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Parallel Evaluation of Circulating Tumor DNA and Circulating Tumor Cells in Metastatic Colorectal Cancer

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Keywords

Colorectal cancer; Tumor heterogeneity; Liquid biopsy; Circulating tumor DNA; Circulating Tumor Cells.

Conflict of interest page

Declarations

Ethics approval and consent to participate – All blood samples for ctDNA and CTCs retrieval and analysis and tumor tissue samples for genotyping were obtained and analyzed through protocol approved by local Ethical Committee at Ospedale Niguarda, Milano, Italy (protocols 1014/09 and 194/2010). All patients signed and provided their informed consent before sample collection.

Consent for publication - NA.

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Authors' contributions - GG performed experiments of CTCs retrieval, participated in data analysis and writing of the manuscript. GM collected patients' samples and performed experiments of CTCs retrieval. GS performed genotyping of ctDNA. CD and JP performed CTCs analysis. FDN participated in data analysis and writing of the manuscript. MDI participated in experiments of CTCs retrieval. AB participated in data analysis and writing of the manuscript. SS participated in data analysis and writing of the manuscript. AS-B collected patients' samples, supervised data collection and analysis and was the major contributor in writing the manuscript. All authors read and approved the final manuscript.

Microabstract

Liquid biopsy, encompassing circulating tumor (ctDNA) and circulating tumor cells (CTCs), is under investigation to overcome spatial and temporal heterogeneity of metastatic colorectal cancer (mCRC). Limited comparative data are available. In a cohort of 20 patients we show that ctDNA was detectable in all cases, whilst CTCs in one third. ctDNA analysis appears readily candidate for clinical application in mCRC.

Abstract

Background: Tissue biopsy is the gold standard for tumor genotyping but it is an invasive procedure providing a single snapshot into tumor heterogeneity. Liquid biopsy approaches, encompassing the analysis of circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs), have been proposed as an alternative with the potential of providing a comprehensive portrait of tumor molecular landscape. In metastatic colorectal cancer (mCRC), both CTCs and ctDNA analysis have been investigated, but comparative analyses are limited.

Methods: We collected blood samples from 20 consecutive mCRC patients with at least one of the following inclusion criteria: high tumor burden (>1 metastasis), intact colonic primary tumor, disease progression at the time of sampling, ≤2 cycles of cytotoxic chemotherapy of current treatment course and time between last chemotherapy cycle ≥4 weeks.

Results: 19/20 samples displayed appropriate quality for CTC analysis. CTCs could be isolated in 7/19 evaluable patients (36.8%). The median number of CTCs was 0 [0 - 73]. In two patients we isolated >1 CTC and in five we found 1 CTC. We retrieved ctDNA in all samples, with a median amount of 732,573 GE/ml [174,774 - 174,078,615 GE/mL]. Concordance between ctDNA and tissue for *RAS*, *BRAF* and *ERBB2* alterations was found in 11/13 cases (84.6%).

Conclusions: In this cohort we show that ctDNA was detectable in all cases, whilst CTCs in one third of cases. ctDNA analysis was achieved with a less amount of blood sampling and allowed molecular characterization. Our data indicate that ctDNA is readily candidate for clinical application in mCRC.

Introduction

Recent technical advances for molecular diagnosis made it possible to obtain molecular information on tumors from peripheral blood through "liquid biopsy". This approach relies mainly on two different sources of circulating genetic information, circulating tumor cells (CTCs) and cell-free tumor DNA (ctDNA).

It has been already established that, in metastatic colorectal cancer (mCRC), the number of CTCs before and during treatment is a strong predictor of survival [1], and enumeration of CTCs using the CellSearch® system (Janssen Diagnostics, LLC; Raritan, NJ, USA) is FDA-approved for monitoring of patients. Advantages of isolation and functional characterization of CTCs include the potential for comprehensive analysis (i.e. protein expression, activation of signaling pathways, quantitative RNA analysis, and cytogenetic characterization) virtually achievable also at the single-cell level [2] and establishment of CTC-derived explants [3], whereas the major drawback of this method is that CTCs are rare, with an estimate of 1 CTC per 106-108 normal blood cells [4]. CTC enrichment methods include an epithelial cell adhesion molecule (EpCAM)-based selection system, which may fail to detect cells that undergo mesenchymal transition [4], and EpCAM-independent isolation systems that are limited by high cell-to-cell variability, necessitating isolation of a large number of CTCs to obtain a representative profile of the individual cancer genome [5].

The other side of the coin is represented by ctDNA, which is an EpCAM-independent, noninvasive biomarker, that can also be isolated from plasma or serum and other body fluids. Its association with prognosis in patients with mCRC has been clearly demonstrated [6], with also the potential for monitoring minimal residual disease in earlier stages [7]. Additionally, several studies from our group and others have already demonstrated concordance of liquid biopsies and tumor-tissue biopsies for molecular characterization of clinically validated biomarkers such as *RAS* mutations [8] [9] and that longitudinal analysis of ctDNA can be used to explore dynamic tumor evolution during targeted treatment in mCRC patients [8,10–12]. However, translation of this knowledge into better patient selection for treatment with molecularly targeted agents is yet to be demonstrated.

Even though both liquid biopsy methods are being increasingly proposed in various tumors including CRC, very limited comparative data are available between analysis of ctDNA and CTCs [6]. With this study we aimed at testing their performance in terms of successful detection as biomarkers in mCRC patients.

Materials and methods

Patients - We collected blood samples from 20 consecutive mCRC patients treated at Niguarda Cancer Center, Milano, Italy, with at least one of the following inclusion criteria: high tumor burden (>1 metastasis), intact colonic primary tumor, disease progression at the time of sampling, ≤2 cycles of cytotoxic chemotherapy of current treatment course and time between last chemotherapy cycle ≥4 weeks. Samples were obtained through protocols approved by local Ethical Committee at Ospedale Niguarda, Milano, Italy. All patients signed and provided their informed consent before sample collection.

Circulating tumor DNA analysis - At least 6 ml of whole blood was collected by blood draw using EDTA as anticoagulant. Plasma was separated within 5 h through two different centrifugation steps (the first at room temperature for 10 min at 1,600g and the second at 3,000g for the same time and temperature), obtaining up to 3 ml of plasma. Only 1 ml of plasma was used for subsequent mutational analysis. Circulating tumor DNA (ctDNA) ctDNA was isolated from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. Mutational analysis was carried out by droplet digital PCR (ddPCR) as follows: isolated ctDNA was amplified using ddPCR Supermix for Probes (Bio-Rad) using KRAS, NRAS, EGFR (custom designed) and ERBB2 CNV. ddPCR was then performed according to manufacturer's protocol, and the results were reported as the percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles or copy number variations. In details, 8–10 µl of DNA template was added to 10 µl of ddPCR Supermix for Probes (Bio-Rad) and 2 µl of the primer and probe mixture. This reaction mix was added to a DG8 cartridge together with 60 µl of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then transferred to a 96-well plate (Eppendorf) and then thermal cycled with the following conditions: 5 min at 95 °C, 40 cycles of 94 °C for 30 s, 55 °C for 1 min followed by 98 °C for 10 min (Ramp Rate 2 °C/s). Droplets were analyzed with the QX200 Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes. Gating was performed based on positive and negative controls, and mutant populations were identified. The ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad) to obtain fractional abundance and copy number variations of the mutated or amplified DNA alleles in the wild-type or normal background. The quantification of the target molecule was presented as number of total copies (mutant plus WT) per sample in each reaction. Fractional Abundance is calculated as follows: F.A. % = $[N_{mut}/(N_{mut} + N_{wt})] \times 100$), where

N_{mut} is the number of mutant events and N_{wt} is the number of WT events per reaction. The number of positive and negative droplets is used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals, as previously shown in [8]. ddPCR analysis of normal control plasma DNA (from cell lines) and no DNA template controls were always included. Samples with too low positive events were repeated at least twice in independent experiments to validate the obtained results.

Genome Equivalent Quantification - ctDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. We used 6 µl of ctDNA as template for each reaction. All samples were analyzed in triplicate. PCR reactions were performed using a 10- µl final volume containing 5 µl of GoTaq qPCR Master Mix, 2× with CXR reference dye) (Promega) and LINE-1 [12.5 µmol] forward and reverse primers. DNA at known concentrations was also used to build the standard curve.

Circulating tumor cells enumeration - Blood (10 ml) was drawn into CellSave tubes (Janssen Diagnostics) for CTC enumeration using the CellSearch platform as previously described [3]. In brief, CTCs were identified as cells co-expressing EpCAM and cytokeratins (8, 18, and 19) without expression of the white blood cell surface marker CD45. The CellSearch Epithelial Cell Kit (Veridex) contains ferrofluid particles coated with anti EPCAM antibodies, two phycoerythrin-conjugated and allophycocyanin-conjugated antibodies specifically directed against cytokeratins and CD45 respectively. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to identify fragmented and condensed nuclei representative of apoptotic cells. The blood was mixed with the dilution buffer, centrifuged (800 x g for 10 min at room temperature) and transferred to the CellTracks AutoPrep system. After incubation with anti EpCAM antibody, unbound cells and remaining plasma were removed and ferrofluid-labeled cells were fluorescently labeled with the anti-cytokeratin antibodies. At the end, unbound reagents were removed and the sample volume was reduced to 300 µl.

Results

A total of 19/20 samples displayed appropriate quality for CTC analysis. CTCs could be isolated in 7/19 evaluable patients (36.8%), whilst ctDNA was retrieved in 20/20 (100.0%) (two-tailed $p < 0.0001$ by Fisher's exact test) (**Table 1**). The median number of CTCs was 0 [0 - 73]. In two patients

we isolated >1 CTC and in five cases we found 1 CTC. The median amount of ctDNA was 732,573 GE/ml [174,774 - 174,078,615 GE/mL]. The two patients having >1 CTCs had a significantly higher average amount of ctDNA (GE/mL) than those without CTCs in their blood draws ($p < 0.005$). In contrast, no statistically significant difference in ctDNA amount was found among patients with ≤1 CTC. Candidate mutation analysis in ctDNA for *RAS*, *BRAF* and *ERBB2* alterations, performed only in those samples in which the respective alteration was known from tissue analysis revealed a concordance in 11/13 cases (84.6%) as compared to tissue analysis. Tissue genotyping was performed on from archival specimens obtained from primary tumor in 15/20 (75.0%) and metastatic sites in 5/20 (25%). Genotyping on CTCs was not carried out because they were detected only in 7/20 (35%) cases and because of the paucity of CTCs isolated using the CellSearch platform (median number=0), overall making the comparison poorly informative and technically challenging without undertaking a single-cell analysis.

Discussion

In this comparative cohort of paired liquid biopsies taken from mCRC patients we show that ctDNA was detectable in all cases, whilst CTCs retrieved by the CellSearch method in about one third of cases. ctDNA analysis was achieved with a less amount of whole blood sampling (6 *versus* 10 mL for ctDNA and CTCs, respectively) and allowed molecular characterization.

Limitations of our study include the lack of comparative molecular characterization with both methods. In our cohort we performed candidate mutation analysis of selected relevant/drugable CRC oncogenes on ctDNA only, confirming that this is a reliable source for genotyping [8]. A recent report has shown that in 15 mCRC patients, using the label-free microfluidic platform Vortex Chip, an enumeration of 0.1 - 29 putative CTCs/mL (mean: 3.4/mL) has been retrieved, being the cutoff for defining positivity set at 0.4 CTCs per mL of blood based on the background noise in healthy donors [13]. In nine of these, a comparative analysis between ctDNA and tissue was performed, showing that in some samples CTCs revealed a mutation that was not detected in ctDNA and viceversa, supporting that both methods are needed to enable optimal surveillance of the course of disease and treatment selection. In contrast, Bettegowda et al. by extracting DNA and performing whole-genome sequencing of tumor DNA from plasma as well as from the cellular compartment of blood obtained after centrifugation, had not identified, in a cohort of 16 patients

including 9 CRC, any cases in which CTCs were detected but in which ctDNA was absent; further, in many cases in which ctDNA was detected (13 of 16; 81%), no CTCs were found [6].

Conclusion

In conclusion our data expand, in a larger comparative series of individual CRC patients than above mentioned studies [6,13], and strictly focused on the metastatic setting, previous observations [6] indicating that ctDNA analysis is readily candidate for clinical application in mCRC.

Clinical practice points

Tumor heterogeneity hampers clinical efficacy of targeted therapies in colorectal cancer (CRC), and tissue biopsy is the gold standard to obtain the tumor molecular make up before initiating treatment. Liquid biopsy, encompassing the analysis of circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs), has been suggested as an alternative with the potential of providing the comprehensive tumor molecular landscape. Both CTCs and ctDNA analysis are under investigation in CRC; however, available comparative data are limited.

In a cohort of 20 metastatic CRC patients, ctDNA was detected in all of them allowing to retrieve main genetic alterations with a high concordance if compared to tissue biopsy. By contrast, CTCs analysis required a higher amount of blood and was successful in only one third on patients.

ctDNA analysis is more likely to be successfully performed than CTCs in metastatic CRC, making it readily candidate for clinical application.

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Tables

Table 1. Comparison between the number of circulating tumor cells and circulating free DNA (genome equivalent, GE) retrieved by liquid biopsy in metastatic colorectal cancer patients.

PATIENT ID	Number of CTCs	cfDNA (GE/mL)	GENETIC ALTERATIONS	
			Tissue	***cfDNA (% mutated fragments)***
#1	0	780090	WT	-
#2	73	174078615	*KRAS* G12D	*KRAS* G12D (54.0%)
#3	0	645082	*ERBB2* ampl.	*ERBB2* 6.05 CNV
#4	0	379836	WT	-
#5	0	174774	*ERBB2* ampl.	*ERBB2* 2.75 CNV
#6	0	1253411	*NRAS* Q61	*NRAS* Q61K (1.7%)
#7	-**	304419	*KRAS* G12R	*KRAS* G12R negative
#8	0	404470	*KRAS* A146T	*KRAS* A146T (24.5%)
#9	0	332217	*KRAS* codon 13	*KRAS* G13D (11.5%)
#10	0	269866	-	-
#11	2	17649043	*ERBB2* ampl.	*ERBB2* 186.5 CNV
#12	0	391527	WT	-
#13	1	1444335	WT	-
#14	1	848815	*KRAS* G13D	*KRAS* G13D (19.0%)
#15	0	896593	*KRAS* G12D	*KRAS* G12D (0.7%)
#16	0	826507	*KRAS* exon 4	*KRAS* A146T negative
#17	1	296838	WT	-
#18	1	797352	WT	-
#19	1	685056	*KRAS* G12D	*KRAS* G12D (14.0%)
#20*	0	3318156	*KRAS* G13D	*KRAS* G13D (2.9%)

cfDNA=circulating free DNA; - = analysis not performed; *Patient affected by metastatic colorectal and breast cancer with bones metastases; ** the quality control did not reach the standards for CTC analysis; GE = genome equivalent (total number of fragments of cfDNA/mL); WT = Wild-Type; ex. = exon; ampl. = amplified; CNV = Copy Number Variation.

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