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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]. During the past decade, this has been achieved by using *KRAS*

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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