

Dual-procedural separation of CTCs in cutaneous melanoma provides useful information for both molecular diagnosis and prognosis

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Abstract

Background: Circulating tumor cells (CTCs) have recently emerged as a new dynamic soluble marker for several malignancies including cutaneous melanoma (CM) and are suitable for prognostic evaluations and treatment monitoring. However, to date many limitations still hamper the wide-scale application of CTCs in CM setting, including the lack of standardized methods as well as both low levels and heterogeneity of these cells.

Methods: We developed a protocol for CTC detection in CM based on immune-magnetic sorting to deplete CD45-, CD31- or CD34-positive cells, followed by dielectrophoretic DEPArray separation according to cell morphology and immunophenotype. To this end, we explored the expression of melanoma stem cell antigens (CD271, ABCB5, and RANK) and the epithelial-to-mesenchymal transition markers (N-Cad, -CD44, and -MCAM/CD146) on CTCs from 17 stage IV CM patients, and investigated their BRAF mutational status by droplet digital PCR.

Results: The number of CTCs isolated from CM patients ranged from 2 to 91 cells (38 ± 6.4) with respect to healthy donors ($p < 0.0002$). To confirm the melanoma origin of isolated cells, we observed an 80% agreement between their BRAF^{V600} mutational status and matched primary tumors. The characterization of the immune phenotype of isolated cells revealed high interindividual and intraindividual heterogeneity that was found to correlate with the clinical outcome.

Conclusions: The dual-step protocol of immune-magnetic sorting and subsequent dielectrophoretic DEPArray separation, turned out to be a suitable method to isolate viable CTCs from stage IV melanoma patients and enabled quantitative and qualitative analyses on these cells, which may deserve prospective evaluation for potential use in the clinical practice.

Keywords: Circulating tumor cells, EMT, liquid biopsy, melanoma, melanoma stem cells

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Introduction

The incidence of cutaneous melanoma (CM) has increased worldwide and a defined improvement of survival has been recently achieved by new treatments primarily including targeted agents and immunotherapy.^{1,2} The prognosis of CM mostly depends on the primary cancer histopathology features while circulating tumor cells (CTCs) are the underpinning resemblance for

metastatic dissemination.^{3,4} On the other hand, based on the tumor spreading risk in performing biopsies of metastatic sites, CTCs are currently considered a real surrogate of tumors. Nevertheless, molecular data from the excised primary CM are perhaps inadequate to reflect the evolution of the genomic landscape particularly in the subclinical progressing disease that evolves with high intratumor heterogeneity.⁵ To this regard, CTCs appear

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highly feasible for genomic and transcriptomic profiles of tumors as CM thus providing insights to the metastatic machinery as well as for monitoring the disease progression in relation to the amounts of CTCs, or for revealing the minimal residual disease by the MCAM/CD146 levels expression.⁶ Furthermore, CTCs have been proposed for the early identification of acquired mutations of resistance to new therapies.^{7,8} Other than CTCs, tumor cell-free (ct)-DNA, microRNAs, and exosomes are actually in intensive investigation in CM for prognostic use and selection of responders to antiprogrammed death (PD)-1 monoclonal antibody (MoAb) treatments.^{4,9,10}

Previous work in breast, colon, prostate and lung cancers has recently proved that CTC levels correlate with treatment outcomes, and their sorting by CellSearch has been previously standardized and translated to clinical use as CTC counts for prognostic purposes.¹¹ Similarly, the number of CTCs in melanoma patients has been correlated with prognosis, and suggests the complete lymph node dissection (CLND) in those with positive sentinel lymph nodes.^{3,7} However, melanoma cells express specific melanoma-associated antigens (MAAs) and are extremely heterogeneous in their genomic and transcriptome aberrations. Thus, it is fundamental to detect multi-MAAs on CTCs to improve their sensitivity and specificity in clinical use though the efficiency of their characterization is frequently denied by both the low number of recoverable cells, and their extended genomic abnormalities.

To optimize the recruitment of CM CTCs, original attempts were based on their capture by detecting specific MAAs such as Melan-A, MART-1, or MAGE-3, while subsequent work focused on the expression of MCAM/CD146 that is up-regulated during the epithelial-to-mesenchymal transition (EMT) process in the metastatic phase of melanoma. Recent reports, however, showed that other techniques including micro manufactured filters,^{12,13} CTC-iChip,¹⁴ Cluster-Chip,¹⁵ and the slanted spiral microfluidic device¹⁶ have been fruitfully adopted to isolate CTCs in CM. In addition, several CTC subsets displaying tumor-initiating or stemness properties are marked by Nestin, ABCB5 (ATP-binding cassette sub-family B member 5), CD133, CD271, and RANK,¹⁷⁻²¹ and their circulating levels have been variably correlated with the disease progression.

Here, we describe a novel method to isolate in a highly specific fashion CTCs from patients with metastatic CM. In addition, by detecting typical melanoma markers, we adopted the DEPArray technology and recorded an apparent linearity of CTC molecular features with the primary tumors as well as a correlation of peripheral counts with clinical outcome.

Methods

Study population

Peripheral blood from 17 hospitalized or out-clinic patients in treatment at the Medical Oncology Unit of the University of Bari Aldo Moro with stage IV CM, and 5 healthy donors were collected after written informed consent (Table 1). The study was carried out in accordance with the Declaration of Helsinki and the protocol was approved by the Independent Ethics Committee (Identification code ID: 44100). Histopathological and clinical data were collected and reported in Supplementary Table 1 (S1). The eligibility criteria for patient recruitment were (a) age ≥ 18 years and (b) diagnosis of metastatic CM and known BRAF^{V600} mutational status of the primary tumor. Enrolled patients were either systemic-treatment naïve or with radiological evidence of disease progression during systemic therapy for metastatic disease; the latter patients were enrolled at least 21 days after the last cycle of treatment. Exclusion criteria included a personal history of other malignancies and patients bearing NRAS or rare BRAF^{V600} (K, N, R) mutations.

Melanoma cell phenotyping

The specific melanoma antigenic profile was evaluated by flow cytometry in several melanoma cell lines, namely LCP, LCM, M-397, and WM115,⁶ and SKMEL2 (American Type Culture Collection, USA). The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin-glutamine (Thermo Fisher Scientific). After gentle cell detachment from culture flasks, the expression of membrane antigens was explored by incubating 1×10^5 cells with 5 μ l fluorescein isothiocyanate (FITC)-conjugated anti-CD271 (Bio-Legend, CA), anti-ABCB5 (Novus Biologicals, MN), and anti-RANK

Table 1. Demographics and major clinical features of enrolled patients.

Patient ID	Sex	Age at recruitment	BRAF status	Sites of distant metastases	Number of metastatic sites (<3; ≥3)	Previous systemic treatment	Number of total CTCs	Number of PE ⁺ CTCs	PFS (months)	OS (months)
231	M	43	V600E	bone, lung, liver, lymph nodes, subcutaneous tissue	≥3	dabrafenib + trametinib	74	56	0.3	0.3
292	F	55	wild type	brain, lung, liver, lymph nodes, subcutaneous tissue	≥3	none	74	27	0.4	0.4
297	F	60	V600E	bone, lung, lymph nodes, subcutaneous tissue	≥3	none	3	2	9.8	16.3
315	M	48	wild type	bone, lung, lymph nodes, muscle	≥3	none	91	3	15.7	15.7
349	M	53	wild type	brain, lung, lymph nodes, muscle	≥3	nivolumab	72	64	3.3	4.1
352	M	58	V600E	brain, liver, bone, lymph nodes, parotid gland	≥3	dabrafenib + trametinib	75	59	7	8.2
369	M	77	V600K	brain, liver, bone, lung	≥3	dabrafenib + trametinib	45	41	1.1	1.1
373	F	69	V600E	liver, lymph nodes	<3	none	10	5	10.9	10.9
376	F	68	wild type	brain, liver, bone, lung	≥3	none	47	42	0.5	0.5
390	M	64	V600K	lung, lymph nodes	<3	none	13	4	11.4	11.4
393	F	80	V600E	brain	<3	none	3	2	1	2.6
416	M	63	V600E	subcutaneous tissue	<3	none	60	34	1.1	4.2
417	M	50	wild type	brain, bone, lung, lymph nodes, subcutaneous tissue	≥3	none	50	32	9.5	9.5
419	M	60	wild type	bone, liver, lymph nodes, adrenal glands	≥3	none	18	13	2.3	14.9
484	M	76	wild type	subcutaneous tissue	<3	none	11	10	1.7	1.7
521	F	38	V600E	brain, bone, lung, subcutaneous tissue, muscle, ovary	≥3	none	2	2	2.6	2.6
526	F	62	V600E	lymph nodes, subcutaneous tissue	<3	none	13	13	2.6	2.6

CTC, circulating tumor cells; OS, overall survival; PFS, progression free survival.

(Novus Biological) MoAbs, as well with phycoerythrin (PE)-conjugated anti-MCAM/CD146 (Becton-Dickinson), anti-N-cadherin (N-Cad; Invitrogen, CA), and anti-CD44 (Bio-Legend) MoAbs. After 30 min of incubation at 4°C, cells were analyzed for their antigenic profile by BD Accuri™ C6-plus (Becton Dickinson, CA). Each experiment was performed in triplicate by determining the positivity with respect to the relative isotype controls.

Melanoma cell line spiking and CTC isolation

A preliminary phase required the instrument set-up that was completed using *in vitro* established cell lines. For this purpose, both LCM and M-397 melanoma cells were used in relation to both expression of MAAs and assessment of specificity and efficiency of the DEPArray-based isolation.²² Briefly, 10, 50, and 100 viable melanoma cells were spiked into 10 ml of peripheral blood from healthy donors and separated by Ficoll density gradient. Then, blood mononuclear cells were depleted by negative immune-magnetic sorting^{23,24} with microbeads conjugated with both anti-CD45 and antiglycophorin (AutoMACS®, Miltenyi, Germany). The next set of experiments was dedicated to select melanoma cells by DEPArray technology (Menarini Silicon Biosystem, Italy) after their incubation with fluorochrome-conjugated MoAbs. For this purpose, two cocktails of MoAbs were used, respectively FITC- (anti-CD271, -ABC5, and -RANK) and PE-conjugated (anti-N-Cad, -CD44, and -MCAM/CD146) while both contaminating mononuclear cells and endothelial cells were excluded by a cocktail of APC-conjugated MoAbs (anti-CD45, -CD3,1 and -CD34; Invitrogen). In particular, 5 µl of each fluorescent antibody were added to the enriched samples, followed by 30 minute-incubation at 4°C.

Nuclei were visualized by 20 µg/ml Hoechst 33342 as a cell-permeable DNA dye that served also to exclude apoptotic and dead cells. Then, specimens were analyzed by dielectrophoretic DEPArray system after sample loading into dedicated cartridges according to the manufacturer's instructions. The cartridge loaded with cells was scanned under an automated fluorescence microscope to generate an image gallery by the Cell Browser Software favoring the selection of cells in relation to specific fluorescence pattern. Selected cells were then transferred throughout dielectrophoretic cages, recovered and collected as pool of

cells. For the CTC isolation, we used 10 ml of blood from patients and healthy donors while the recovered CTCs were numbered and stored at -80°C for molecular analysis.

Molecular characterization of CTCs

The mutational status of putative CTCs was investigated by Droplet Digital polymerase chain reaction (ddPCR) by using the ddPCR Supermix for probes (no dUTP, Bio-Rad) as well as ddPCR Mutant Assay BRAF^{V600E} and BRAF^{wild type} (Bio-Rad). The amplification was completed in the T100 thermal cycler (Bio-Rad) while the fluorescence intensity of individual droplets was investigated by the QX200 droplet and data analyzed by the QuantaSoft software. DNA was purified by Single Shot Cell Lysis Kit (Bio-Rad) according to the manufacturer's instructions. Detection of BRAF V600E mutation by ddPCR was carried out by using ddPCR Supermix for Probes (no dUTP, Bio-Rad) and validated Prime PCR ddPCR Mutation Assays including BRAF MUT V600E-FAM probe (Id: dHsaCP2000027, Bio-Rad) and BRAF WT V600E-HEX probe (Id: dHsaCP2000028, Bio-Rad). ddPCR reaction consisted of 10 µl 2X ddPCR Master Mix (Bio-Rad), 1 µl lBRAF MUT V600E-FAM probe, 1 µl BRAF WT V600E-HEX probe, 5 µl sample DNA and 3 µl deionized water. This solution was converted to droplets with the QX200 droplet generator (Bio-Rad). Amplifications were performed in T100 thermal cycler (Bio-Rad) with the following cycling conditions: 1 × (95°C for 10 min), 40 × (94°C for 30 s, 55°C for 1 min), 1 × (98°C for 10 min) with 2°C/s ramp rate. After PCR, the fluorescence intensity of individual droplets was analyzed with the QX200 droplet (Bio-Rad) and the data analysis was performed with Quanta Soft analysis software (Bio-Rad).

Statistical analysis

The Kruskal–Wallis (nonparametric) test explored the difference of total CTC levels between groups of patients stratified for clinical parameters or BRAFV600 status. The Kaplan–Meier analysis of survival was based on the number of CTCs per 10 ml of blood at baseline. For all survival analyses, the progression-free survival (PFS) was calculated as intervals between the first dose of targeted or immunotherapy after the enrolment and the date of disease progression, death, or the last follow-up visit, while overall survival (OS) was the interval between the treatment and death or the

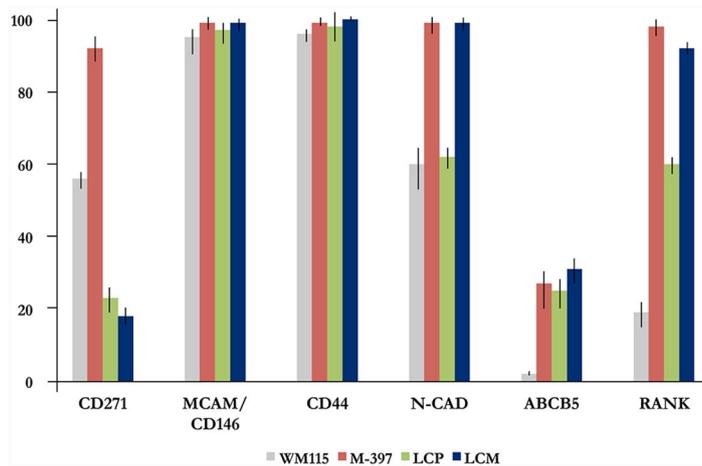


Figure 1. Phenotypic characterization of melanoma cell lines. Metastatic (M-397 and LCM) and not-metastatic (WM115 and LCP) cell lines were analyzed for the expression of melanoma associated antigens. The panel shows the high expression of both MCAM/CD146 and CD44 (>95%) by most cells. The marker of stemness CD271 was over-expressed in M-397 (on average 92%) and moderately present in the other cell lines, whereas highest levels of RANK occurred in metastatic cells (>95%) in a fashion almost similar to N-Cad. In addition, the ABCB5 antigen had low expression except for WM115, which was negative. Histograms and standard deviations were calculated from biological triplicates.

last follow-up visit. Survival curves were compared with the use of log-rank testing. To select a cut-off of CTCs that most clearly distinguished patients with different prognosis, thresholds of 2–91 total baseline CTC levels were systematically correlated with OS for the 17 patients enrolled in the study. Then, median OS among patients with levels above each threshold was calculated, while hazard ratio served to select the appropriate cut-off able to stratify patients with different prognosis, as already described.^{11,25} The statistical analyses were completed with the Medcalc software (version 12.7.0.0) accepting as statistically significant $p < 0.05$.

Results

Patients

Overall, patients were followed up for a median period of 4.8 months. The male-to-female ratio was 1.42, while median age at the time of CTC collection was 60 years. At the time of enrollment all patients had a stage IV CM, while at the time of melanoma diagnosis their clinical stage ranged from IB to IV. Most patients (8/17) had a nodular melanoma that also exhibited polypoid features in 2 out of 8 cases, whereas a superficial spreading melanoma occurred in seven patients. The BRAF^{V600E} mutation was detected in 10 out of 17 patients. The most frequent primary tumor sites were trunk and extremities, while a single patient

had an unknown primary melanoma. At least three different anatomical sites of distant metastases were described in 63% of patients. A total of 13 out of 17 patients were naïve from systemic therapies, three patients had received anti-BRAF and anti-MEK agents whereas one patient progressed from anti-PD1 immunotherapy (Tables 1 and S1).

Melanoma cell phenotype and spiking experiments

Preliminary experiments were completed in melanoma cell lines to define the expression levels of melanoma-related molecules as well as to select those useful for the CTC isolation. As shown in Figure 1, not metastatic (WM115 and LCP) and metastatic (M-397 and LCM) melanoma cells showed different levels of those antigens. Both CD44 and MCAM/CD146 were highly expressed by all populations ($96.1 \pm 2.3\%$) while the expression of the stem cell marker CD271 was high in M-397 (92% on average) and similarly lower in the other cells ($21.3 \pm 18.5\%$). Moreover, RANK was mostly detected in metastatic melanoma cells as compared with the others, and ABCB5 was undetectable in WM115 cells and expressed in 25%, 27%, and 31% of LCP, M-397, and LCM cells, respectively. Finally, melanoma cells exhibited a mesenchymal phenotype with N-Cad expression detected in more than 90% of LCM and M-397 cells, whereas lower levels were

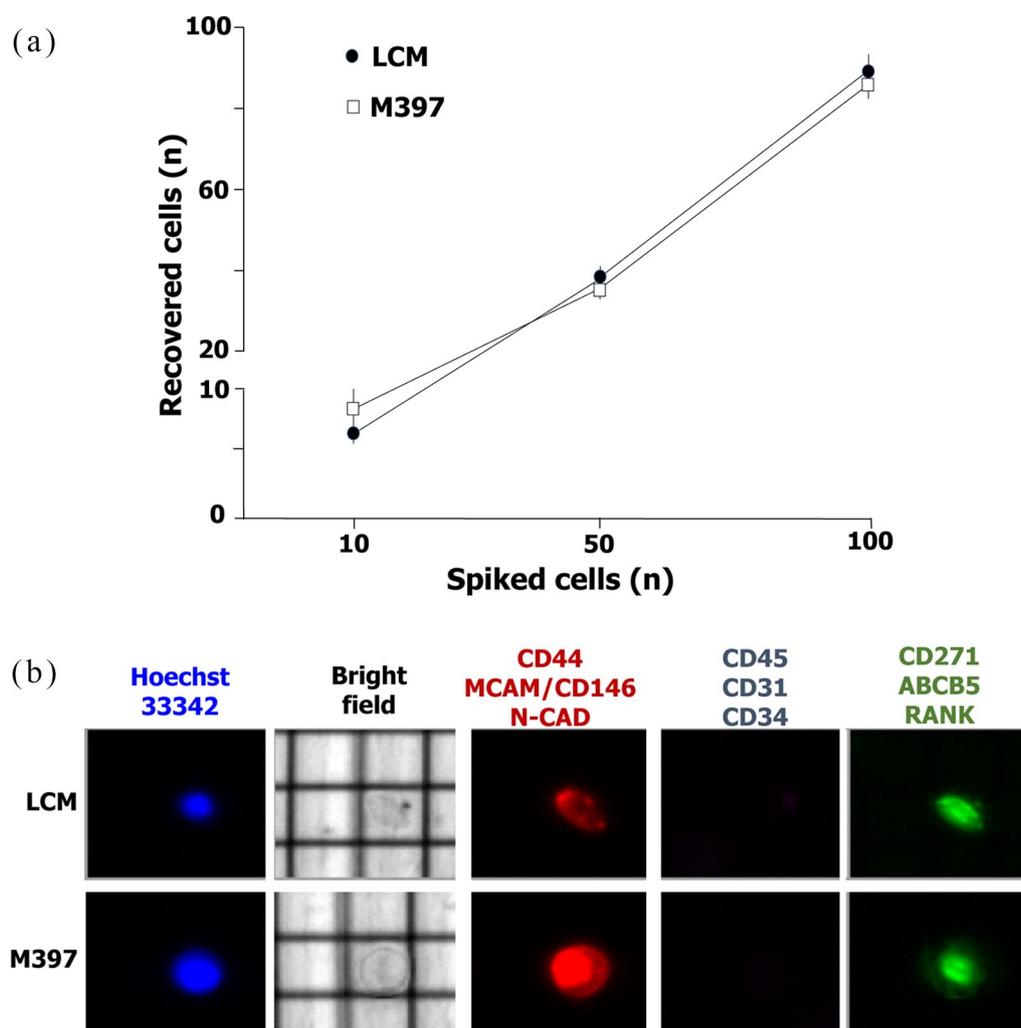


Figure 2. Detection of melanoma cell lines by DEPArray technology. Based on the melanoma associated antigen profile, both LCM and M-397 were selected for the spiking experiments required for the evaluation of performance in circulating tumor cell detection. As shown in (a), we detected $79 \pm 7\%$ of spiked ($n = 10, 50, 100$) M-397 (empty box) and $81 \pm 5\%$ of LCM (black box). After capturing by DEPArray, melanoma cells were characterized (b) in relation to their size as well as nucleus/cytoplasm ratio and nuclear integrity by Hoechst-33342 staining. Representative cell lines were negative for CD45, CD31 and CD34 expression. By contrast, both cell lines were stained by a cocktail of PE-conjugated (anti-CD44, -MCAM/CD146 and -N-CAD) or FITC-conjugated (anti-CD271, -ABCB5 and -RANK) monoclonal antibodies.

demonstrated in WM115 and LCP (59% and 63%). Based on the prevalent expression of these markers by LCP and M-397 cells, they were selected for next experiments.

The evaluation of the DEPArray performance in capturing rare CTCs permitted to detect at least $79 \pm 7\%$ of spiked M-397 and $81 \pm 5\%$ of LCM (Figure 2) and the recovery rate was independent of the number of spiked cells. The viability of spiked melanoma cells from lines was evaluated by nuclear staining with Hoechst in parallel with the previously mentioned surface antigens (Figure 2b), whereas Hoechst⁺ cells expressing at least

one of them among CD45, CD34 and CD31, were defined as peripheral mononuclear cells. As shown, the phenotypic features of melanoma cells allowed their efficient capture by combining MoAbs specific²⁶ for mesenchymal (CD44, MCAM/CD146 and N-Cad) or melanoma stem cell antigens (RANK, ABCB5 and CD271) that resulted in them being over-expressed.

Melanoma CTC recovery by DEPArray technology

After capturing by DEPArray, melanoma cells were further identified in relation to their

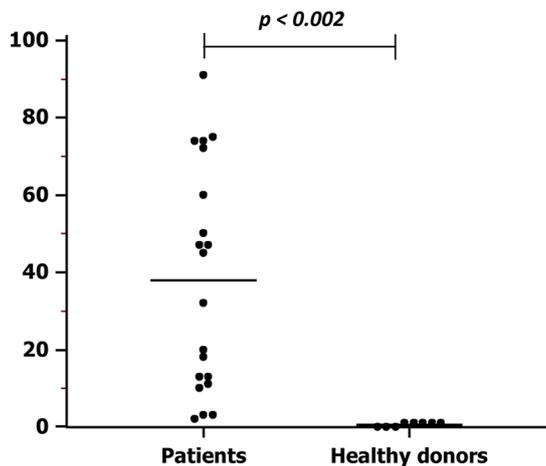


Figure 3. Numbers of circulating tumor cells in melanoma patients and healthy donors. The average number of CTCs isolated from patients with metastatic melanoma was higher (mean: 38.0 ± 6.4 cells; range 2–91 cells) with respect to that obtained from healthy subjects (mean: 0.62 ± 0.52 cells; range 0–1 cells) by using the same cocktail of monoclonal antibodies ($p < 0.002$).

morphological features such as size, high nucleus/cytoplasm ratio, nuclear integrity, and negative expression of CD45, CD34 and CD31. Most isolated CTCs showed a variable size (diameter range: $8.6\text{--}13.3\mu\text{m}$) that was, however, smaller with respect to spiked melanoma cell lines (diameter range: $11.1\text{--}17.4\mu\text{m}$). As shown in Figure 3, the number of CTCs purified from melanoma patients ranged from 2 to 91 cells (mean 38 ± 6.4), thus being significantly higher as compared with healthy donors whose circulating cells were isolated with the same panel of antigens. In fact, the range was up to one single cell (mean 0.62 ± 0.32 ; $p < 0.0002$).

Furthermore, the phenotypic characterization of CTCs showed high heterogeneity of melanoma CTCs (Figure 4a and b). In all patients we identified CTCs expressing at least one marker belonging to the CD44/MCAM-CD146/N-cad cocktail, in the absence of RANK, ABCB5 and CD271 (PE⁺ CTCs; mean number/ml: 2.4; range/10ml: 1–64). In a single patient (#349) we found FITC⁺ CTCs with the RANK/ABCB5/CD271 positive phenotype. Moreover, in 10 out of 17 patients we identified ‘double positive’ (FITC⁺/PE⁺) CTCs, namely cells expressing at least one marker from both antibody mixtures (mean number of CTCs/ml: 1.3; range/10ml: 1–88), whereas 11 patients harbored ‘double negative’ (FITC⁻/PE⁻) CTCs (mean number of CTCs/ml: 1; range/10ml: 1–26).

In order to confirm the melanoma derivation of isolated CTCs, we searched the BRAF^{V600E} mutational status that showed an 80% of concordance with respect to the primary tumors. The BRAF mutational status was confirmed in all subgroups of collected CTCs in 4 randomly selected BRAF^{V600E} mutated patients. Moreover, in all CTC-enriched samples from BRAF^{wild type} patients, no mutant copies of the gene were detected by ddPCR. Figure 4c shows representative panels of BRAF^{V600wt} (left) and BRAF^{V600E} (right) CTCs.

Correlation between CTC features and clinical-pathological parameters

We next sought to determine any potential correlation among CTC characteristics, clinical and pathological features as well as the patients’ outcome. By arbitrarily defining 60 years as the cut-off for age, we found a statistically significant association of higher numbers of CTCs in younger than in over-sixty patients ($p < 0.05$), whereas no correlations were detected with either the BRAF^{V600E} mutation or peculiar histopathology aspects of the primary tumor. Similarly, we did not find any association between CTC counts and serum melanoma biomarkers, as lactate dehydrogenase and S100B (data not shown).

With respect to the prognostic role in melanoma, the number of metastatic sites was investigated for the potential correlation with CTC number, and more viable CTCs were observed in patients suffering of at least three different metastatic sites, although no statistically significant values were detected (data not shown). Moreover, the potential influence of systemic treatments on both CTC count and phenotype distribution was also evaluated. As shown in Figure 5 (a), the total CTC number was higher in pretreated patients ($p = 0.06$) who also harbored significantly higher numbers of PE⁺ CTCs (Figure 5b) than treatment-naïve ones ($p = 0.005$). The latter patients were then defined as responders or nonresponders (NR) to subsequent first line therapy with the purpose to reveal potential differences between these groups in terms of CTC counts and phenotype distribution. The preliminary analysis showed a trend to higher amounts of both total and PE⁺ CTCs in NR patients (Figure 5c), which might deserve further investigation for predictive purposes.

Finally, we investigated the association between CTC number and clinical outcome in terms of

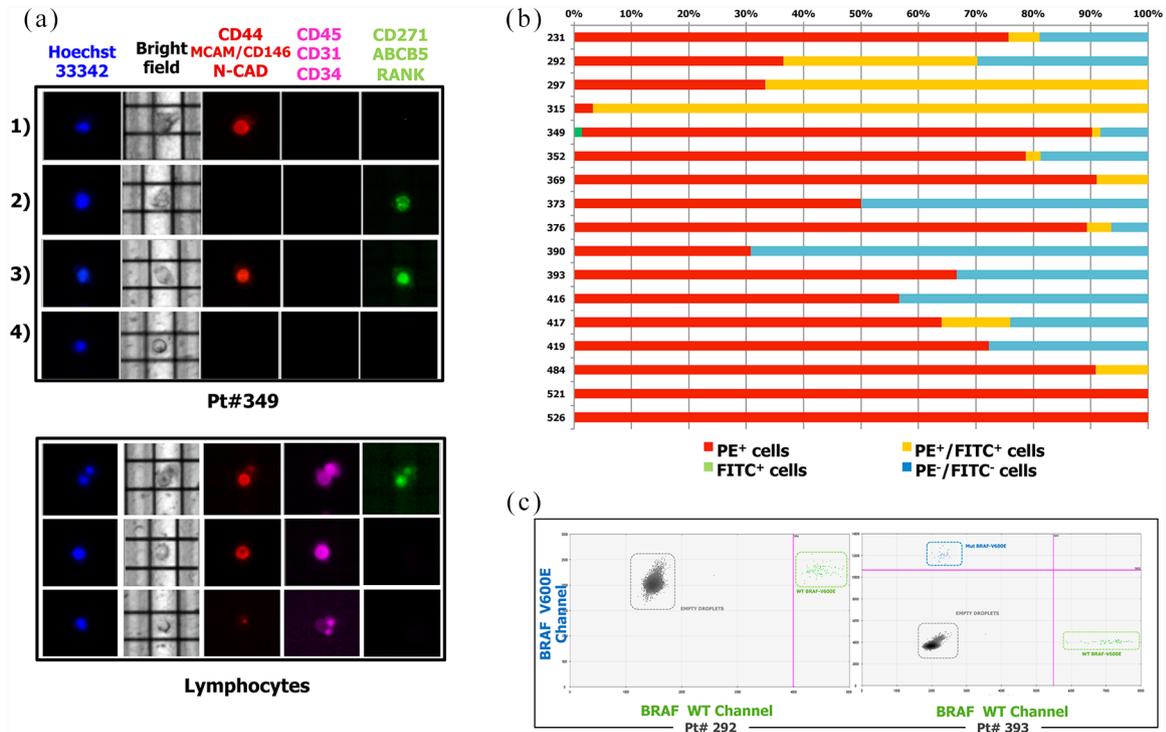


Figure 4. Detection of circulating tumor cells (CTCs) in melanoma patients. (a) shows representative patterns of CTCs isolated from a patient with metastatic melanoma (#349). As shown, nuclei were not damaged (Hoechst-33342 positive cells) while all isolated populations were negative for the expression of CD45, CD31 and CD34. Line 1 reveals PE⁺ CTCs (CD44⁺ or CD146⁺ or NCAD⁺), line 2 the positivity for the FITC-conjugated monoclonal antibodies (CD271 or ABCB5 or RANK), line 3 the double positivity (PE⁺/FITC⁺) and line 4 the CTCs negative for all the mentioned antigens. The bottom panel in (a) shows the negative control represented by lymphocytes detected by the expression of CD45 or CD31 or CD34. (b) shows the phenotypic profile of CTCs isolated from the 17 patients with metastatic melanoma. (c) is representative of the mutational assessment by Droplet Digital polymerase chain reaction (ddPCR) of CTCs from two patients previously investigated for the presence of BRAF^{V600E} mutation in primary tumor. The ddPCR analysis produced the same results obtained in primary melanoma specimens.

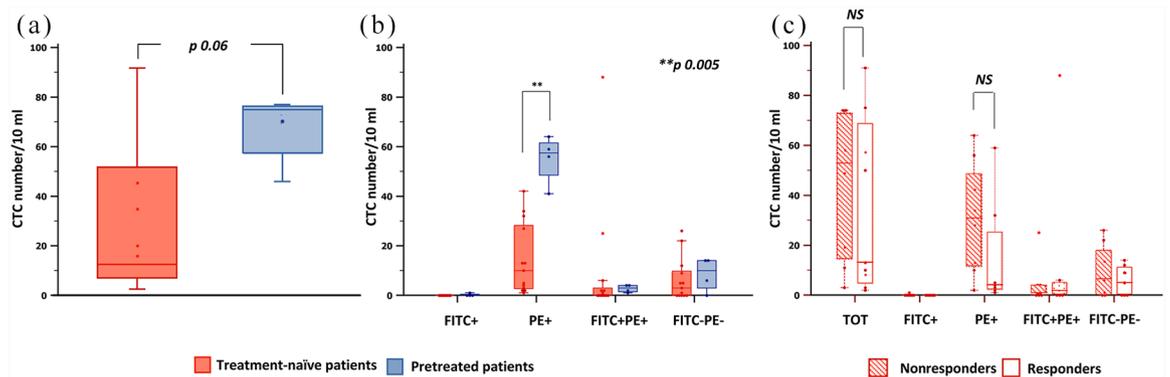


Figure 5. Correlation of circulating tumor cell (CTC) number with clinical and laboratory parameters. As shown in (a), the total number of CTCs was higher in pretreated patients with respect to the naïve ones ($p < 0.05$) and showed higher numbers of PE⁺ CTCs ($p < 0.05$; b). By dividing patients into responders (R) and nonresponders (NR) following the first line therapy (c), a trend to the increase of PE⁺ CTCs was demonstrated.

both PFS and OS, respectively (Figure 6). The median OS among patients with levels above or below each threshold differed for each cut-off of CTCs and reached a plateau at approximately 20

CTCs per 10 ml of blood. Thus, a cut-off of 20 CTCs was arbitrarily used to separate patients with unfavorable from those with favorable prognosis. We observed that the total CTC count was

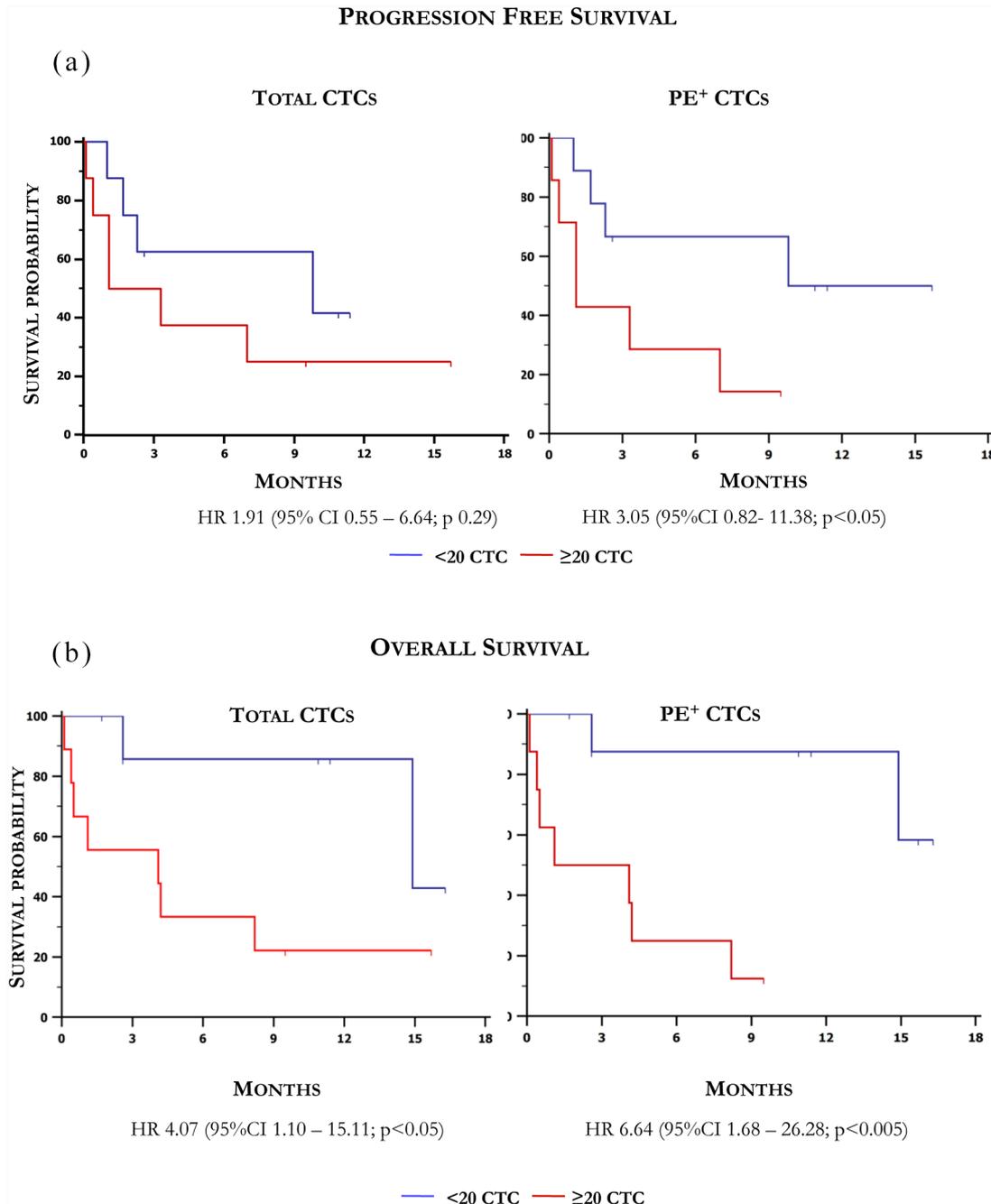


Figure 6. Correlation of circulating tumor cell (CTC) number with outcome. To verify the impact of CTC release on both PFS and overall survival (OS), a cut-off of 20 CTCs per 10 ml of blood was arbitrarily applied. As shown in (a), the CTC count was not prognostic for the calculation of the PFS (right), whereas the presence of a number of PE⁺ CTC > 20 was associated with a worse outcome ($p < 0.05$; left). Moreover, a number of CTC higher than 20 was correlated with shorter OS (b), which was further worsened in patients whose CTCs expressed a PE phenotype ($p < 0.05$). CI, confidence interval; HR, hazard ratio.

not prognostic for PFS, while the presence of at least 20 PE⁺ CTCs was associated with worse outcome (median PFS 1.1 months *versus* not reached, HR: 3.05, 95% CI: 0.82–11.38; $p < 0.05$) (Figure 5, panel a). Moreover, the

presence of ≥ 20 cells significantly correlated with shorter median OS, as compared with smaller amounts (4.1 *versus* 14.9 months, HR: 4.07, 95% CI: 1.10–15.11; $p < 0.05$). However, by limiting the analysis on PE⁺ CTC subset (Figure 5, panel

b), we estimated a median OS of 2.6 months in patients with at least 20 cells/10 ml as compared with those with fewer PE⁺ CTCs, for whom the median OS was not reached (HR 6.64, 95% CI: 1.68–26.28, $p < 0.005$).

Discussion

Metastasis onset is a hallmark of cancer and liquid biopsy became an innovative tool for the characterization of genetic signature of CTCs, thus providing predictive and prognostic information as well as options for tailored therapeutic strategies. We standardized a new method for the isolation of CTCs in metastatic melanoma based on dual immune-magnetic selection and DEPArray separation and propose peculiar CTC phenotypes for potentially prognostic purposes.

The prognostic role of CTCs has been well established in several malignancies since the CTC enumeration is being used for monitoring the outcome in advanced, refractory, and early solid cancers as well as for identifying indolent or aggressive tumor genotype. CTCs act as biomarker for the disease progression in breast and metastatic castration-resistant prostate cancer and have been suggested as mirror of the tumor heterogeneity in lung, prostate, and colon cancer.^{27–30} A similar contribution emerged in other neoplasms including noncutaneous and CM although the results have not been translated into the clinical practice. A modest predictive role has been demonstrated in CM and few studies suggest a possible application of CTC enumeration in patients bearing stage III of the disease. By contrast, a prognostic role in the metastatic setting emerged in the majority of studies, with a variability that regards the optimal cut-off. Furthermore, a peculiar association between CTC enrichment in peripheral blood and liver metastasis has been revealed in uveal melanoma.^{8,31–35}

The lack of consensus on the best method to be employed for CTC detection in melanoma still hampers their routine application in the clinical setting. To this regard, many technologies have been attempted, including filtration-based, microfluidic, and dielectric procedures to obtain CTCs according to their physical properties, while immune phenotyping of these cells has also been proposed for their detection and enrichment.^{36,37} In this context, Hong and colleagues have recently developed a 19-gene RNA

signature applied to microfluidically-enriched CTCs, for the longitudinal monitoring of stage IIIC/IV melanoma patients receiving immunotherapy and described a significant correlation between variations of the ‘CTC score’ and clinical outcome.³⁴ In addition, the detection by CellSearch Circulating Melanoma assay of at least 1 CTC per 7.5 ml of blood has been significantly correlated with poor PFS in metastatic melanoma.³³ However, a disagreement on the cut-off to be applied for prognostic purposes is today persistently considerable.^{3,31} Further challenges to be faced include the rarity of CTCs in the bloodstream and their heterogeneity, which may provide incomplete or inaccurate information.^{26,36–38}

In the last decade, EMT has emerged as one of the key mechanisms involved in cancer progression and metastasis^{39–43} and several researchers investigated its activation state by monitoring CTC phenotype, based on the expression of epithelial or mesenchymal markers, and their variations over time.^{38,44–46} Furthermore, recent evidence suggests that the acquisition of a mesenchymal phenotype by CTCs is often associated with stemness properties, which might result in both tumor-initiating capability and resistance to anticancer drugs.^{47–49}

Melanoma CTCs with stemness features have been described in both early stage and metastatic melanoma patients.²⁶ The latter apparently exfoliate more circulating melanoma cells with heterogeneous phenotypic profile, thus underlying the importance of multiple antigen detection for their characterization in the clinical setting. In this context, a phase III multicenter trial demonstrated the prognostic meaning of multiple MAA expression in CTCs from stage III melanoma patients after CLND.⁸

In our work, following the depletion of blood and endothelial cells by immune-magnetic sorting, the dielectrophoretic DEPArray system was employed for CTC detection mostly based on morphological and immunophenotypical features. In particular, we mutually detected CD271, ABCB5 and RANK stem cell antigens, while mesenchymal markers including N-cad, CD44 and MCAM/CD146 were investigated through a separate antibody mixture. In agreement with previous reports in melanoma and other malignancies,^{26,50–52} the majority of patients spread CTCs with mesenchymal features either alone or

in combination with stemness properties, that might account for their enhanced invasiveness and self-renewal potential.

In order to confirm the presence of melanoma cells in our CTC-enriched fractions, we tested them for BRAF^{V600E} mutation by ddPCR, due to the high sensitivity of this method.^{53,54} Hence, we verified whether or not both CTC detection and enumeration by DEPArray could be appropriate to establish any potential correlation between clinical-pathological parameters and CTC count, to be applied in subsequent studies on a wider patient series. Interestingly, we observed a significant association between CTC number and patient age, whose prognostic role in CM is still debated^{40,55,56} while, in accordance with previous reports,^{3,33} no correlation was found between the number of viable CTCs and primary tumor features, including BRAF^{V600E} status. By contrast, exploring a potential relationship with other well-known prognostic factors in CM as the number of metastatic sites we observed that CTC counts reflected the extent of metastatic disease.^{31,57-59} However, our small sample size did not reach statistical significance.

To the best of our knowledge, this is the first report in which immune-magnetic sorting and DEPArray technology were combined to detect and isolate viable CTCs in stage IV melanoma patients with the purpose to simultaneously investigate amounts, morphological patterns (e.g. nucleus/cytoplasm ratio, cell size, and nuclear integrity), phenotype, and molecular properties in melanoma. Our highly sensitive method also enabled the prognostic stratification of patients by using the cut-off of 20 CTCs/10ml, and suggested that, alongside the total CTC count, phenotypical features of these cells should be carefully evaluated, since the occurrence of at least 20 PE⁺CTCs with mesenchymal properties was associated, in our cohort, with a worse outcome in terms of PFS and OS and may deserve further investigation on a wider population. This is in line with current studies that support the role of EMT in melanoma metastasis and progression⁴¹ since subjects enrolled in our study were characterized by high disease burden in relation to their status of treatment naïve or relapsed metastatic patients.

In conclusion, here we described a dual-step procedure for the CTC recruitment in CM that may also validate this modality of liquid biopsy for the molecular characterization of this tumor. We

proved that the phenotype pattern of the CTCs is apparently correlated with prognosis in line with the evidence that their number reflects both PFS and OS in metastatic patients. These data are, however, preliminary and mostly based on a small sample size without longitudinal sampling in the same patients but in line with the most innovative research in melanoma. However, a larger study could be of great benefit for a definite validation of CTC enumeration by this new method with the final aim of monitoring the CM evolution including the development of drug resistance, thus permitting timely variations of the therapeutic strategies.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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