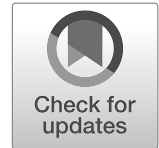
Cellular Oncology
https://doi.org/10.1007/s13402-020-00536-2

SHORT COMMUNICATION



Selective capture of plasma cell-free tumor DNA on magnetic beads: a sensitive and versatile tool for liquid biopsy

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Received: 30 November 2019 / Revised: 6 May 2020 / Accepted: 15 May 2020
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Abstract

Purpose Recently, 'solid tumor biopsies' have been challenged by the emergence of 'liquid biopsies', which are aimed at the isolation and detection of circulating cell-free tumor DNA (ctDNA) in body fluids. Here, we developed and optimized a method for selective capture of ctDNA on magnetic beads (SCC-MAG) for mutation detection in plasma of patients with colorectal cancer (CRC).

Methods Blood and tissue samples from 28 CRC patients were included for the detection of *KRAS* mutations. For the tissue samples, mutation analysis was conducted by high resolution melting (HRM) analysis and sequencing. For the SCC-MAG method, ctDNA was isolated from 200 μl plasma from patients with a mutant *KRAS* gene. For comparison, ctDNA extraction was carried out using a silica membrane-based method, after which mutations were detected using Intplex allele-specific PCR.

Results The mean ctDNA integrity index in plasma samples of cancer patients was 1.03, comparable with that of silica membrane-derived ctDNA (1.011). Notably, the limit of detection for the SCC-MAG approach was lower than that of the silica membrane method and measured 2.25 pg/ml ctDNA in plasma. Our analyses showed that while the silica membrane-based approach was capable of collecting ctDNA from two out of six CRC patient samples (average Cq 34.23), the SCC-MAG captured ctDNA from all samples with an average Cq of 29.76.

Conclusions We present a robust, reproducible, and highly sensitive method for the analysis of mutation statuses in liquid biopsies. The SCC-MAG method can readily be applied to any nucleic acid target for diagnostic purposes upon careful design of the specific capture probes, and can be multiplexed by several probes to identify multiple targets.

Keywords Cell-free tumor DNA extraction · ctDNA · Colorectal cancer · *KRAS* mutations · Plasma · Serum

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13402-020-00536-2) contains supplementary material, which is available to authorized users.

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Published online: 03 June 2020

1 Introduction

Detection of cancer-related mutations (e.g. *KRAS* in lung cancer) or epimutations [e.g. *Septin 9* methylation in colorectal cancer (CRC)] is widely performed after tumor tissue biopsy. Recently, this "gold standard" method has been challenged by the 'liquid biopsy' method, a term adopted for the analysis of ctDNA in body fluids such as blood [1, 2]. Tissue biopsy has several limitations, including difficulty to repeat, invasiveness, impracticality for periodic monitoring of treatment response, failure to detect metastasis at distant sites [3] and may not represent the molecular profile of a tumor due to spatial heterogeneity [4]. Liquid biopsy may overcome many of these limitations, including the dynamic monitoring of cancer recurrence. However, minute quantities of circulating tumor biomarkers and high background signals released from normal tissues or blood cells are some of the challenges that should be tackled in liquid biopsies [5].

The liquid biopsy method includes two phases, namely the isolation (pre-analytic) phase and the detection (analytic) phase. It was recently shown that with respect to pre-analytic variables, the selection of a ctDNA extraction method may affect the detection of oligonucleosomes [6]. During the isolation phase, liquid biopsies may contain a plethora of potential PCR inhibitors, which may affect the outcome of the detection phase. Moreover, hemolysis of blood samples during or before plasma preparation may cause an additional release of inhibitors disturbing the results [7]. The result of a gene-based test from a liquid biopsy is constrained not only by the lack of identified mutations/epimutations for some tumors, but also by the quantity of the tumor-derived DNA recovered from plasma. Circulating cell-free DNA is extremely fragmented, of which the total ctDNA comprises only a minor fraction that may be as small as 0.01% [8]. Thus, to enhance the sensitivity of detecting DNA alterations in an asymptomatic population for cancer screening, maximizing target cell-free DNA recovery is critical. An insufficient recovery would result in a possible lack of mutant DNA molecules in the diagnostic PCR product, leading to false-negative outcomes [9].

The aim of the present study was to establish a sensitive approach for solving the biological and technical problems of ctDNA extraction from liquid biopsies. We developed and optimized a method for mutation detection (in the *KRAS* gene) by specific capture of ctDNA using magnetic beads (SCC-MAG) in plasma from CRC patients.

2 Materials and methods

2.1 Cell lines and detection limit analysis

Human colorectal cancer (CRC) cell line SW-480 (ATCC® CCL-228™), as a positive control (with *KRAS* G12V mutation) and HeLa (ATCC® CCL-2™) as a negative control (without mutation in the *KRAS* gene) were purchased from the Pasteur Institute, Tehran, Iran. Cells were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO2 and 88% humidity.

For ctDNA analysis, DNA was extracted from SW-480 or HeLa cells using Quick-DNA Kits (Zymo Research, Orange, CA, USA) and sheared using the DNA fragmentation enzyme *Alu*I (NEB, New England Biolabs, Hitchin, UK), yielding fragments of mostly 120 to 400 base pairs (bp) in size (supplementary Fig. 1). The limit of detection (LoD) for the SCC-MAG method was determined using the *Alu*I-digested DNA from SW-480 cells spiked into healthy donor plasma. To prepare the spiked digested DNA, the isolated DNA from SW-480 cells was quantified by measuring absorbance at 260 nm using an Epoch Microplate Spectrophotometer (Winooski, Vermont, USA), and various concentrations ranging from 0.625 to 50 pg/ml were prepared and digested. To determine the effect of the DNA concentration on the DNA recovery from plasma samples, the indicated concentrations of the spiked DNAs in plasma were prepared, extracted and analyzed by qPCR. An independent experiment was performed with 5 and 2.25 pg/ml mutant DNA in plasma using a silica membrane-based extraction method to compare its LoD with that of the SCC-MAG method.

2.2 The pre-analytic phase

2.2.1 Patients and sample processing

Colonoscopy screening was scheduled for all patients included in this study. All patients gave written informed consent to store and use their samples for purposes of this study. All procedures and protocols in the present study were approved by the ethical committee of Mashhad University of Medical Sciences, Mashhad, Iran. Both plasma and tissue samples were obtained from each patient. Biopsies were taken for histological routine examination and research purposes during colonoscopy. In total 28 CRC patients were included in this study and five plasma samples from healthy individuals were included as controls. *KRAS* mutation analysis by high-resolution melting (HRM) was also conducted for the healthy controls. All peripheral blood samples were collected in 10 ml K_2EDTA tubes (BD Biosciences, UK) and stored at 4 °C (for a maximum of 1 h) until DNA or plasma extraction. All plasma samples were stored for at least 24 h at -80 °C. DNA was extracted from 5 to 20 mg fresh tumor tissues using a QIAamp Fast DNA Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA was eluted in 100 µl elution buffer and stored at -80 °C until further analysis. To examine the performance of the SCC-MAG method for hemolyzed samples, we collected blood from healthy individuals and induced *in vitro* hemolysis by passing the blood through a 26-

gauge needle followed by quantification of cell-free DNA using the reference gene beta-actin (*ACTB*).

2.2.2 Mutation analysis in tumor tissue

Mutation analysis in tissue was conducted by HRM analysis followed by Sanger sequencing. The HRM method was performed for the screening of *KRAS* mutations in tissue samples using a LightCycler® 96 Real-Time System (Roche Diagnostics, Germany). LightCycler® 96 Gene Scanning Software Version 1.1 (Roche Diagnostics, Germany) was used for data analysis. Sanger sequencing was conducted on DNA extracted from tissue samples to detect the type of *KRAS* codon 12 or 13 mutations. To this end, a 202-bp PCR fragment of the *KRAS* gene, encompassing codons 12 and 13, was amplified using primer pairs *KRAS* forward; 5'-TTAACCTT ATGTGTGACATGTTCTAA-3' and *KRAS* reverse: 5'-TGCATATTAAAACAAGATTTACCTCTA-3'. The sequencing reactions were performed on the purified PCR products. The obtained sequencing files were aligned and analyzed for the presence of mutations in the *KRAS* gene using Chromas software, version 2.6.6 [10] and the sangerseqR package [11].

2.3 Circulating nucleic acid extraction methods

2.3.1 Selective capture of ctDNA on magnetic beads (SCC-MAG)

For SCC-MAG, *KRAS* DNA fragments were pulled downed from total nucleic acid preparations by performing oligonucleotide-based hybrid capture. Two oligonucleotides targeting the *KRAS* and *ACTB* genes were selected, where the latter served as an internal control. To extract cell-free DNA, 200 µl plasma from each sample was mixed with an equal volume of lysis buffer (60 mM Tris-HCl, pH 7.4, 30 mM Na_2EDTA, 6 M guanidine-HCl, 10% Tween 20 and 2% Triton X-100) and 20 µl of 20 mg/ml proteinase K, and incubated for 30 min at 56 °C. For each sample, hybrid capture reactions were performed in triplicate. The capture reaction was carried out by adding 420 µl of the mixture of plasma and the lysis buffer to an equal volume of 6 M guanidine isothiocyanate (GITC) solution containing a biotinylated sequence-specific capture probe at 20 pmol (the probe sequences are available upon request). The mixture was heated at 95 °C for 10 min, cooled to room temperature (RT), and incubated overnight at RT on a roller mixer with gentle speed. Next, 5 µl streptavidin-coated magnetic beads (Dynabeads™ M-280 Streptavidin, Invitrogen) were added to the solution, and the tubes were incubated for an additional 3 h at RT on a roller mixer. The bead/capture probe complexes were subsequently washed two times with washing buffer, and the sequence-specific captured DNA was eluted into 20 µl ddH_2O by heat denaturation (72 °C for 3 min), and immediately used as a template for Intplex PCR (Fig. 1).

2.3.2 Silica membrane-based extraction

For the silica membrane-based extraction method, ctDNA was isolated from 200 µl plasma adjusted to a volume of 1 ml with 800 µl PBS, using a QIAamp circulating nucleic acid kit (QIAGEN, Germany) according to the manufacturers' protocols. The ctDNA was eluted with 20 µl elution buffer.

2.3.3 ctDNA integrity index measurement

DNA integrity of ctDNA was calculated as the ratio of qPCR results, qPCR-Alu247/qPCR-Alu115, where qPCR-Alu115 and qPCR-Alu247 are the Alu qPCR products obtained with the ALU115 and ALU247 primer sets, respectively. The sequences of these primers are as follows: ALU115 forward: 5-CCTGAGGTCAGGAG TTCGAG-3; ALU115 reverse: 5-CCCGAGTAGCTGGG ATTACA-3; ALU247 forward: 5-GTGGCTCACGCCTG TAATC-3; ALU247 reverse: 5-CAGGCTGGAGTGCA GTGG-3. Because the annealing sites of ALU115 are inside the ALU247 annealing sites, the DNA integrity value will be 1.0 when the template DNA is not truncated and 0.0 when the template DNA is fragmented into sequences smaller than 247 bp. Since the ALU115 primers can amplify most of the circulating DNA, the absolute amount of DNA is represented by the qPCR-Alu115 results. The absolute equivalent amount of ctDNA in each sample (plasma sample and silica membrane-based extracted ctDNA) was determined by the use of a calibration curve with serial dilutions (10 ng-0.001 ng) of genomic DNA obtained from SW-480 cells. A negative control lacking the template was simultaneously run in each reaction.

2.4 The analytic phase

2.4.1 Mutation analysis in plasma

Intplex allele-specific PCR for the detection of *KRAS* exon 2 and 3 mutations in plasma was performed according to a clinically approved assay with minor modifications: qPCR amplifications were carried out in duplicate in a reaction volume of 10 µl on a LightCycler® 96 Real-Time System. Primer sequences were designed, and thermal cycling and data analysis were conducted as described by Thierry et al. [12]. Each run contained positive and negative controls to ensure specific detection of the targeted mutation.

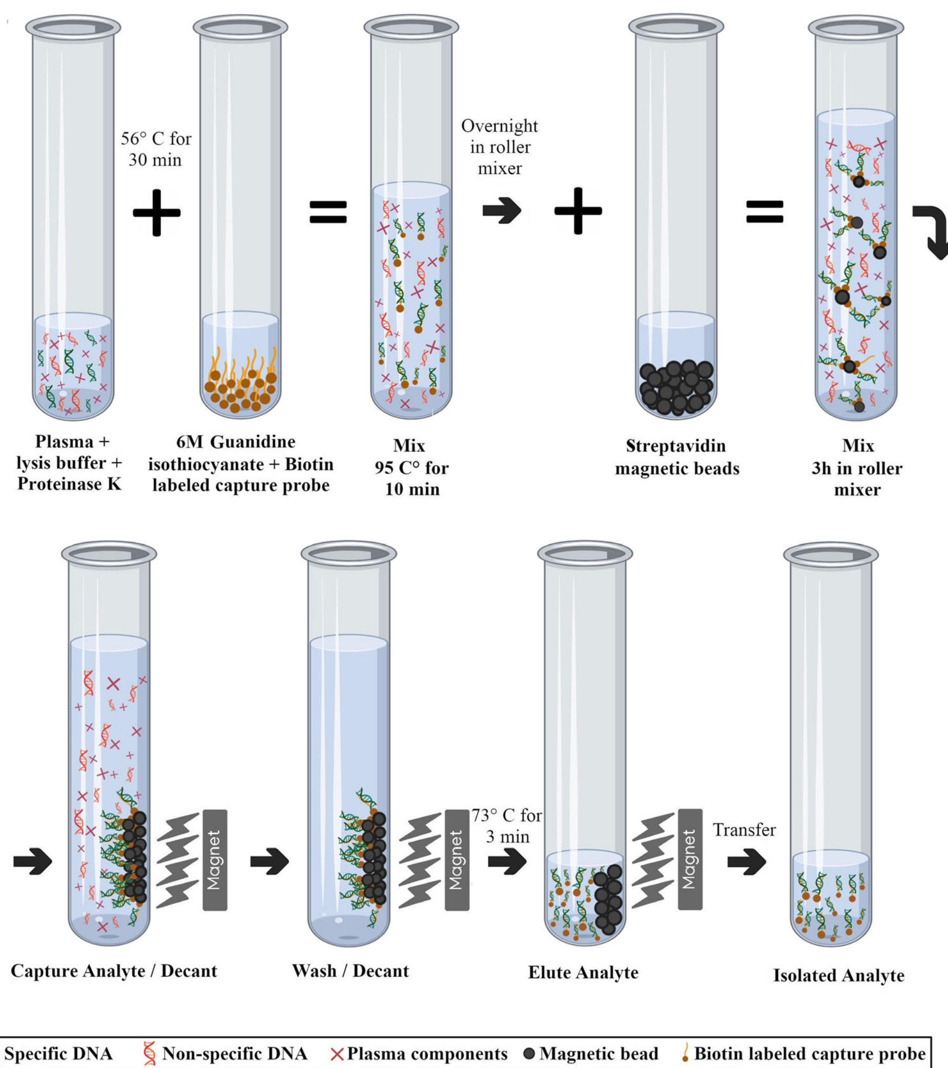

Fig. 1 Scheme showing the procedure for sequence-specific capture of ctDNA by streptavidin-coated magnetic beads. (For interpretation of the references to the colors in this figure legend, the reader is referred to the Web version of this article)

3 Results and discussion

The LoD of the SCC-MAG method was first evaluated, based on analysis of replicate standard curves [13], with sheared *KRAS* mutant cell line (SW-480) DNA spiked into a wild-type cell-free DNA background prepared from a healthy donor plasma sample. The prepared blends were spiked into the plasma at 50, 10, 5, 2.5, 1.25, 0.625, and 0 pg/ml. The result of the initial qPCR (Fig. 2A) allowed us to determine a narrow range of DNA concentrations (2.5, 2.25, 2, 1.75, and 1.5 pg/ml), in order to measure a precise value for LoD (Fig. 2B). Three replicates of each concentration were tested in duplicate by qPCR for LoD determination. Both experiments were independently analyzed and the results were combined using the ggplot2 package [14] and presented in Fig. 2. The LoD was defined as the lowest copy number that yields a detectable PCR amplification product with a probability of 95% [13]. The results indicated that the *KRAS* mutation could be detected with as low as 2.25 pg/ml of the mutant DNA in plasma (mean Cq: 32.54). Furthermore, a comparison of the results obtained for the SCC-MAG method with those of a silica membrane method showed that the silica membrane-based method was not able to detect the *KRAS* mutation at 2.25 pg/ml. It, however, detected the mutation at a concentration of 5 pg/ml (Cq: 37.21), consistent with a previously reported LoD of 4.7 pg/ml [15]. Thus, SCC-MAG proved to be a more reliable technique for detecting low copy numbers of mutant ctDNA than the silica membrane method, and it is recommended when maximum sensitivity is necessary. It may also offer the advantage of increased specificity by removing non-target (background) oligonucleosomes.

A summarized overview of the patient's characteristics is presented in Table 1. The presence and the type of *KRAS* mutations were assessed in colon tissues from all 28 CRC patients by HRM and sequencing (supplementary Fig. S2). Overall, four types of *KRAS* mutations (G12D, G12V, G12A, or G12R) were identified in 6 out of 28 tumor tissue samples (Table 1; Fig. 3). The mutated samples were enrolled

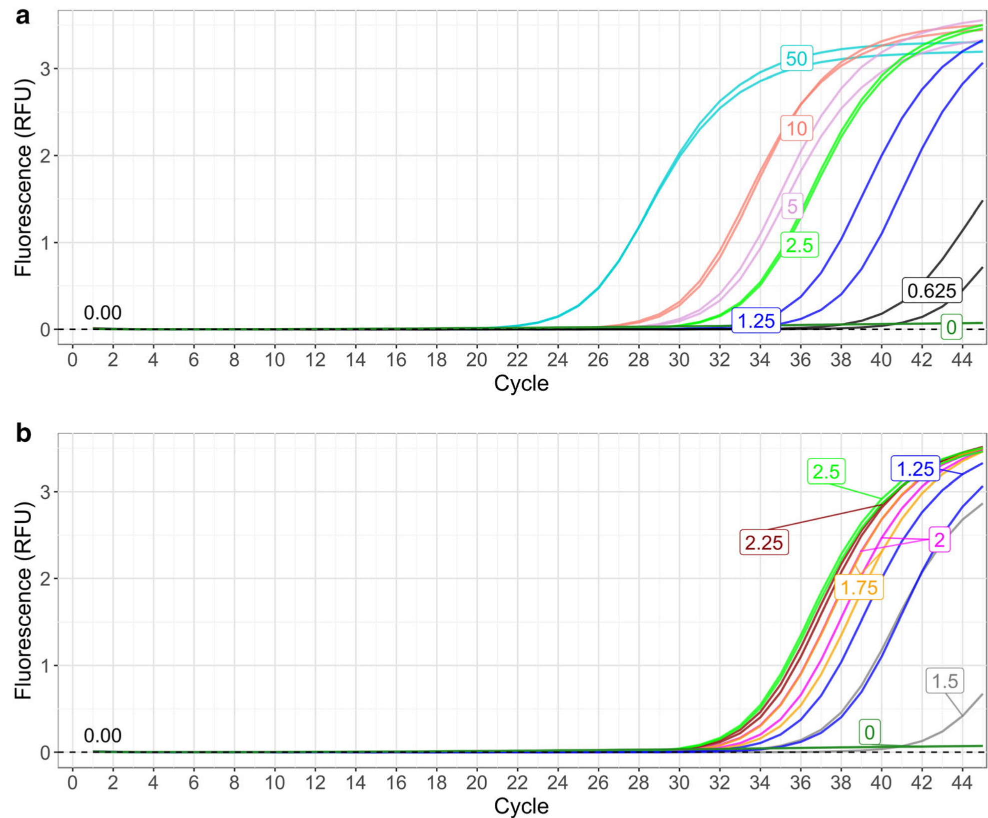

Fig. 2 Analysis of the limit of detection (LoD). Fractions of positive reads obtained by qPCR in samples containing 50 to 0.625 (**a**) or 2.5 to 1.5 (**b**) pg mutant DNA per 1 ml plasma. At 95% confidence level, LoD was recorded as 2.25 pg/ml. (For interpretation of the references to the colors in this figure legend, the reader is referred to the Web version of this article)

in the study for the detection of *KRAS* mutations in ctDNA isolated from plasma, using Intplex PCR. Sequencing of the DNA extracted from the CRC tissues was able to detect only 5 out of 6 samples positive of Intplex PCR (supplementary Table S1).

We next examined the degree of ctDNA integrity called ctDNA integrity index, which measures the ratio of long versus short ctDNA fragments. To this end, plasma samples were collected and processed from the 6 selected CRC patients. Direct plasma and the extracted DNA using a silica membrane-based method were analyzed by qPCR for Alu115 and Alu247 (see materials and methods Sect. 2.4). Data obtained from the qPCR assays were analyzed, and ctDNA values and the integrity index were calculated (Table 2). The integrity indexes for ctDNA in plasma samples and the extracted ctDNA by the silica membrane-based method from cancer patients were ranging from 0.95 to 1.21 and 0.88 to 1.12, respectively, with an average of 1.03 and 1.011. The square of the correlation coefficient (R^2) was higher than 0.99 for both Alu standard curves.

We next sought to compare the two nucleic acid extraction methods using a low volume of plasma samples (200 µl) from the 6 CRC patients. To this end, the efficiency and reproducibility of detection of *KRAS* mutations was compared between the plasma samples and the extraction methods using Cq values (supplementary Table S2). Strikingly, the SCC-MAG approach revealed a significantly higher performance than the silica membrane-based method (Fig. 3A). Notably, in contrast to the silica membrane-based method, which was able to extract ctDNA from only two samples (average Cq 34.23), the SCC-MAG protocol captured ctDNA from all samples with an average Cq of 29.76. Moreover, despite the varied amounts of input ctDNAs in the SCC-MAG approach (0.042 ng/µl − 1.202 ng/µl; Table 2), the outputs of the assay were similar between the samples (based on the Cq values: 29.38–30.18), indicating the sensitivity and reproducibility of the method (Fig. 3A). Figure 3B shows the results of Intplex PCRs for the G12V mutation with ctDNAs isolated by the SCC-MAG method, while Fig. 3C shows the same assays with ctDNAs isolated by a silica membrane-based extraction method (using QIAamp circulating nucleic acid kit).

Taken together, these results show that, although the amounts of input ctDNAs applied to the magnetic beads approach was different (Table 2), the performances of the samples were comparable (Fig. 3). Moreover, the repeatability of ctDNA extraction was shown to differ between the two methods, with the SCC-MAG method having a coefficient of variation (CV) of 1.06% compared to that of the silica membrane method, which was more variable (CV 9.25%). These results are in agreement with previous reports showing that the magnetic beads are most efficient in overall yield and purity of isolated ctDNA with the smallest range of variation [16].

It is widely accepted that most of the variability observed between samples results from biological variations. There are

Table 1 Patients and colon tumor characteristics

		Case	Control
Number		28	5
Gender	Male	19 (68%)	3 (60%)
	Female	9 (32%)	2 (40%)
Age at diagnosis (year)	Median	67	59
	Range	[44–82]	[45–63]
Pathology report	Signet ring carcinoma	3 (11%)	--
	Moderately differentiated adenocarcinoma	19 (68%)	--
	Ulcero Infiltrative adenocarcinoma	1 (3%)	--
	Invasive adenocarcinoma	1 (3%)	--
	Well differentiated adenocarcinoma	3 (11%)	--
	Poorly differentiated adenocarcinoma	1 (3%)	--
Tumor location	Anal	0 (0%)	--
	Rectum	10 (36%)	--
	Sigmoid	6 (21%)	--
	Transverse colon	0 (0%)	--
	Descending colon	1 (3%)	--
	Ascending colon	3 (11%)	--
	Cecum	8 (29%)	--
	Entire colon	0 (0%)	--
TNM stage	I	5 (18%)	--
	IIA	6 (22%)	--
	IIB	2 (7%)	--
	IIC	1 (4%)	--
	IIIA	3 (10%)	--
	IIIB	7 (25%)	--
	IIIC	4 (14%)	--
	IVA	0	--
	IVB	0	--
KRAS mutation		6 (21.4%)	--
	G12D	2	--
	G12A	1	--
	G12V	2	--
	G12R	1	--

also several technical factors that result in a broad spectrum of discrepancies in measurement. Our data confirm that one of the principal causes of technical variability arises from the choice of isolation method, where the yield and efficiency of ctDNA extraction from the same plasma samples can differ based on the technique of isolation. By comparing the SCC-MAG with a silica-based method, we show that this method generates a higher yield than the silica membrane kit (see Fig. 3 and supplementary Table S2) [17, 18].

The SCC-MAG technique has several other advantages over the silica membrane-based method. Hemolysis can lead to an increased amount of wild-type DNA fragments due to a release of cellular DNA from disrupted blood cells into plasma. Hence, ctDNA can be masked by a large amount of background DNA fragments leading to false-negative results [19]. Since the SCC-MAG method isolates sequence-specific ctDNA, the contaminating inhibitors and nonspecific DNA fragments from blood cells are significantly lower than in other ctDNA extraction methods, resulting in a higher performance. Indeed, assays with hemolyzed samples showed that while the silica membrane-based method failed to extract ctDNA, the SCC-MAG technique was capable of isolating ctDNA with high-efficiency from these samples (mean Cq 27.12). In addition, sample material may be scarce in a clinical environment. In contrast to common ctDNA extractions performed with plasma volumes ≥ 1 ml, we found that the SCC-MAG method is capable of ctDNA mutation detection in small-volume plasma samples (~ 200 µl) [20].

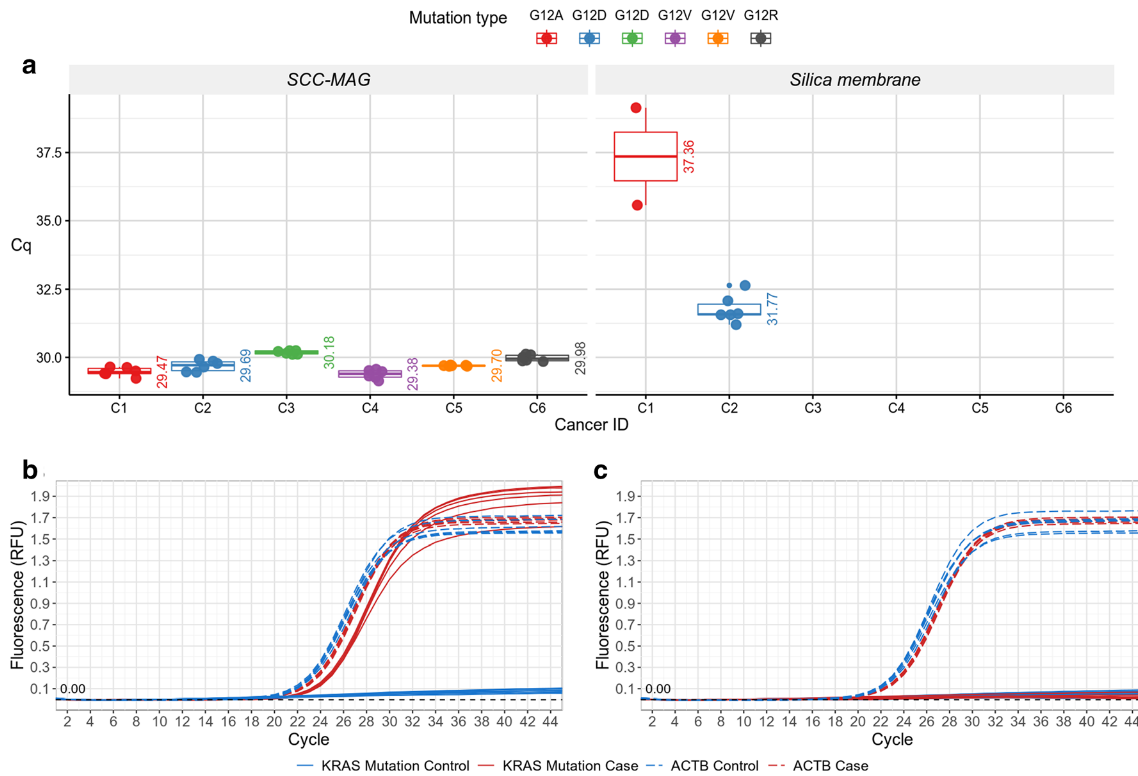

Fig. 3 Comparison of Cq values for *KRAS* mutation detection between the two ctDNA extraction methods from plasma. The silica membrane-based method was not able to extract sufficient amounts of ctDNA in 4 out of 6 CRC patient plasma samples **(a)**. Detection of *KRAS* (G12V) mutation (C4) by Intplex PCR using ctDNA extracted by the SCC-MAG method **(b)** or by a silica membrane method (QIAamp Circulating Nucleic Acid Kit) **(c)**. Case (patient ctDNA; red line) and control (healthy control ctDNA; blue line). *ACTB*: beta-actin as internal control. (For interpretation of the references to the colors in this figure legend, the reader is referred to the Web version of this article)

In conclusion, we report a robust, reproducible, and highly sensitive method for the analysis of mutation statuses in liquid biopsy assays. To the best of our knowledge, this is the first methodology reported for selective capturing of ctDNA directly from plasma with the potential of clinical use. This technology can be applied to any nucleic acid sequence for quantification and diagnostic purposes, upon careful choice of specific capture probes. A limitation of this study is the number of patient samples. More extensive studies with a larger number of cancer mutations are required to further evaluate the efficiency of ctDNA extraction from plasma by the SCC-MAG method.

Table 2 Levels of cfDNA in plasma samples and extracted cfDNA from CRC patients

Type of cfDNA	Cancer ID	Mean Cq for *Alu*115	Mean Cq for *Alu*247	Ratio *Alu*247/115	Alu115 Conc. ng/ml	Alu247 Conc. ng/ml	Conc. Ratio *Alu*247/115
Plasma	C1	10.51	12.74	1.21	1.202	1.002	0.83
	C2	9.89	10.56	1.07	1.356	1.205	0.89
	C3	15.78	16.24	1.02	0.041	0.102	0.002
	C4	13.03	12.68	0.97	0.192	1.003	5.22
	C5	14.38	13.73	0.95	0.125	0.971	0.007
	C6	11.77	11.5	0.97	0.572	1.140	1.99
Silica membrane	C1	10.12	11.02	1.09	1.394	1.725	2.40
	C2	9.56	8.41	0.88	2.010	10.203	5.07
	C3	10.23	10.14	0.99	1.410	1.920	1.36
	C4	20.1	22.63	1.12	0.001	0.0004	0.4
	C5	14.2	13.51	0.95	0.129	0.902	6.99
	C6	11.47	12.01	1.04	0.601	1.012	1.68

Acknowledgements We would like to thank Ehsan Shams Davodly for designing and preparing the figures, and the charity of Reza Radiotherapy and Oncology Center for supporting this study. This work is in memorial of Mrs Robabeh Aminian for her contribution to cancer research studies.

Funding information This study was supported financially by the Reza Radiotherapy and Oncology Center; Mashhad University of Medical Sciences (Grant number: 961467) and the Iran National Science Foundation (INSF) (Grant number: 93048371).

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval The current study was approved by the Mashhad University of Medical Sciences (MUMS) ethics committee (approval number: 961467) and all analyses were performed in accordance with the relevant guidelines and regulations of MUMS.

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