

Single-Cell Phenotypic and Molecular Characterization of Circulating Tumor Cells Isolated from Cryopreserved Peripheral Blood Mononuclear Cells of Patients with Lung Cancer and Sarcoma

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BACKGROUND: The isolation of circulating tumor cells (CTCs) requires rapid processing of the collected blood due to their inherent fragility. The ability to recover CTCs from peripheral blood mononuclear cells (PBMCs) preserved from cancer patients could allow for retrospective analyses or multicenter CTC studies.

METHODS: We compared the efficacy of CTC recovery and characterization using cryopreserved PMBCs vs fresh whole blood from patients with non-small cell lung cancer (NSCLC; $n = 8$) and sarcoma ($n = 6$). Two epithelial cellular adhesion molecule (EpCAM)-independent strategies for CTC enrichment, based on Parsortix[®] technology or immunomagnetic depletion of blood cells (AutoMACS[®]) were tested, followed by DEPArray[™] single-cell isolation. Phenotype and genotype, assessed by copy number alterations analysis, were evaluated at a single-cell level. Detection of target mutations in CTC-enriched samples from frozen NSCLC PBMCs was also evaluated by digital PCR (dPCR).

RESULTS: The use of cryopreserved PBMCs from cancer patients allowed for the retrospective enumeration of CTCs and their molecular characterization, using both EpCAM-independent strategies that performed equally in capturing CTC. Cells isolated from frozen PBMCs were representative of whole blood-derived CTCs in terms of number, phenotype, and copy number aberration profile/target mutations. Long-term storage (≥ 3 years) did not affect the efficacy of CTC recovery.

Detection of target mutations was also feasible by dPCR in CTC-enriched samples derived from stored PBMCs.

CONCLUSIONS: Isolating CTCs from longitudinally collected PBMCs using an unbiased selection strategy can offer a wider range of retrospective genomic/phenotypic analyses to guide patients' personalized therapy, paving the way for sample sharing in multicenter studies.

Introduction

Detection and enumeration of circulating tumor cells (CTCs) can predict patient prognosis (1), enable real-time monitoring of tumor treatment response and offer the ability to implement targeted therapies in the absence of tissue biopsies (1–3). Although CTCs have the potential to reveal the molecular and phenotypic complexity of tumors (3–5), the main impediments to their study are CTC rarity and heterogeneity (5, 6).

Currently, the CellSearch[®] System (Menarini-Silicon Biosystems), based on positive selection of epithelial cellular adhesion molecule–positive (EpCAM+) CTCs, is the only Food and Drug–approved system for CTC enumeration in metastatic breast, colon, and prostate cancer (1). However, even in the case of epithelial tumors, cancer cells traveling in the circulation can display mesenchymal traits (7). Thus, EpCAM-based strategies for CTC detection may fail to detect CTCs shed

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from mesenchymal tumors, such as sarcomas, but also CTCs from epithelial tumors that have undergone epithelial to mesenchymal transition, as seen for non-small cell lung cancer (NSCLC) (8). In fact, in NSCLC, epithelial marker-independent strategies have made it possible to capture a greater number of CTCs in a greater percentage of patients than the CellSearch[®] System (9, 10). Therefore, relying on EpCAM-independent strategies for CTC enrichment overcomes the challenges raised by different tumor types.

Several epithelial-marker independent enrichment strategies have been tested to select CTCs, but there is still no consensus on which strategy is the most effective (11). Among the EpCAM-independent strategies available, Parsortix[®] technology (Angle plc) leverages microfluidics-based technology to separate rare cells based on their distinct size and deformability (12). The Parsortix[®] system allows a target cell recovery rate greater than 70% and the harvesting of viable cells suitable for downstream analyses (13). Immunomagnetic depletion of hematopoietic cells to enrich for CTCs has also been extensively tested, utilizing anti-CD45 microbeads, followed by cell separation on magnetic columns and cocktails of antibodies against different hematopoietic markers (RosetteSep[™]) (14, 15). The recovery rate of CTCs, even with mesenchymal phenotype, was reported to reach up to 99.98% efficacy, but this method had the disadvantage of leaving a large number of contaminating leukocytes after blood sample processing (11, 15–17).

Regardless of the enrichment strategy, almost all CTC detection methods share a limitation due to the need for rapid blood processing after collection from patients, which represents an obstacle to implementing CTC studies in multicenter clinical trials (4, 6). The CellSearch[®] System-associated CellSave Preservative Tubes (4) allows blood storage at room temperature but only up to 96 h, a time period not compatible with retrospective studies. A few attempts have been made to enable true long-term blood storage for CTC studies, including cryopreservation of vitally frozen peripheral blood mononuclear cells (PBMCs). In sarcoma patients and metastatic prostate cancer patients, the comparison of fresh whole blood samples (WBS) vs corresponding frozen PBMCs showed that cryopreservation did not interfere with enumeration of CTCs identified by immune fluorescence or fluorescence in situ hybridization analysis (18, 19). Similarly, in patients with gastroesophageal cancer, no relevant differences in CTC counts were reported in fresh samples or frozen PMBCs processed using the IsoFlux system (Fluxion Biosciences), based on EpCAM+ CTC enumeration (20).

Although preliminary studies support the feasibility of identifying CTCs in cryopreserved PBMCs, they are limited to CTC identification by phenotypic characteristics without any molecular evidence demonstrating

their tumor origin. We addressed this gap by developing a pipeline for EpCAM-independent enrichment and phenotypic and molecular characterization of single CTCs isolated from frozen PBMCs from patients with advanced stage NSCLC and sarcoma.

Materials and Methods

PATIENTS AND WHOLE BLOOD SAMPLE COLLECTION

All enrolled patients provided written informed consent prior to blood collection (INT 210/18 and INT 77/18 studies). Twelve patients were diagnosed with advanced stage NSCLC (IIIb–IV) at Fondazione IRCCS Istituto Nazionale Tumori of Milan. Patients underwent different treatments before blood sampling, as reported in online Supplemental Table 1. Six patients with a histologically confirmed diagnosis of soft tissue sarcoma, with no evidence of distant metastasis, were recruited at Fondazione IRCCS Istituto Nazionale Tumori before undergoing surgery (Supplemental Table 2). WBS (20 mL) were collected in K₂EDTA tubes: 1 tube (10 mL) was immediately processed for CTC enrichment and 1 (10 mL) was used to derive PBMCs.

PROCESSING OF FROZEN SAMPLES

For the comparison study between fresh and cryopreserved blood, WBSs (10 mL) were processed with the SepMate[™] kit (STEMCELL Technologies) for collection of PBMCs following the manufacturer's instructions. PBMCs were washed with PBS + 2% fetal bovine serum, resuspended in fetal bovine serum + 10% DMSO and stored at –80°C for 2 weeks. For the long-storage study samples, PBMCs were collected as previously described and stored for 1 to 3 years (Supplemental Fig. 1). Frozen PBMCs were thawed and resuspended in 10 mL of PBS + 1% BSA + 2 mmol/L EDTA, and then processed with Parsortix[®] or AutoMACS[®] for CTC enrichment, following the same protocol used for WBS.

CTC ENRICHMENT BY PARSORTIX[®]

WBSs (10 mL) were processed following the manufacturer's instruction using the PX_S99F protocol and the Parsortix[®] GEN3D6.5 Cell Separation Cassettes (critical gap size of 6.5 μm, Angle plc). At the end of the processing step, enriched cells were washed out of the cassette with 200 μL of PBS into a 1.5 mL tube for downstream analysis.

CTC ENRICHMENT BY AUTOMACS[®]

WBSs (10 mL) were incubated with Lysis Buffer (Miltenyi Biotec) in a ratio of 1:10 for 10 min at room temperature. Blood was then washed in PBS twice and incubated with Tumor Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instruction for 15 min at 4°C. After a washing step, samples were

resuspended in 500 μ L of PBS + 0.5% BSA + 2 mmol/L EDTA and separated using the “DepleteS” program of the AutoMACS[®] Pro Separator (Miltenyi Biotec). Fractions of CTC-enriched cells (depleted from blood cells) were eluted and used for downstream analysis.

SPIKE-IN EXPERIMENTS

CME-1 (synovial sarcoma), H1299 (large cell lung carcinoma), and LT73 (primary lung adenocarcinoma) cell lines were stained with CellTracker[™] Green CMFDA Dye (Thermo Fisher) following the manufacturer’s instructions. For each cell line, 10 fluorescent cancer cells were manually picked using a micropipette and spiked into 2 K₂EDTA tubes with 10 mL of blood from a healthy donor: 1 tube was immediately processed, and 1 was cryopreserved for two weeks, as previously described. After the enrichment steps, collected fluorescent cancer cells were counted under a fluorescence microscope.

CTC IDENTIFICATION AND COLLECTION OF SINGLE CELLS

After enrichment, the samples from NSCLC patients were incubated with anti-CD133, anti-EpCAM, and antihematopoietic markers (CD45, CD14, and CD16) in PBS + 0.5% BSA + 2 mmol/L EDTA and then fixed for 20 min at room temperature with 2% paraformaldehyde and stained with diaminopyrolyindole 4,6-diamino, 2-pyrolyindole solution.

Cells from sarcoma patients’ samples were collected in PBS after enrichment and immediately stained in suspension with fluorescently labeled antibodies against EpCAM, EGFR, cell-surface vimentin, and hematopoietic markers (CD45, CD14, and CD16). Samples were then fixed with 2% paraformaldehyde for 20 min at room temperature, permeabilized using Inside Perm (Miltenyi Biotec), incubated with anticytokeratins (anti-CKs) fluorescently labeled antibody and stained with diaminopyrolyindole 4,6-diamino, 2-pyrolyindole solution. Further information regarding all antibodies is reported in the online [Supplemental Methods](#).

Single putative CTCs were selected and isolated using the DEPAArray[™] platform (Menarini-Silicon Biosystems), based on their morphology and negativity for hematopoietic markers (21, 22).

MOLECULAR CHARACTERIZATION OF ISOLATED CTCs AND BIOINFORMATICS ANALYSIS

Recovered single cells and pools of white blood cells were subjected to whole genome amplification (WGA) followed by a PCR-based quality control (Ampli1[™] WGA+Ampli1 QC kit, Silicon Biosystems), which allows evaluation of the genomic integrity index (23). Only samples with a genomic integrity index ≥ 1 were subjected to a low-pass whole genome sequencing to detect copy

number aberrations (CNA). Barcoded libraries were prepared using the Ampli1[™]Low Pass kit for Ion Torrent (Silicon Biosystems), pooling 16 or 24 samples depending on the amplified DNA quality or the Ampli1[™]Low Pass kit for Illumina (Silicon Biosystems, pools of 96 samples). Libraries were subjected to sequencing with the IonTorrent Ion S5[™] system (Thermo Fisher) using the Ion530 chip as per the manufacturer’s instructions or with HiSeq system (Illumina). Quality checks were performed with Torrent_Suite 5.10.0 and fastQC for Ion Torrent and Illumina samples, respectively.

WGS sequences were aligned to the Human Reference Genome (hg19) using tmap (Torrent_Suite 5.10.0) and bwa aligner tool for Ion Torrent and Illumina samples respectively. CNAs were predicted by using QDNAseq, following already published settings (24, 25). All postprocessing analyses were performed using R software (<https://www.R-project.org/>). More details on bioinformatics analysis and CNA computation are available in the [Supplemental Methods](#). Correlation between different groups of CTCs was computed using the Pearson coefficient and submitted to hierarchical cluster analysis considering Euclidean distance and the complete linkage method (26).

REALTIME PCR ANALYSIS FOR MUTATION DETECTION

RealTime PCR for MET p. T1010I mutation was performed on amplified genomic DNA from single cells using TaqMan[®] Mutation Detection Assay (MET_707_mut) (Thermo Fisher). One microliter of WGA reaction DNA (V_f 50 μ L) was mixed with TaqMan[™] Universal Master Mix II, no UNG (2 \times) and MET_707_mut assay or MET gene reference (MET_rf) (10 \times) in a final volume of 20 μ L. The amplification was performed into 96-well plates run on QuantStudio[™]6 Flex Real-Time PCR Systems (Thermo Fisher) according to preset Standard Curve program and analyzed using QuantStudio[™] Real-Time PCR software.

CHIP-BASED DIGITAL PCR

DNA from CTC-enriched samples was extracted using the Maxwell[®] RSC Blood DNA Kit (Promega). Reaction mix for digital PCR (dPCR) was composed of 2 μ L of DNA+ 8 μ L QuantStudio[™] 3D Digital PCR Master Mix+ 0.8 μ L of TaqMan[™] Liquid Biopsy dPCR Assay (EGFR_6223 p. E746_A750deIELREA, 20 \times containing 2 primers and 2 probes for EGFR wt and mutant allele) + 5.2 μ L nuclease-free H₂O.

Fifteen microliters of the mix were loaded onto a 20K Chip Kit and run on QuantStudio[™] 3D Digital PCR (all from Thermo Fisher), according to the thermal profile reported in the [Supplemental Methods](#). QuantStudio[™] 3D Analysis Suite Cloud Software provided the copies number of VIC and FAM probes,

which were next corrected according to total volume of loaded mix and DNA dilution.

Results

FEASIBILITY OF CTC ISOLATION FROM CRYOPRESERVED PBMCs FROM PATIENTS WITH SARCOMA AND NSCLC USING DIFFERENT ISOLATION STRATEGIES

CTCs were isolated with the same efficiency from whole blood and cryopreserved PBMCs from cancer patients using Parsortix[®]+DEPArrayTM strategy. A preliminary assessment of the Parsortix[®] technology efficiency in enriching CTCs from cryopreserved PBMCs was performed through spike-in experiments using a synovial sarcoma cell line (CME-1). Sarcoma cells (n = 10) were spiked into healthy donor blood samples, which were then processed in parallel as WBS or frozen PBMCs. The mean recovery of cancer cells was 86.7% in freshly processed WBSs and 53.3% in cryopreserved PBMCs (Supplemental Table 1).

We then combined Parsortix[®] technology enrichment with DEPArrayTM (13, 27–29), to compare CTC recovery from blood samples collected from patients with advanced stage NSCLC (n = 4) or sarcoma (n = 6) (Supplemental Tables 2 and 3). Samples were processed in parallel as fresh WBS and cryopreserved PBMCs (Supplemental Fig. 1).

In NSCLC patient samples, 59% and 73% of the 78 and 51 cells isolated from fresh and frozen samples, respectively, were rated adequate for CNA analysis based on genomic integrity index and bioinformatics sequencing reads quality control (QC; low-pass whole genome sequencing-QC), and in both sample types, 7 cells were called as CTCs due to their aberrant CNA profiles (Fig. 1A) (Supplemental Fig. 2, A-D).

In sarcoma patients we rated as adequate for downstream CNA analysis 82% and 89% of the 122 and 181 cells recovered from fresh and frozen samples, respectively. The total number of confirmed CTCs was 11 in fresh samples and 15 in frozen samples (Fig. 1B).

Unsupervised hierarchical clustering of CNA profiles from CTCs did not separate cells from fresh and frozen samples, for either NSCLC or sarcoma patients, indicating that the freeze and thaw procedure did not introduce bias in CTC genomic characterization (Supplemental Fig. 2E).

CTCs were isolated with the same efficiency from whole blood and cryopreserved PBMCs from patients with NSCLC using AutoMACS[®] + DEPArrayTM strategy. Next, in NSCLC we evaluated an alternative CTC enrichment strategy based on immunomagnetic depletion of hematopoietic cells (Tumor Cells Isolation Kit + AutoMACS[®] Pro Separator) (Supplemental Fig. 1).

First, we performed spike-in experiments with 2 lung cancer cell lines (H1299 and LT73) to assess

AutoMACS[®] performance in cryopreserved PBMCs. Overall, the mean recovery of cancer cells was 75% in freshly processed blood and 72.5% in cryopreserved PBMCs (Supplemental Table 4).

Thereafter, AutoMACS[®] enrichment was combined with DEPArrayTM analysis to compare CTC recovery efficiency in fresh WBS and corresponding cryopreserved PBMCs (1-month storage) collected from 4 NSCLC patients (Supplemental Fig. 1). A total of 110 putative CTCs were isolated from fresh samples and 84 from corresponding frozen samples. Quality controls demonstrated that only 44% of cells isolated from fresh blood and 80% of cells obtained from frozen samples were considered suitable for CNA analysis, indicating that AutoMACS[®] procedure can be stressful on cells, especially in fresh samples.

Genomic analysis confirmed 16 CTCs from fresh WBS and 20 CTCs from frozen PBMCs (Fig. 2).

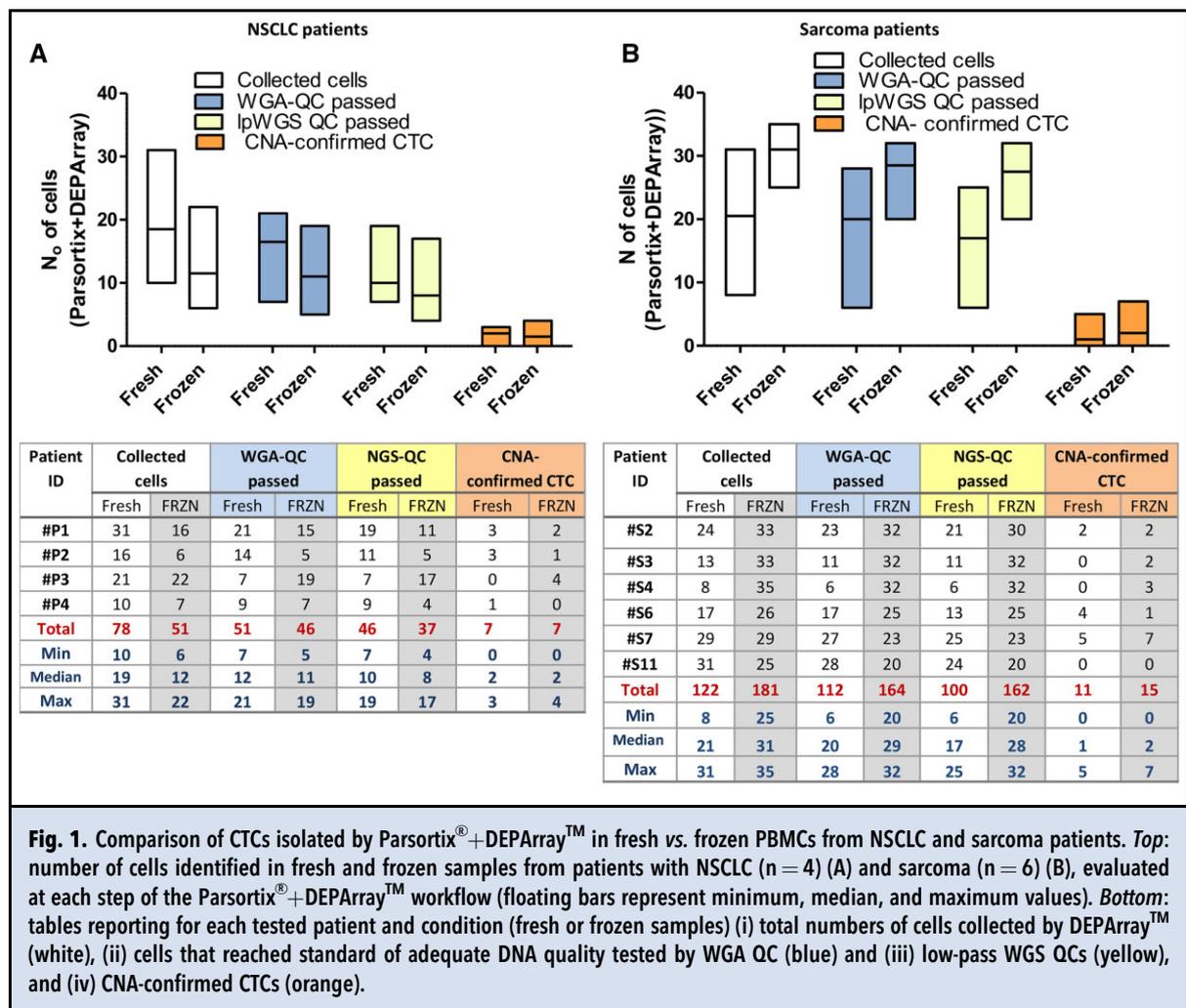
Comparison of AutoMACS[®] vs Parsortix[®] strategies in long-term stored PBMCs from patients with NSCLC. Finally, to assess the impact of long-term storage on the tested workflows we selected 3 NSCLC patients for whom 2 aliquots of PBMCs stored for more than 3 years were available and investigated in parallel the CTC recovery efficiency of Parsortix[®]+DEPArrayTM and AutoMACS[®] + DEPArrayTM strategies (Supplemental Fig. 1).

After the QC steps, 54% of the 68 cells isolated with AutoMACS[®] + DEPArray and 75% of the 44 cells collected with Parsortix[®] + DEPArrayTM were suitable for CNA analysis. Six cells with aberrant DNA (CTCs) were detected in samples processed by AutoMACS and 7 in those enriched with the Parsortix (Fig. 3).

CTC PHENOTYPES IN CRYOPRESERVED PBMCs VS FRESH SAMPLES

CTCs with different phenotypes were detectable in both fresh WBSs and cryopreserved PBMCs from NSCLC patients. In NSCLC, in addition to epithelial markers (EpCAM), we investigated the expression of CD133, a marker identifying cancer stem cells, the population with the highest ability to sustain primary tumor growth and initiate metastasis (Supplemental Fig. 3A) (30, 31).

In fresh and frozen samples EpCAM+ cells represented, respectively, 5% and 3% of total selected cells. After the QC steps, we confirmed by CNA analysis only 1 EpCAM+ CTC in 1 patient's fresh WBS (Table 1). Notably, in the few patients undergoing surgical resection, we demonstrated that primary NSCLC tumors were positive for EpCAM expression, whereas no EpCAM+ CTCs were detected in the blood (Supplemental Fig. 3B).



Interestingly, CD133+EpCAM-cancer stem cells were detected in 7/8 and 5/8 patients in fresh and frozen samples, respectively, representing 14% and 19% of the total selected cells. After the QC steps, CNA analysis confirmed that CD133+CTCs represent 5% and 15% of total CTCs identified in fresh and frozen samples, respectively (Table 1). Notably, these frequencies are significantly higher than those previously reported in NSCLC primary tumors (CD133+ cells = 0.3%) (30).

Generally, we found that a majority of selected cells were negative for both EpCAM and CD133 markers: “nonepithelial” cells represented 80% and 78% of total selected cells in fresh and frozen samples, respectively. Among these, CNA analysis confirmed 21 CTCs in fresh samples and 23 CTCs in frozen samples (Table 1).

Similarly, CTCs detected in long-term frozen samples confirmed that “nonepithelial” cells represented 82% and 92% of detected cells in AutoMACS[®] and Parsortix[®] enriched samples, respectively. We confirmed

7 “nonepithelial” CTCs in AutoMACS[®]+DEPArray[™] selected cells and 9 CTCs in Parsortix[®]+DEPArray[™] selected cells (Supplemental Table 5).

CTC with different phenotypes were detectable in both fresh blood and cryopreserved PBMC samples from sarcoma patients. In sarcoma patient samples, we investigated the epithelial (EpCAM+, EGFR+, or CK+), mesenchymal (cell-surface vimentin positive) and nonconventional (lacking epithelial, mesenchymal, and leukocyte markers) phenotypes (Supplemental Fig. 4). Among 16 putative epithelial CTCs collected in fresh samples and 34 isolated in frozen PBMCs, CNA analysis confirmed 5 CTCs in frozen samples only (Table 2).

CNA analysis allowed confirming as mesenchymal CTCs 5 out of 59 cells isolated from fresh samples and 7 out of 111 cells isolated from frozen samples. Nonconventional CTCs, confirmed by CNA analysis, were 6 out of 47 collected cells and 3 out of 36 cells collected in fresh and frozen samples, respectively (Table 2).

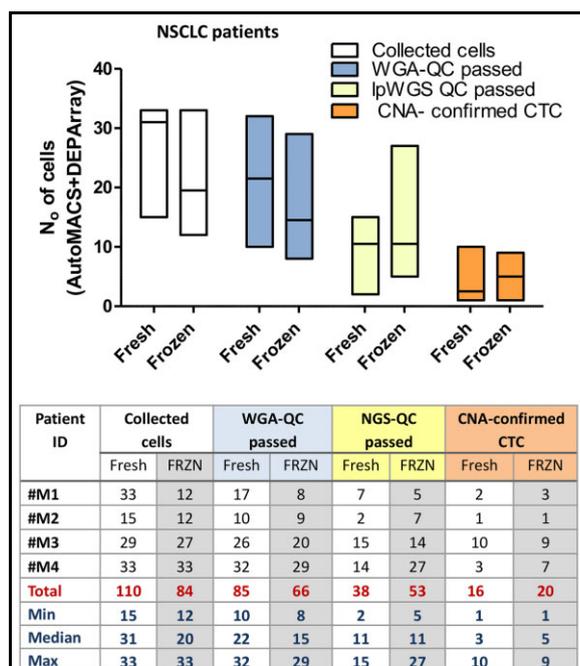


Fig. 2. Comparison of CTCs isolated by AutoMACS[®]+DEPArray[™] in fresh vs frozen PBMCs from NSCLC patients. *Top*: number of cells identified in fresh and frozen samples from patients with NSCLC (n = 4), evaluated at each step of AutoMACS[®]+DEPArray[™] workflow (floating bars represent minimum, median, and maximum values). *Bottom*: tables reporting for each tested patient and condition (fresh or frozen samples) (i) total numbers of cells collected by DEPArray[™] (white), (ii) cells that reached standard of adequate DNA quality tested by WGA QC (blue) and (iii) low-pass WGS QCs (yellow), and (iv) CNA-confirmed CTCs (orange).

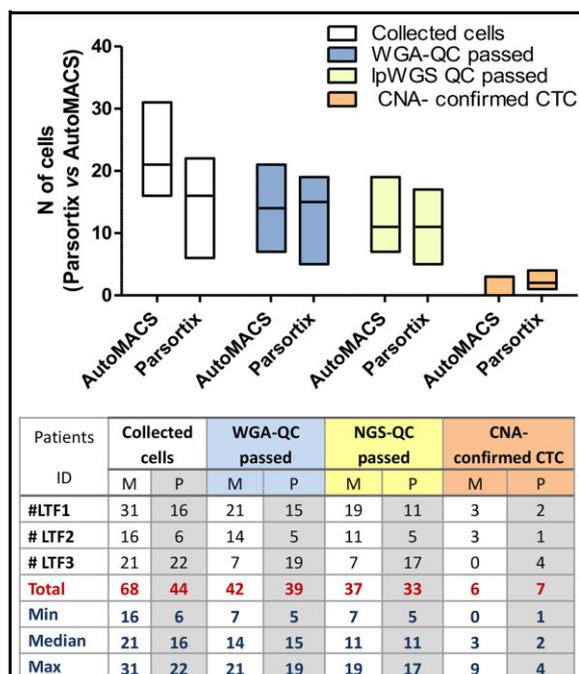


Fig. 3. Comparison of CTCs isolated by AutoMACS[®] or Parsortix[®]+DEPArray[™] from long-term stored PBMCs from NSCLC patients. *Top*: number of cells identified in long-term stored PBMCs from patients with NSCLC (n = 3), evaluated after either AutoMACS[®] or Parsortix[®]+DEPArray[™] workflows (floating bars represent minimum, median and maximum values). *Bottom*: table reporting for each tested patient and condition [AutoMACS[®] (M) or Parsortix[®] (P)]: (i) total number of cells collected by DEPArray[™] (white), (ii) cells that reached standard of adequate DNA quality tested by WGA QC (blue) and (iii) low-pass WGS QCs (yellow), and (iv) CNA-confirmed CTCs (orange).

MOLECULAR CHARACTERIZATION OF CTCs IN LUNG CANCER

To assess the analytical and clinical validity of the developed strategy, we tested the feasibility of detecting target mutations in CTCs isolated from WBS and stored PBMCs from NSCLC patients exploiting real-time PCR and dPCR (Supplemental Fig. 5).

We analyzed CNA-confirmed CTCs isolated from a NSCLC patient harboring the MET-T1010L mutation (detected by Sanger sequencing in primary tumor DNA). Real-Time PCR analysis detected the mutant allele in 3 out of 11 CTCs (27%) isolated from fresh WBS and in 4 out of 15 CTCs (27%) from frozen PBMCs. Notably, dPCR analysis of primary tumor DNA showed that MET-T1010L mutation allele frequency was 30%, the same frequency of mutant cells detected in the circulation (Supplemental Fig. 6),

supporting the possible use of CTCs isolated from cryopreserved PBMCs to track primary tumor mutations.

Taking advantage of the high sensitivity of dPCR to detect target mutations at low frequency, we also tested samples enriched for CTCs obtained both by Parsortix[®] and AutoMACS[®] from 4 EGFR-mutated (Del E746-A750) patients, from whom 2 aliquots of long-term stored PBMCs were available (Supplemental Table 6, Supplemental Fig. 5). First, to assess the sensitivity of our strategy and to possibly estimate the number of CTCs in our pre-enriched samples, we generated a standard curve by spiking into 8 mL of healthy donor blood cells from the H1650 NSCLC cell line, harboring EGFR Del E746-A750 mutation, at scalar doses (50, 20, 5 cells). Spiked-in samples were then processed with AutoMACS[®] and tested by the dPCR technique, which detected the

Table 1. Phenotype of cells isolated from blood samples of NSCLC patients, at each step of the Parsortix® or AutoMACS® + DEPArray™ workflows.

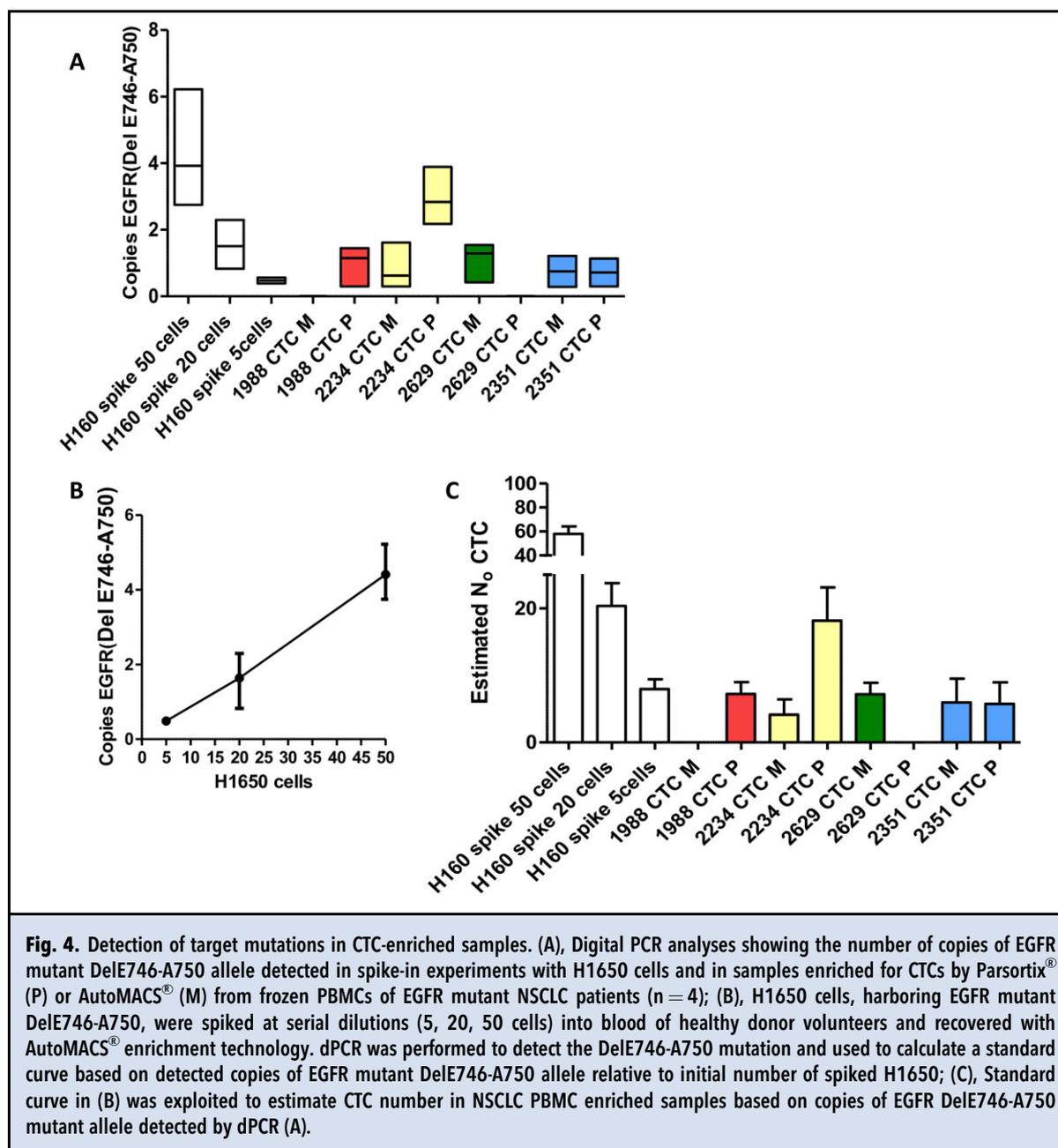
Patient ID	Collected		QCs passed		CTCs		QCs passed		CTCs		QCs passed		CTCs						
	Epithelial cells		Epithelial cells		CSC ^a cells		CSC cells		CSC		Nonepithelial cells		Nonepithelial cells						
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen					
#P1	4	0	1	/	0	/	7	2	0	1	/	0	0	20	14	18	10	3	2
#P2	0	0	/	/	/	/	1	0	1	/	1	/	1	15	6	10	5	2	1
#P3	4	0	2	/	0	/	3	3	0	1	/	0	14	19	5	16	0	4	
#P4	0	0	/	/	/	/	1	0	0	/	/	/	9	7	9	4	1	0	
#M1	0	1	/	0	0	4	1	0	0	0	0	0	29	10	7	5	2	3	
#M2	0	0	/	/	/	0	0	/	/	/	/	/	15	12	2	7	1	1	
#M3	2	3	1	1	1	0	6	8	1	2	0	1	21	16	13	11	9	8	
#M4	0	0	/	/	/	/	5	12	1	9	0	3	28	21	13	18	3	4	
Total	10	4	4	1	1	0	27	26	3	13	1	4	151	105	77	76	21	23	
Median	0	0	1	1	0	0	4	2	0	1	0	0	18	13	10	9	2	3	

^aCancer stem cell.
/ = no cells available for analysis.

Table 2. Phenotype of cells isolated from blood samples of sarcoma patients, at each step of the Parsortix® or AutoMACS® + DEPArray™ workflows.

Patient ID	Collected		QCs passed		CTCs		QCs passed		CTCs		QCs passed		CTCs					
	Epithelial cells		Epithelial cells		Mesenchymal cells		Mesenchymal cells		Mesenchymal		Nonconventional cells		Nonconventional cells					
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen				
#S2	0	0	/	/	8	22	7	19	0	2	16	11	14	11	2	0		
#S3	3	6	2	6	0	3	23	3	22	0	2	7	4	6	4	0		
#S4	1	6	1	5	0	2	5	24	4	22	0	0	2	5	1	5	0	1
#S6	4	13	2	13	0	7	12	5	11	3	1	6	1	6	1	1	0	
#S7	5	8	3	5	0	3	16	9	15	8	2	2	8	12	7	10	3	2
#S11	3	1	2	1	0	0	20	21	17	16	0	0	8	3	5	3	0	0
Total	16	34	10	30	0	5	59	111	51	98	5	7	47	36	39	34	6	3
Median	3	6	2	5	0	0	8	22	6	18	0	2	8	5	6	5	1	0

/ = no cells available for analysis.



target EGFR mutation in all samples, indicating sensitivity up to 5 CTCs/sample (Supplemental Fig. 7A).

Next, we performed dPCR analysis on 4 patients' PBMCs, enriched in parallel by Parsortix® and AutoMACS®. Overall, we were able to detect EGFR mutant alleles in all patients, of which 3/4 using Parsortix® and 3/4 using AutoMACS® enriched samples. In 2/4 patients, we identified EGFR mutation in both samples enriched by Parsortix® and AutoMACS® (Fig. 4A, Supplemental Fig. 7B, Supplemental Table 6).

Using H1650 cell-based standard curve, we inferred CTC numbers from detected EGFR mutant copies, and we estimated a median value of n = 6 CTCs (0–23) in CTC-enriched samples (Fig. 4, B and C).

Discussion

In the current study we found that enumeration and phenotypic and molecular characterization of CTCs at the single-cell level is feasible from cryopreserved

PBMCs by using marker-independent (Parsortix[®]) or negative marker (AutoMACS[®]) selection strategies.

We focused on samples from patients with sarcoma and NSCLC, 2 clinical settings where EpCAM+ CTC enumeration based on the Food and Drug Administration–approved CellSearch[®] system has been ineffective so far in providing clinically useful information (8, 32). NSCLC CTCs may evade EpCAM-based detection due to the acquisition of mesenchymal traits and loss of epithelial markers (9, 10, 33), whereas primary sarcomas could undergo mesenchymal to epithelial transition to enhance their metastatic potential, thus releasing into circulation both mesenchymal and epithelial CTCs (34). Therefore, EpCAM-independent enrichment may represent the best approach to capture the entire spectrum of CTC subpopulations.

Here, both Parsortix[®] and AutoMACS[®] CTC-enrichment strategies were coupled with downstream DEPAarray[™] technology to investigate CTC heterogeneity at the single-cell level. Single putative CTCs were sorted according to the lack of expression for hematopoietic markers and possible expression of epithelial, mesenchymal, or cancer stem cell markers and then subjected to low-pass whole genome sequencing to confirm their tumor origin. Even if slightly lower isolation yields were observed from frozen samples in spike-in experiments with cancer cell lines, in blood samples from cancer patients no striking differences were observed in numbers of CTCs isolated from fresh vs frozen samples as well as in CTC genotype and phenotypes.

These data should be interpreted with caution due to the limited number of analyzed samples and to the potential strong impact of sampling errors when dealing with rare events. Additionally, the different staining strategies and selection criteria used for CTC identification in NSCLC and sarcomas do not allow a direct comparison to evaluate whether freezing procedure could specifically impact CTC recovery in different tumor types.

Our study provides preliminary evidence that these 2 strategies are equally effective in recovering CTCs in long-term stored PBMCs, although AutoMACS[®] separation may result in samples with lower quality DNA.

Both strategies, regardless of fresh or frozen protocols, can inform on the heterogeneity of CTCs in NSCLC and sarcoma. Interestingly, our data suggest that EpCAM- (mesenchymal) and CD133+ (stem-like) NSCLC cells may have a greater advantage in entering the circulation than EpCAM+ epithelial differentiated tumor cells, data consistent with those reported in different types of cancer (7, 35).

Compared with previously reported results supporting the possibility to detect CTCs in cryopreserved PBMCs by relying on CTC phenotypic definition only (18–20), our study moves a step forward demonstrating

the feasibility of characterizing single CTCs at the phenotypic and genomic level.

Importantly, our stringent criteria for CTC definition based on presence of genomic alterations (CNA) highlight that a low percentage of cells selected according to their phenotype are bona fide CTCs. This result underlines the need for molecular characterization of CTCs defined solely on the basis of negativity for hematopoietic markers and/or positivity for EpCAM/CKs since not all of these cells are effectively malignant. Indeed, the detection of CD45-/EpCAM+/CK+ normal circulating epithelial cells has already been reported in patients with lung cancer, chronic obstructive pulmonary disease, or benign inflammatory colon diseases (36–38).

As further support for the clinical relevance of using frozen PBMCs for CTC characterization, we found it possible to detect by dPCR clinically relevant mutations directly in DNA extracted from Parsortix[®] or AutoMACS[®] CTC-enriched samples, avoiding the costly and time-consuming steps of single-cell isolation and WGA. Preliminary data suggest a good concordance of EGFR mutation detection in samples from cryopreserved PBMCs and primary tissue. This strategy, even though not informative on mutation frequency, may aid clinical assessment of treatment decisions by tracking druggable mutations in CTCs that are directly involved in metastatic dissemination.

In conclusion, despite the limited number of samples analyzed, this study demonstrates the feasibility of CTC analyses in cryopreserved PBMCs and represents an advance in blood sample management for CTC studies, allowing for a better selection of informative time points to longitudinally investigate tumor progression/response to therapy thereby enabling retrospective studies.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: CTCs, circulating tumor cells; EpCAM, epithelial cellular adhesion molecule; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cells; WBS, whole blood sample; CKs, cytokeratins; WGA, whole genome amplification; CNA, copy number alterations; dPCR, digital PCR; QC, quality control.

Human Genes: *EGFR*, epidermal growth factor receptor; *MET*, MET proto-oncogene, receptor tyrosine kinase.

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