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Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: *KRAS* mutation status in pure CTCs

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ABSTRACT

The characterization of circulating tumor cells (CTCs) could substantially improve the management of cancer patients. However, their study is still a matter of debate, often due to lymphocyte contamination. In the present paper, an investigation of CTCs was carried out for the first time using DEPArray, a dielectrophoresis-based platform able to detect and sort pure CTCs. Analyses were conducted on peripheral blood (PB) samples from patients with metastatic colon cancer. After 100% pure cell recovery and whole genome amplification, *KRAS* gene mutation of CTCs was screened and compared to gene status in the primary tumor tissue. CTCs were found in 21 colon cancer patients (52.5%), with more than three tumor cells per 7.5 ml. *KRAS* gene mutation analysis, showed a mutational concordance between CTCs and primary tumor in 50% of matched cases. The present study demonstrates for the first time the feasibility of analyzing at the molecular level pure CTCs avoiding lymphocyte contamination using an innovative instrumentation, and a *KRAS* discordance between CTCs and primary tissue. Our results present dielectrophoresis-based procedures as a new standard in single cell analysis and recovery and invite careful reflection on the value of CTCs characterization.

1. Introduction

The study of circulating tumor cells (CTCs) has become a central area of research in oncology. The biological significance of CTCs in cancer stems from their potential role in the metastatic process which, in turn, is extremely important since the majority of cancer patients die from their metastases [1,2]. The timing of CTC shedding from the primary tumor, their potential genetic and phenotypical plasticity, evasion of therapeutic interventions and, finally, ability to give rise to new metastatic lesions in specific sites are important interconnected CTC features. It is very tempting to imagine that in the near future CTC detection and characterization could be considered as a "liquid biopsy" able to monitor disease progression and of defining the tumor at the molecular level through a simple blood sample. Such progress could substantially improve the management of cancer patients, paving the way for the personalization of targeted therapy strategies [3,4].

However, there is still no general consensus about the most accurate method to use for the detection of these rare cells. The perfect marker for CTC selection has yet to be identified, as has the best technical strategy for characterizing them. Moreover, the role of CTCs in the clinical setting has not been fully validated. In fact, although the number of CTCs would seem to be strongly correlated with overall (OS) and progression-free survival (PFS) in metastatic patients with different kinds of tumors [5,6], this parameter differs among studies and its relevance is still a matter for debate especially with regard to disease monitoring. It has been suggested that the biomolecular characterization of CTCs could prove to be a more effective tool than the investigation of CTC numbers for personalized treatments and clinical surveillance, patient stratification and monitoring of disease status, better understanding of tumor biology and aggressiveness and identification of therapeutic targets. Thus, new methods for detecting and sorting pure CTCs from whole blood samples of cancer patients and for performing specific molecular assays avoiding normal cell contamination are undoubtedly worthy of being evaluated.

A better accurate identification of patients with metastatic colon cancer (mCRC) who could benefit from therapy is needed [7].

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During the past decade, this has been achieved by using *KRAS* mutational status of the tumor to predict patient response to cetuximab [8,9]. The possibility of studying CTC molecular profile in mCRC could be a further crucial step towards achieving a better and personalized management of patients.

In the present study we evaluated, for the first time, the feasibility of studying a specific molecular feature of pure CTCs thanks to an innovative di-electrophoresis-based platform able to handle small numbers of cells. The device provides the operator with the possibility of specifically evaluating and sorting rare cells thanks to an image-based selection process and to the entrapment of cells inside di-electrophoretic cages. Selected cells can be individually moved by software controlled modulation of electrical fields and eventually recovered for downstream molecular analysis. Our main aim was to carry out an investigation of this technological platform to identify and study rare tumor cells in whole blood samples from patients with mCRC. In particular, it was planned to evaluate *KRAS* gene mutation to find out whether mutations were present and whether gene status remained the same as that observed in the primary tumor tissue.

2. Materials and methods

2.1. Cell culture and spiking experiments

Studies were performed on A549 cell line derived from a human lung adenocarcinoma harboring a *KRAS* mutation, obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained as a monolayer at 37 °C and subcultured twice weekly. Culture medium was composed of HAM F12K supplemented with fetal calf serum (10%), glutamine (2 mM) (Mascia Brunelli) and insulin (10 mg/ml) (Sigma Aldrich). Cells were used in the exponential growth phase in all the experiments. To identify the percentage of tumor cell recovery of our method and to verify the ability of the DepArray system to recover cells, we spiked a specific number of A549 cells in 15 ml of peripheral blood (PB) from healthy donors for each test. Spiked cell number ranged from 5 to 300 cells per ml. (All spiking experiments were performed in duplicate to test the reproducibility of the cell recovery rate.)

2.2. Patients, tissue and blood collection

A total of 40 patients with metastatic colorectal cancer (mCRC) were recruited from the multicenter ITACA study (Eudract number: 2007-004539-44). Paraffin-embedded sections obtained from histological specimens of primary tumor or metastatic lesions were used for *KRAS* characterization. Peripheral blood (PB) samples were obtained at baseline, *i.e.*, before the start of treatment. Patients were classified on the basis of cancer subtype, age, time of surgery, histology, tumor size, lymph node status and tumor grade (Table 1). Analyses were carried out on samples of 15–20 ml of fresh PB collected from 10 healthy donors and from 40 mCRC patients recruited at IRST. Blood was drawn in EDTA tubes (BD Vacutainer; Becton Dickinson) and processed immediately. The first 3 ml of PB were discarded to avoid contamination with cytokeratin-positive normal skin epithelial cells from the site of needle puncture. Blood from patients was always collected at least 4 weeks after surgery, before the beginning of chemotherapy regimen. Study participants were informed of the investigational nature of this analysis and gave informed consent in accordance with institutional guidelines. The study protocol was approved by the ethical committee of our institute.

2.3. Tumor cell enrichment and staining

CTC enrichment was achieved by density gradient centrifugation using OncoQuick (Greiner BioOne), performed in accordance with the manufacturer's instructions. Cells were then fixed in 2% paraformaldehyde (Miltenyi Biotec, Bergisch Gladbach, Germany) and stained with antibodies directed against CD45 (Miltenyi Biotec) and a panel of cytokeratins (4, 5, 6, 8, 10, 13 and 18; clone C11, Aczon, Bologna, Italy). Cell nucleus counter-stain was accomplished with Hoechst 33342 (Invitrogen). Preliminary experiments performed in our laboratory demonstrated that our procedure was reproducible in detecting cancer cells suggesting a fairly high degree of specificity. In fact, using other tumor specific-antibodies, results were super-imposable with those obtained with C11 clone (data not shown).

2.4. Cell evaluation and recovery

Each enriched sample was manually transferred to an A300 K cartridge (Silicon Biosystems) consisting of a silicon chip directly interfaced to a microchamber containing cells. The cartridge was inserted into the DepArray system (Silicon Biosystems), scanned through an automated fluorescence microscope, and captured images were presented in a software-generated gallery. A cell was classified as CTC when its morphologic features (round or oval morphology with a round or oval visible nucleus within the cytoplasm) and staining patterns were consistent with those of an epithelial cell (Hoechst positive/CK pos/CD45 neg). CTCs then were moved to another part of the cartridge and recovered by routing the DEP cages through specific inbuilt software. At the beginning of the study, cells detected in the first few samples were recovered in small aliquots (5–10 cells). After having observed differences in the mutational status between cell groups in some patients, it was decided to recover at least 5 single cells and thereafter aliquots containing 5–10 cells. Cells with the highest mean positivity for panCK, measured as mean fluorescence intensity (PhycoErythrin), were selected as "single recoveries". Cells with lower positivity were recovered in aliquots.

Table 1
Clinical characteristics of patients.

		CTC presence (%)	
		Positive	Negative
Age (years)			
Average	63	21 (52.5)	19 (47.5)
>65	17 (42.5)	12 (70.6)	5 (29.4)
⩽65	23 (57.5)	9 (39.1)	14 (60.9)
Gender			
Male	24 (60.0)	11 (45,8)	13 (54,2)
Female	16 (40.0)	10 (62.5)	6 (37.5)
Site			
Colon	29 (73.2)	16 (55.2)	13 (44.8)
Rectum	11 (26.8)	5 (45.5)	6 (54.5)
Grade			
1	3 (7.5)	2 (66.7)	1 (33.3)
2	15 (37.5)	6 (40.0)	9 (60.0)
3	11 (27.5)	6 (54.5)	5 (45.5)
Unknown	11 (27.5)	7 (63.3)	4 (36.4)
T			
2	3 (7.5)	2 (66.7)	1 (33.3)
3	19 (47.5)	9 (47.4)	10 (52.6)
4	14 (35.0)	9 (64.3)	5 (35.7)
1 or X (unknown)	4 (10.0)	2 (50.0)	2 (50.0)
Node status			
–	8 (20.0)	3 (37.5)	5 (62.5)
+	32 (80.0)	18 (56.3)	14 (43.8)

Positive = three or more CTCs/7.5 ml PB.

2.5. KRAS sequence analysis

For CTC, **after** cell recovery, cells underwent whole genome amplification (Ampli1 Whole Genome Amplification Kit, Silicon Biosystems). Amplified DNA was used for *KRAS* gene analysis performed by direct sequencing and pyrosequencing methodologies. In the former, DNA was amplified for *KRAS* exon 2 with specific primers. PCR products were purified using the MiniElute PCR purification kit (Qiagen) and sequenced using BigDye Terminator 3.1 Reaction CycleSequencing kit (Applied Biosystems). Sequence reaction was purified using DyeEx 2.0 Spin kit (Qiagen) and separated by capillary electrophoresis with laser-induced fluorescence detection (3100 Genetic Analyzer, Applied Biosystems). Pyrosequencing analysis of exon 2 of the *KRAS* gene was performed on PyroMark Q96 ID (Qiagen) using anti-EGFR MoAb response (*KRAS* status) (Diatech), according to the manufacturer's instructions. For histological samples, areas containing at least 50% of tumor cells were identified in hematoxylin-eosin-stained tissue sections, after which 5-μM sections of the corresponding areas were macrodissected and collected in specific tubes. Cells were lysed in 50 mM KCl, 10 mM Tris–HCl pH 8.0, 2.5 mM MgCl2 and Tween-20 0.45% supplemented with proteinase K at a concentration of 1.25 mg/ml overnight at 56 °C. Proteinase K was inactivated at 95 °C for 10 min after which samples were centrifuged twice to eliminate debris. The supernatant was assessed for DNA quality and quantity by Nanodrop (Celbio) and then submitted to PCR amplification as described above.

3. Results

3.1. DEPArray evaluation, specificity and recovery assessment

Cell count, analysis and recovery were performed through the DEPArray device as shown in Fig. 1. During the assay, each dielectrophoretic cage generally contained single cells, a lymphocyte or a

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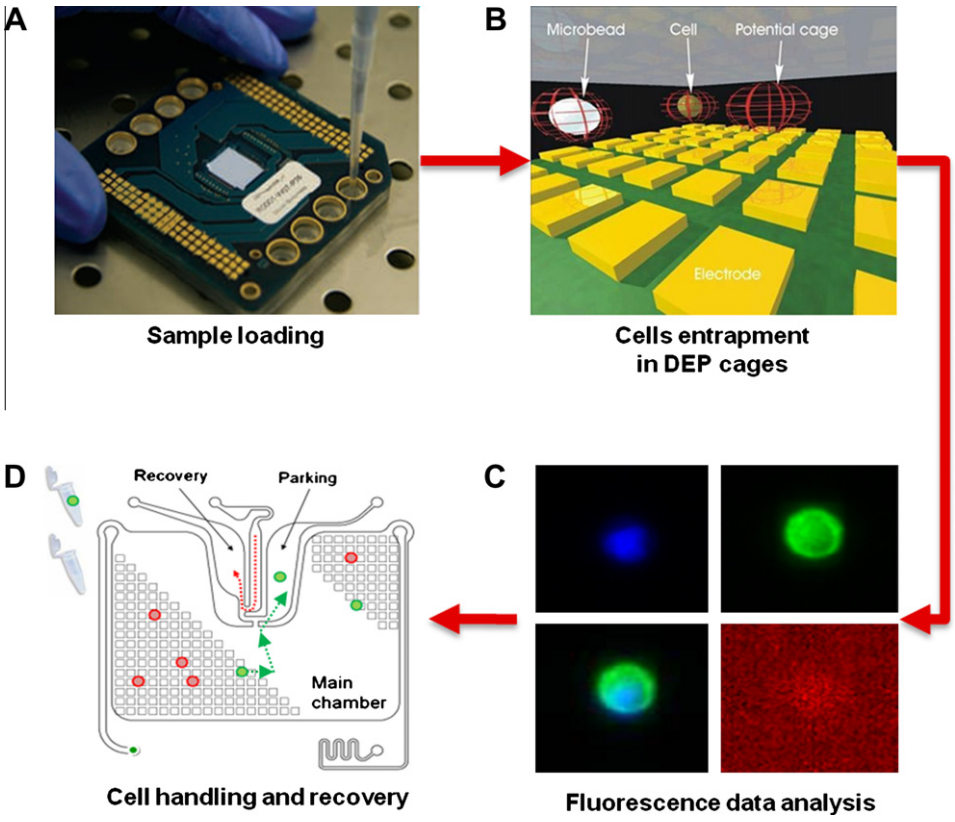

Fig. 1. Exemplificative diagram of DEPArray modus operandi. (A) Cell loading cartridge. (B) Schematic representation of the microchamber containing cells and of cell entrapment. (C) An A549 cell observed through different single fluorescent filters and filter merging. (D) Illustration of the microchamber containing cells and of the recovery path followed by selected cells.

CTC. When a cell was classified as a CTC, it was recovered as previously described. Specificity was tested by analyzing 15 ml of PB from 10 healthy donors with no history of malignant disease, which did not highlight more than 1 cell per ml with characteristics similar to that of epithelial cell (i.e. CK staining and absence of CD45 positivity) samples. The "probable" circulating epithelial cells found in the blood of healthy controls also showed very low CK staining intensity.

We evaluated the CTC detection percentage by performing 20 experiments in which a specific number of cells from the lung cancer cell line A549 were spiked into PB (range 5–300 spiked cells/ml PB). Overall, the cell recovery percentage was directly dependent on the number of spiked cells and ranged from 11.6% to 86.0%. However, spiked cells were detected in every tested sample. Independently of these considerations, we were always successful in sorting and recovering single A549 cells with 100% purity. An example of cells observed through the DEPArray is shown in Fig. 2 *KRAS* mutational analysis was performed on recovered A549 cells. DNA amplification was possible in more than 70% of single cell recoveries and the original *KRAS* mutation was always found in amplified samples.

3.2. Patients

Forty mCRC patients, irrespective of line of treatment received, were consecutively enrolled onto this preliminary prospective study. Clinical characteristics are reported in Table 1. Twenty-nine received first-line treatment, while eleven were recommended for second-line regimens.

3.3. CTC screening

CTCs were found in 21 (52.5%) patients (Table 2) with 3 or more CTCs/7.5 ml of PB. The mean number of CTCs per ml of PB was 7 (range 0.4–60). Interestingly, we also detected cells that had atypical nuclear and/or cytoplasmatic morphology in 12 (30.0%) patients. These cells showed a multinucleated nucleus with apparently condensed chromatin and/or dotted cytokeratin staining and, although not initially considered as true CTCs, they were also recovered. We plan to analyze them at the molecular level to identify their true nature (Fig. 2). No significant correlations were found between CTC number and clinical characteristics. Only a trend towards an association with age (>65 years) was observed.

3.4. KRAS sequence analysis in CTCs and tissue

KRAS gene mutation analysis was performed in 21 CTC-positive cases. Five CTC samples were not assessable due to unsuccessful DNA amplification. In the remaining 16 samples, mutational concordance was found in eight (50.0%) cases. Among the other eight cases, *KRAS* wild type CTCs where found in seven patients with mutations in the primary tumor. *KRAS* mutated CTCs were found in one patient harboring a wild type *KRAS* primary tumor (Table 2). Interestingly, different mutations were present in three groups of CTCs recovered from the same patient. Two mutations were found in one sample, one concordant with that of the primary tumor (G12D) and the second dissimilar (G12C). A third mutation was also found in a further sample from the same patient (G13D). This last result was confirmed in two independent assays.

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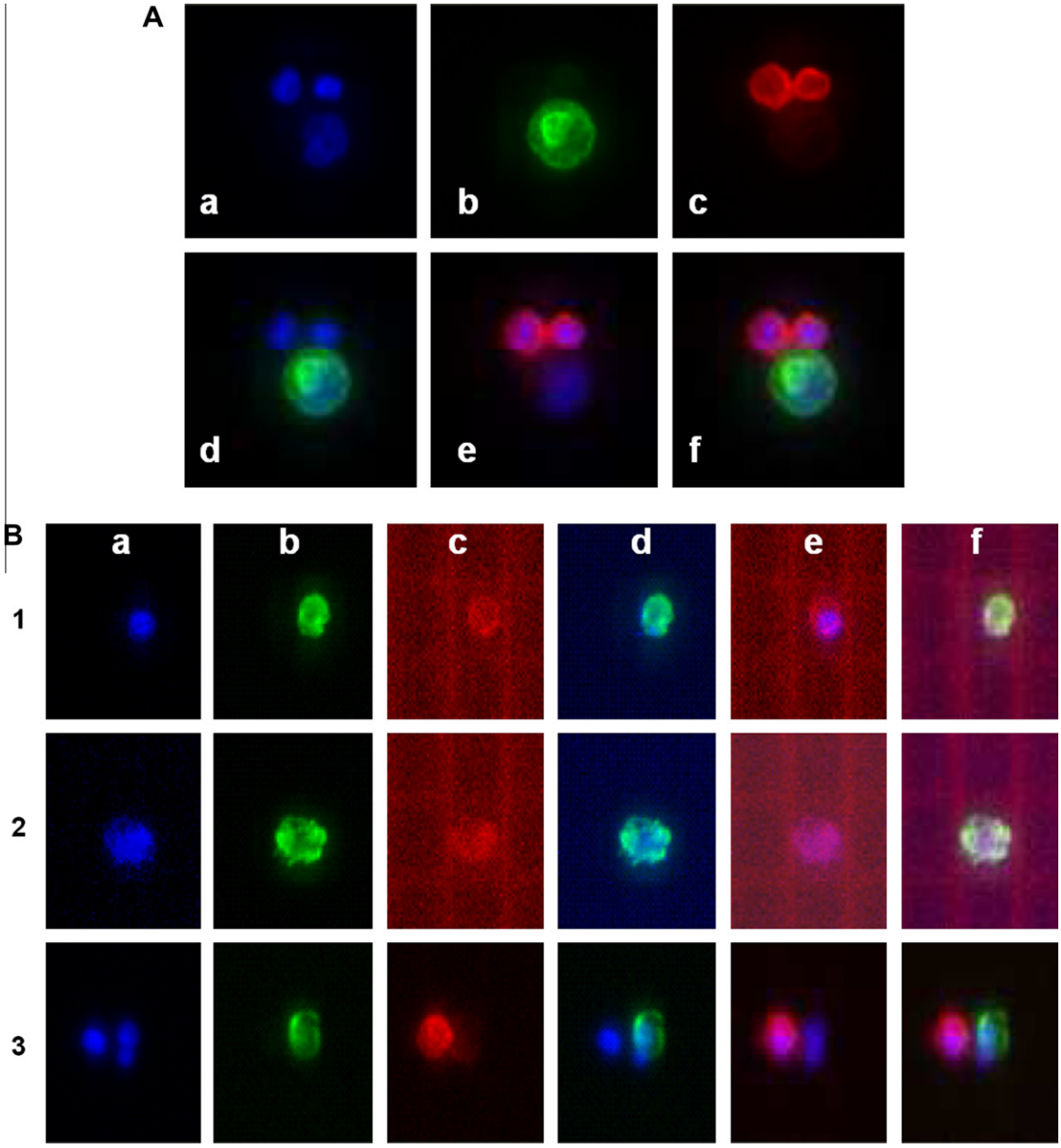

Fig. 2. (A) Exemplificative images of cells observed through the DEPArray software. The images depict an A549 cell together with two normal lymphocytes observable through different single fluorescent filters (a–c) and fluorescent filter merging (d–f). (B) Exemplificative images of CTCs observed through the DEPArray. The images depict three CTCs observable through different single fluorescent filters (a–c) and fluorescent filter merging (d–f). Line 1: single CTC; line 2: atypical CTC (see text); line 3: CTC with atypical nucleus plus lymphocyte. Blue fluorescence corresponds to nuclear staining, green to cytoplasmatic pan-CK staining and red to membrane CD45 staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Of note, in two of the eight discordant cases the primary tumor had been excised and *KRAS* mutation analyzed one year or more before the CTC assay was performed. The other six cases were analyzed one month after surgery. The examination of additional primary tumor blocks is planned to evaluate tumor heterogeneity in patients with *KRAS* mutation discordance between primary tumor and CTCs.

Finally, 12 (57%) of the 21 CTC-positive patients showed *KRAS* mutation in the primary tumor, whereas this was observed in only 6 (31.6%) of the 19 patients without CTCs. Moreover, three cases with *KRAS* mutation but without CTCs were positive for atypical epithelial-like cells (atypical nuclear and/or cytoplasmatic morphology).

4. Discussion

In the last few years, improvements made in technological platforms and techniques have turned CTC investigation into a reality [8]. However, numerous methodological and theoretical problems remain to be solved. CTC number would appear to be an important issue, but with a limited impact on clinical outcome. Moreover, we cannot be completely sure that every single cancer cell present in peripheral blood is detected with currently available methods, which is probably a result of primary tumor heterogeneity and marker expression changes that can occur during the metastatic process, or that putative CTCs are actually cancer cells. Thus, the need for molecular profiling has emerged.

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Table 2
KRAS status in primary tissue and CTCs.

Patient no.	*KRAS* status	
	Primary	CTC
32	**G12D**	**G13D**; **G12D**; **G12C**
40	**G12D**	**G12D**
2	**G13D**	WT
5	**G13D**	WT
7	**G13D**	WT
25	**G12C**	WT
29	**G12A**	WT
31	**G12D**	WT
33	**G12V**	WT
28	**G12V**	ne
14	**G12D**	ne
38	**G12D**	ne
11	WT	WT
22	WT	WT
24	WT	WT
30	WT	WT
36	WT	WT
37	WT	WT
39	WT	**G12D**
16	WT	ne
26	WT	ne

ne, not evaluable.

The procedure described in the present study for the first time proved able to recover about 10–80% of cancer cells present in a PB sample, which was a function of the number of tumor cells in the starting sample. We always succeeded in recovering a sufficient number of cells at the end of each experiment. Thus, this DEPArray-based method was shown to be able to isolate and sort enriched rare cells. Moreover, single or multiple cell sorting was possible avoiding lymphocyte contamination, making it easier to perform accurate downstream gene sequencing assays.

Interestingly, we observed a fairly elevated number of positive patients with moderately high CTC numbers per milliliter of PB, more than some of the previously reported value in the literature. The percentage of patients with three or more CTCs almost doubled with respect to percentages reported by other authors and the median cell yield per ml increased at least twofold [10–12]. Multiple factors could have caused this difference. One explanation could be the enrichment method used. The Onco-Quick is probably less selective than an antigen-based selection toward EpCAM.

However, our results are fairly in line other studies, quite recently published also [13–18] and the cell density-based system was used assuming that a density gradient would recover a fair amount of CTCs without the potential bias produced by an antigen-specific method. Secondarily, it might be thought that differences could be due to the antibody utilized to detect CKs. Nevertheless, the antibody utilized in this research provided reproducible results in the experimental set-up phase in detecting actual cancer cells. Third, some of the detected CTCs could be not true cancer cells, but they were selected on the current generally accepted definition of CTC [19]. Finally, it has to be noted that our strategy is rather different from those already tested and therefore it is not completely correct to make direct comparison with the recovery percentage obtained with other methodologies.

Although it is clear that any method can have pitfalls, in particular in the enrichment phase of actual CTCs, our approach proved able to obtain enough cells to be analyzed through the DEPArray. Notwithstanding the improvements that could be obtained using different enrichment processes and more specific DEPArray-based selection strategies to find real CTCs with a higher grade of precision, the procedure was clearly successful and advantageous in detecting, recovering and analyzing single pure CTCs.

The *KRAS* gene mutation status of mCRC CTCs was also studied to find out whether mutations were present or whether gene status remained the same as that observed in primary tumor tissue. Although preliminary and based on a small number of cases, our results highlighted unexpected mutational discordance between *KRAS* primary tumor tissue and CTC status, revealing *KRAS* WT (wild type) CTCs in patients harboring mutated primary tumor, but also the opposite. Interestingly, three different mutations were detected in CTC samples from a single patient, with just one mutation similar to that observed in the primary tumor (G12D). G12D mutation was also found in the metastatic tissue of this patient (unpublished data), suggesting that more than one cell clone had shed from the primary tissue and were present in the bloodstream, but that only one had established a detectable metastasis. In support of this hypothesis, more than one half of the CTC-positive patients had *KRAS* mutated primary tumors, whereas only one third of negative patients showed this gene mutation. It can thus be presumed that patients who harbor *KRAS* mutations are more likely to develop metastases than WT subjects.

The unexpected CTC-primary tissue discordance could have a number of explanations, first and foremost, the presence of intratumor heterogeneity. The potential presence of different cancer sub-clones in the PB of patients is a major problem of this research, such as of every other study on CTCs. This is a strongly emerging feature and a number of papers are depicting this scenario [16,18–22]. Cellular heterogeneity is widely reported in epithelial malignancies and it is reasonable to assume that CTCs also show this condition [23–25]. Important genetic and phenotypic differences in cancer initiation-/progression-driver genes can also be expected between primary lesions and CTCs. It is still not known whether metastases always originate from the most "advanced" cell clone, which is also the most widely expressed in the primary tumor according to a stepwise progression model. A second hypothesis, based on the parallel progression of primary and metastatic tumor, suggests that it may be possible to have multiple metastatic clones which are disseminated very early during disease progression and remain dormant for years. The results presented here would seem to be in agreement with the latter theory. For example, a *KRAS* WT clone may emerge from the primary tumor earlier than the expansion of a dominant *KRAS* mutated cell population in the primary tissue, remaining silent or undetectable to conventional diagnostic techniques. It is not known whether this hidden clone will ever give birth to perceptible metastases, even when other evident metastases, *KRAS* mutated as the primary tumor, are detected. Clearly, the completely opposite situation may also occur. It must be pointed out that the CTC assay for our study was performed when the metastases were already present, sometimes years after the surgical resection which provided the material for *KRAS* sequence analysis. Thus, we cannot be absolutely certain about the actual origin (primary tissue or metastatic lesion) of the recovered CTCs.

Other potential explanations for the reported discordance could be the detection of normal circulating epithelial cells or unspecific expression of CKs in normal blood cells. For example, it has been shown that normal epithelial cells may be found circulating in the peripheral blood of patients with chronic, noncancer-related diseases [26]. At the same time, it has been found that some normal blood cells can illegitimately express CKs [27]. Obviously, it is not possible to exclude any of these hypotheses, indicating that further studies are needed to address these issues, in particular taking into account future clinically relevant decisions. Such a topic certainly deserves further separate investigations.

Although the concordance between *KRAS* mutation in primary and metastatic lesion in mCRC has been demonstrated by a number of authors [28,29], other recently published papers challenge this assumption, indicating that the discordant results reported

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in our paper may not be so unusual [30–33]. Moreover, the data presented here support the importance of performing gene mutation analyses on pure tumor cells. In fact, lymphocyte and/or normal epithelial cell contamination can undoubtedly alter the results of such analyses. Unpublished data performed in our laboratory confirmed that normal cell contamination can substantially alter results, especially when working with small numbers of cells, concealing the actual mutational status of examined cells. Therefore caution must be used when interpreting results from experiments where a really pure cell sorting has not been performed.

In conclusion, despite improvements that will inevitably be achieved by using different and more specific enrichment and identification strategies, the method we present demonstrates great potential for the molecular characterization of CTCs. Our results indicate that this kind of di-electrophoresis-based procedure represents the gold standard to detect, handle and recover single pure CTCs. Further studies are clearly needed to deal with issues such as the identification of the most accurate panel of markers to discriminate between normal epithelial, mesenchymal and tumor cells, or between aggressive and dormant cancer cells. Although it was not the principal goal of this paper, we are already working on implementing our detection strategy using markers typical of EMT, i.e. vimentin and/or N-cadherin, and stemness, i.e. CD133, ALDH1 and ABC-G2.

However, the OQ-DEPArray-based procedure proved to be extremely versatile in this field, introducing new possibilities such as 100% pure cell retrieval and reinforcing its potential as a new strategy to help resolve these problems. The present study also highlighted the importance of molecular characterization of single CTCs. Once confirmed, our findings could have a substantial impact on the monitoring of disease status, choice of treatment and understanding of tumor biology. In particular, this study directly suggests that treatment choice could be greatly influenced using CTC analysis. For example, the choice of the best suited biologically targeted therapy could be assisted monitoring disease progression and molecular changes by CTC study. To decide treatment options with a single blood drawn could represent an enormous step forward therapy personalization and health cost reductions.

At the same time, however, this investigation poses several questions, especially with regard to the real value of such an analysis. Taking into account previously mentioned tumor heterogeneity and genotypic and phenotypic cancer cell changes, how useful is the evaluation of single CTCs? How many cells should be tested? Which cells require more in depth analysis?

Further studies are clearly needed to answer these questions.

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