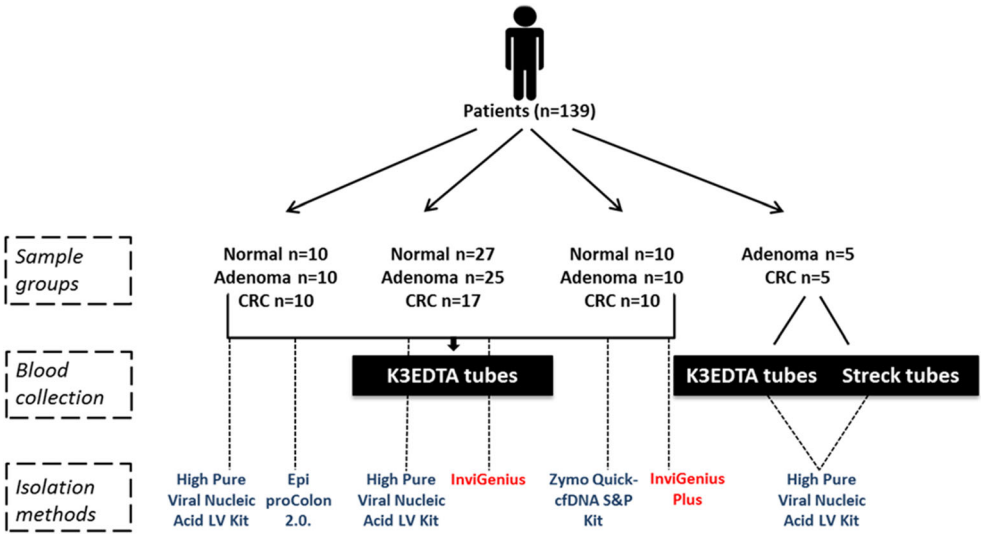

Fig. 1 Experimental design. Blood samples were collected in K3EDTA and Streck Cell-Free DNA BCT® tubes. After plasma separation 3 manual (High Pure Viral Nucleic Acid Large Volume Kit; Epi proColon 2.0 Kit; Quick-cfDNA™ Serum & Plasma Kit) and 2 automated (InviGenius; InviGenius PLUS) cfDNA isolation methods were tested

cfDNA was determined from samples isolated with High Pure Viral Nucleic Acid Large Volume Kit. The average quantities of cfDNA were found to be 16.7 ± 4.76 ng, 49.23 ± 13.03 ng and 69.64 ± 74.47 ng of healthy control, AD and CRC samples, respectively. The methylation levels of the four markers were above 0.1% in 10–30% of healthy, 50–100% of adenoma and 70–90% of CRC samples using HP isolation method. In the case of EpC isolation, we found lower methylation frequencies in almost all cases (Table 1/A). The average methylation percentages of the markers were higher in all CRC samples using HP isolation (Table 1/B).

Comparison of Manual and Automated cfDNA Isolation Methods: High Pure Viral Nucleic Acid Large Volume Kit vs. InviGenius

Automated isolation method was tested using InviGenius system and was compared to HP manual DNA extraction method on 27 normal, 25 AD and 17 CRC plasma samples. The amount of cfDNA was slightly higher using InviGenius in all sample groups (Fig. 2a). Continuous increase could be observed along adenoma-carcinoma sequence, and the highest cfDNA concentration was found in late (Dukes C, D) cancer stages using automated isolation. Table 2 shows the frequency of DNA methylation in the different samples groups, we found quite diverse results comparing the two methods. In the case of plasma from adenoma patients, *SFRP2* and *SDC2* gene promoters were found to be methylated in more plasma samples using manual isolation method than the InviGenius system. However, the methylation frequencies of *SFRP1* and *PRIMA1* were higher after using the automated isolation. Analyzing CRC samples, 3 genes (*SFRP2, SDC2*, and *PRIMA1*) showed aberrant DNA methylation with higher frequency using HP DNA extraction. The average methylation level in healthy controls was lower than 1% in all groups except for the *PRIMA1* gene promoter after InviGenius isolation. In adenoma samples, *SFRP1*, *SFRP2*, and *SDC2* presented raised methylation level with HP isolation, than the InviGenius system. In the case of plasma from CRC patients, all four genes showed elevated methylation level using manual DNA extraction (24.6%, 6.67%, 16.37%

Table 1 Methylation frequency (A) and average methylation (B) of *SFRP1*, *SFRP2*, *SDC2,* and *PRIMA1* using manual High Pure Viral Nucleic Acid Large Volume Kit (HP) and Epi proColon 2.0 (EpC) kit in normal, adenoma and colorectal cancer (CRC) samples

A	Methylation frequency (%)	
	HP	EpC
	SFRP1	
Normal	30	30
Adenoma	100	70
CRC	90	90
	SFRP2	
Normal	10	0
Adenoma	50	10
CRC	90	30
	SDC2	
Normal	20	10
Adenoma	90	30
CRC	80	50
	PRIMA1	
Normal	30	40
Adenoma	100	90
CRC	70	80
B	Average methylation (%)	
CRC	HP	EpC
SFRP1	5.5	2.4
SFRP2	1.4	0.4
SDC2	4.5	0.8
PRIMA1	22.8	14.6

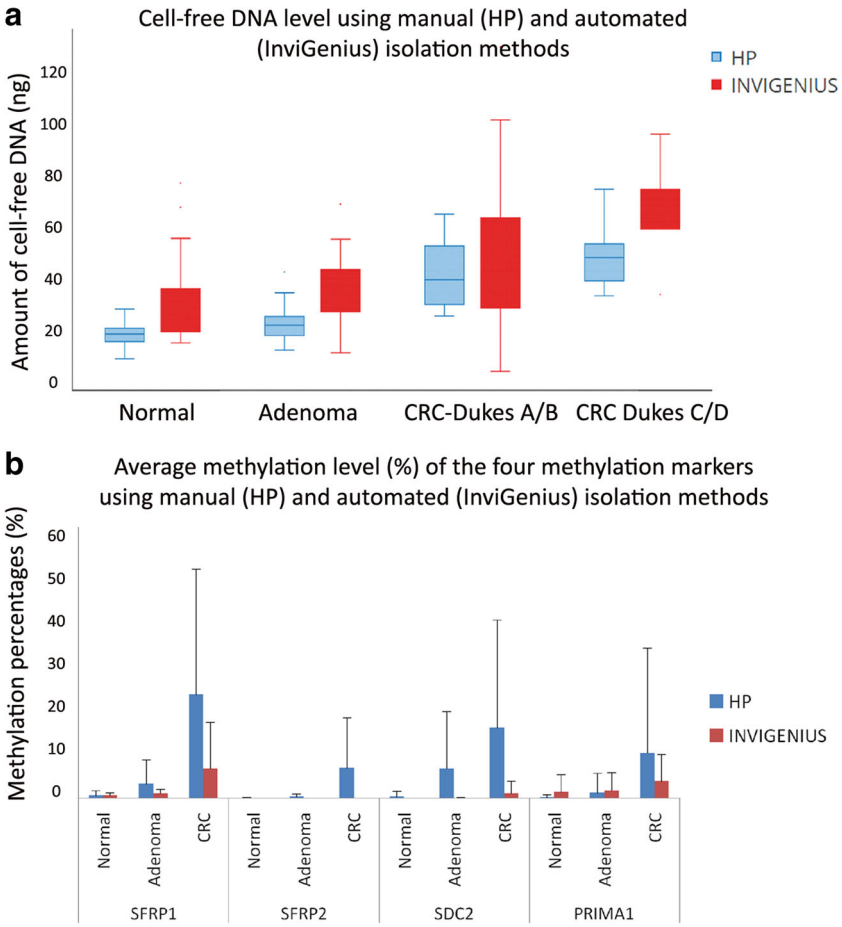

Fig. 2 CfDNA yield and DNA methylation level of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* in plasma samples isolated with High Pure Viral Nucleic Acid Large Volume Kit (*HP*) manually and with InviGenius automated cfDNA extraction system

and 9.91%) than the automated method (6.55%, 0.02%, 1.03% and 3.66%) (Fig. 2b).

Table 2 Methylation frequencies of *SFRP1*, *SFRP2*, *SDC2*, and *PRIMA1* using manual High Pure Viral Nucleic Acid Large Volume Kit (HP) and InviGenius automated cfDNA isolation system in plasma of normal, adenoma and colorectal cancer (CRC) patients

	Methylation frequency (%)	
	HP	InviGenius
	SFRP1	
Normal	48	78
Adenoma	72	88
CRC	76	82
	SFRP2	
Normal	30	4
Adenoma	52	0
CRC	94	6
	SDC2	
Normal	11	4
Adenoma	40	8
CRC	76	35
	PRIMA1	
Normal	4	15
Adenoma	16	24
CRC	53	47

Comparison of Two Large Plasma Volume Optimized Kits: Quick-cfDNA™ Serum & Plasma Kit vs. InviGenius PLUS

The new version of automated cfDNA extraction system, InviGenius PLUS was compared to a column-based manual isolation method. The starting plasma volume was 4 ml in both cases, and cfDNA concentration was determined. Higher cfDNA quantity was observed in all samples groups with the use of Quick-cfDNA™ Serum & Plasma Kit. The highest cfDNA level was detected in adenoma samples, and interestingly, CRC samples showed quite low cfDNA amount with both methods (Fig. 3a). The average methylation level of the four markers in normal samples was lower using manual isolation than automated extraction method (Fig. 3b). Analyzing adenoma samples we found that *SFRP1*, *SDC2*, and *PRIMA1* markers showed elevated methylation level isolated with QcD kit compared to the automated system. In CRC samples a similar tendency could be observed except for *SFRP1* gene, which presented higher average methylation percentage in case of InviGenius PLUS. *SFRP2* gene indicated very low methylation level in all sample groups after both isolation methods.

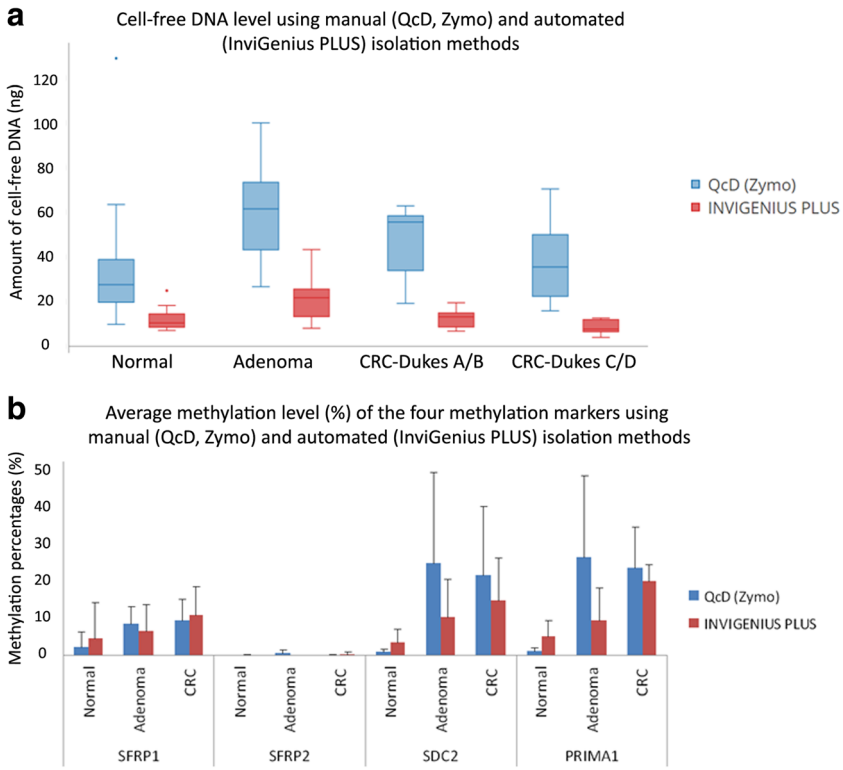

Fig. 3 CfDNA yield and DNA methylation level of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* in plasma samples isolated with Zymo Quick-cfDNA™ Serum & Plasma Kit (*QcD*) manually and with InviGenius PLUS automated cfDNA extraction system

Blood Collection Tube Comparison: K3EDTA Tubes vs. Cell-Free DNA BCT®

According to our observations, High Pure Viral Nucleic Acid Large Volume Kit was proven to be the most reliable isolation kit in term of cfDNA yield and methylation levels. In order to increase the efficiency even more, Cell-Free DNA BCT® (Streck) tube was tested in comparison to K3EDTA tube, what promises to preserve the stability of plasma DNA. In the case of K3EDTA collection tubes, plasma separation was done within 4 hours; in contrast, using Streck tubes, the plasma separation was performed after 48 hours. In terms of cfDNA level, similar quantities were found after the two different sample collection methods (Fig. 4a), there was no significant difference between the groups ($p = 0.86$). The average methylation levels of the markers in adenoma samples did not show substantial differences between the groups. However, higher methylation of all markers was observed in the plasma from CRC patients collected in K3EDTA tubes, and the differences were significant in the case of *SFRP1* and *SDC2* ($p < 0.05$) (Fig. 4b).

Discussion

Several studies demonstrated that tumor-derived cfDNA can be an ideal biomarker for cancer screening, since it carries tumor-specific changes, such as DNA mutations and aberrant promoter methylation [9, 23–25]. Previously we reported four biomarker candidates (*SFRP1*, *SFRP2*, *SDC2*, and *PRIMA1*), which applied as a biomarker panel offers the possibility to discriminate CRC patients from controls using plasma samples providing a potential non-invasive diagnostic test [21]. Using multiple logistic regression analysis, we detected highly sensitive and specific differentiation of CRC (91.5% sensitivity, 97.3% specificity) and adenoma (89.2% sensitivity and 86.5% specificity) plasma samples from healthy controls based on the methylation levels of the markers. In that study we collected blood samples in K3EDTA tubes, and used manual cfDNA isolation method. To increase the cfDNA yield and stability, and to enhance the reliability of our biomarker panel, we have performed several additional experiments. In the present work, we tested various manual and automated cfDNA extraction methods, and compared two types of blood collection tubes in order to assess whether these factors influence our previous results. At first, we compared a column-based (HP) and a magnetic bead-based (EpC) isolation method. The Epi proColon 2.0 kit is the first commercially available and FDA-approved blood pre-screening test for CRC what detects aberrantly methylated *SEPT9*. However, it can be used for other markers analysis, as Schmidt et al. used the isolation kit for mSHOX2 analysis in non-small cell (NSCLC) and small-cell lung cancer (SCLC) patients [26]. CfDNA extraction and bisulfite conversion steps are closely linked in the protocol; therefore the concentration measurement was not possible with this method. After isolation with HP kit, the level of cfDNA in the sample groups was similar to our

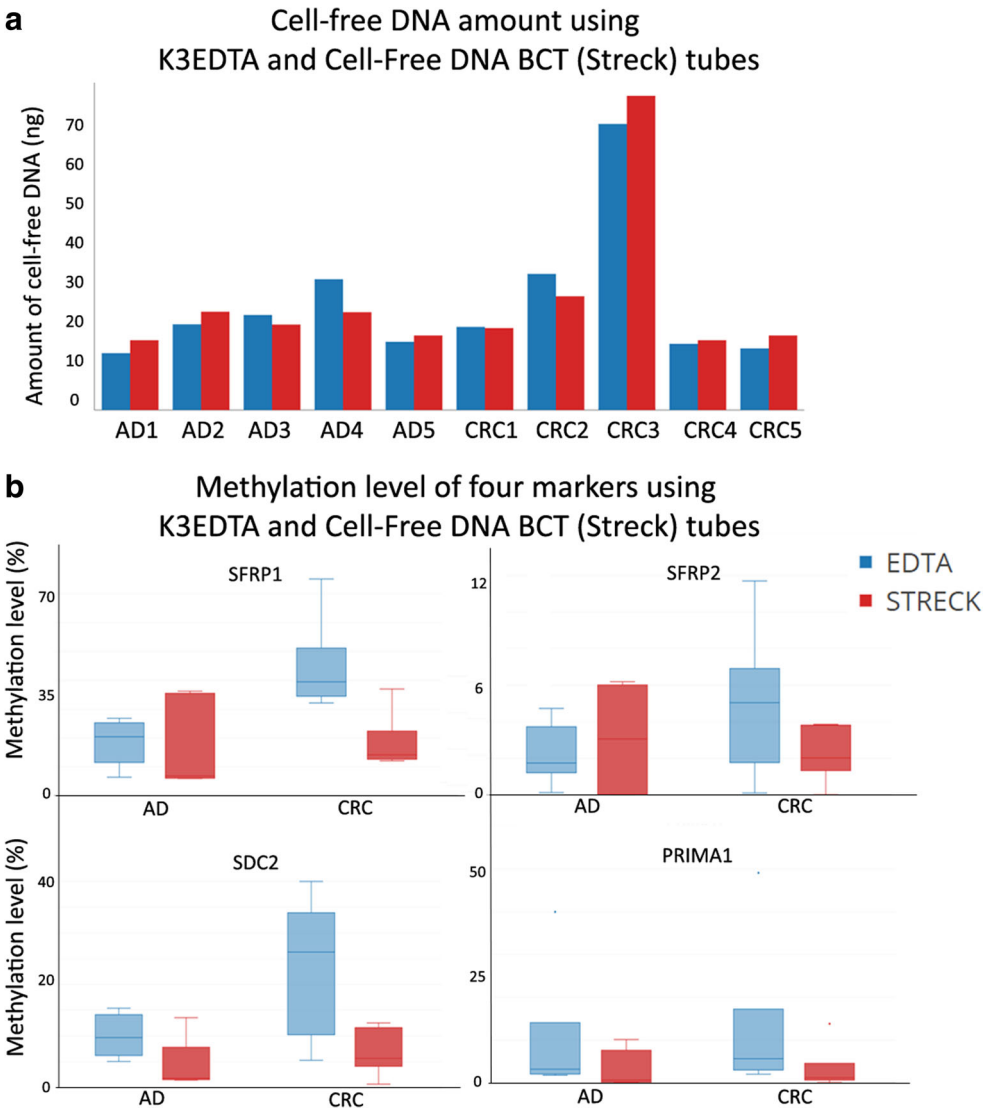

Fig. 4 CfDNA amounts of 5 adenoma and 5 CRC samples using K3EDTA and Cell-Free DNA BCT (Streck) blood collection tubes and DNA methylation level of *SFRP1*, *SFRP2*, *SDC2,* and *PRIMA1* in plasma samples

previous work [21]. Interestingly, the methylation frequency of *SFRP1*, *SFRP2*, *SDC2*, and *PRIMA1* was lower using the EpC kit in adenoma samples, and in the case of two genes was quite low also in CRC samples. Moreover, the average methylation level was higher using HP kit in CRC samples in all four genes. The technical background behind the differences between the two methods is not clear, but it may be caused by the way of the cfDNA binding.

We tested the Epi proColon 2.0 kit on InviGenius automated cfDNA extraction system as well, which also separates DNA with magnetic beads, and compared to HP manual isolation, higher cfDNA level was observed in all sample groups using InviGenius. Fleischhacker et al. have applied two manual and one automated cfDNA extraction methods using MagnaPure™ LC Instrument (Roche Life Science), and they have also observed higher DNA yield with the automated system [27]. Another study analysed the cell-free foetal DNA extracted from maternal plasma, and using the automated system (MagnaPure™ LC) they found 40.7% more cell-free foetal DNA than with manual isolation [28]. Despite the larger amount of DNA what we detected with InviGenius, after MethyLight analysis, lower methylation frequencies and levels were noticed in the case of all biomarkers in comparison to the manual protocol.

In order to test another column-based cfDNA isolation method, we examined the newly developed and introduced Zymo Quick-cfDNA™ Serum & Plasma Kit, and compared it to the new version of the automated isolation system, InviGenius PLUS. In this comparison, manual method resulted in larger cfDNA quantity, but the cfDNA amount was a bit lower in late stage CRC samples than after HP isolation. Analyzing the methylation level of the four markers, *SDC2* and *PRIMA1* revealed increased methylation percentages with Zymo protocol in adenoma and CRC plasma samples, *SFRP1* showed similar methylation with both methods and in the case of *SFRP2* we observed very low methylation level. These

results indicate that not only the manual and automated isolation, but also the different manual extraction methods result in diverse methylation pattern of the markers.

After we have found that HP is the most reliable method for our research, due to increased cfDNA stability in blood, Streck tubes containing preservative reagents were tested. Kang et al. also compared K3EDTA and Streck tubes, and measured the cfDNA and wild-type genomic DNA amount in plasma of breast cancer patients [29]. Tubes were kept at 4 °C and room temperature, and plasma separation was done after 2, 6, and 48 houres. They observed that cfDNA was stable up to 6 hours, independently from the collection tubes, but for longer periods of storage, Streck tubes appeared consistent, especially stored at room temperature. Several further studies examined the stability of cfDNA in Streck tubes, and they also obtained similar results [30–32]. We have not found significant differences between the tubes, though 6 out 10 plasma samples showed higher cfDNA level when using Streck collection tubes instead of the conventional K3 EDTA tubes. In the aspect of methylation level, adenoma samples did not show differences between the storage conditions, but in plasma from CRC patients, moderately higher average methylation was observed in blood collecting K3EDTA tubes.

Taken together, Streck collection tubes are proposed for longer storage due to minimizing cfDNA degradation even with extended time frame before plasma separation; however, this time period and ingredients may influence the methylation status of DNA molecules. Furthermore, we tested diverse DNA extraction and blood collection methods, and according to our results, the level of cfDNA and the methylation pattern of specific genes are influenced by these factors. The automated isolation systems are less labor-intensive and time-consuming techniques, but perhaps due to the magnetic bead-based isolation method we have found quite varied results, therefore, further developments are needed.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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