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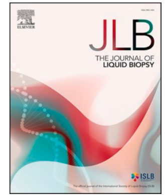
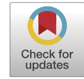
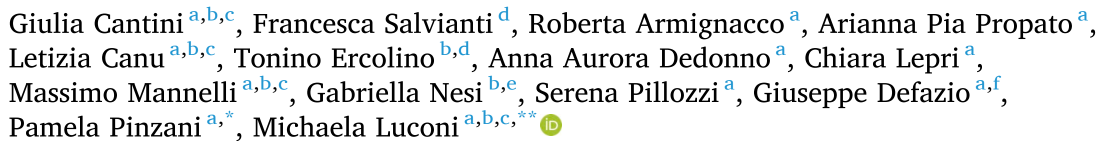




An innovative nuclear antigen-based approach for single-cell isolation of circulating tumor cells in adrenal cortical carcinoma



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A R T I C L E I N F O

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A B S T R A C T

Adrenal cortical carcinoma (ACC) is a rare and aggressive endocrine tumor that originates from the adrenal cortex. Radical tumor resection remains the most effective therapy since survival dramatically drops when metastases are present at diagnosis. Hence, there is an urgent need for reliable biomarkers to enable early diagnosis, monitor minimal residual disease (MRD), and assess chemotherapy response. Circulating tumor cells (CTCs) detected via blood liquid biopsy may represent a valuable oncological marker in ACC patients. However, CTC isolation methods so far applied to ACC patients lack specificity, reproducibility and standardization, thus preventing the potential application of CTCs in the management of these patients.

In this study, we present a novel method for the specific and reproducible detection and analysis of single CTCs in ACC patients. This approach combines size- and mechanical property-based enrichment via the Parsortix® system with immunofluorescent detection and isolation of single CTCs using DEPArray® technology, targeting the steroidogenic factor-1 (SF1) nuclear adrenal cortex marker. Isolated CTCs undergo low-pass copy number alteration (CNA) analysis. This is the first report of a nuclear antigen-based method for isolating single CTCs in suspension, overcoming the limitations of membrane and cytokeratin markers commonly used in other solid tumors. By exploiting the large nuclear size of CTCs, this strategy provides an alternative and more standardized approach for single-cell isolation in tumors lacking specific surface markers.

1. Introduction

Adrenal cortical carcinoma (ACC) is a rare endocrine malignancy originating in the cortex of the adrenal gland. Despite advances in understanding its biology, effective and specific therapies remain limited. Currently, radical resection (R0), combined with adjuvant mitotane treatment, is the main therapeutic option for ACC patients. The risk of disease progression and mortality significantly increases when the tumor is diagnosed at an advanced or metastatic stage [1,2]. Consequently, there is an urgent need for early diagnostic and prognostic informative markers that are minimally invasive, easily accessible, and independent of tumor tissue availability, in order to enable close patient monitoring and personalized therapeutic strategies. Circulating tumor cells (CTCs) have been identified in ACC using membrane filtration techniques. However, the lack of standardized detection methods and variability across protocols limits the clinical applicability of CTCs as reliable biomarkers [3,4]. In particular, the lack of expression of the epithelial cell adhesion molecule (EpCAM) and cytokeratins, generally employed for antibody-based CTC detection in other tumor types, hampers the use of the CellSearch® system, the only FDA-approved and

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clinically validated platform for CTC detection and enumeration in colorectal, breast and prostate cancer. Moreover, size-based CTC isolation methods, which exploit the larger dimensions of CTCs compared to other blood cells, are often technically complex, introduce analytical biases and lack standardization. These limitations also affect the feasibility of downstream molecular analysis on filter-isolated CTCs.

Here, we have developed a novel approach for single CTC capture in ACC patients, based on a standardized workflow for the enrichment, isolation, and analysis of CTCs in suspension. This workflow enables single-cell copy number alteration (CNA) analysis of CTCs and lymphocytes from the same blood sample, paving the way for the implementation of liquid biopsy-based single-cell genomics in ACC.

2. Materials and methods

2.1. Cell cultures

The H295R cell line [5] was purchased at ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in DMEM/F12 medium supplemented with 10 % fetal bovine serum (FBS) (Euroclone, Pero, Milan, Italy), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, Milan, Italy), enriched with a mixture of insulin/transferrin/selenium (ITS, Sigma-Aldrich). For all experiments, the cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2.

2.2. ACC patients and ethical approval

A total of 5 adult patients diagnosed with ACC were included in this study designed and conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the local committee (Prot. 2011/0020149, Rif CEAVC Em. 2019-201 November 26, 2019), and all patients provided written informed consent prior to inclusion in the study.

Patients were followed at Careggi University Hospital in Florence, Italy. Histological diagnosis of ACC was made according to the European Network for the Study of Adrenal Tumors (ENSAT) guidelines [2]. All patients underwent surgical removal of the tumor except one, for whom ACC diagnosis was made instrumentally based on computed tomography (CT) tumor characteristics.

2.3. Blood sample collection

Peripheral blood samples (10 mL) were collected from ACC patients before surgery for CTC enrichment, using the Parsortix® system (ANGLE Europe, UK). Blood was drawn into Cell-Free DNA BCT® tubes (Streck, USA), which contain K_3-EDTA anticoagulant and a cell preservative that stabilizes CTCs in whole blood for up to 7 days at 15–25 °C. On the same day, an additional 6 mL-blood sample was collected in standard ethylenediaminetetraacetic acid (EDTA) tubes for CTC detection using the ScreenCell® Cyto system (ScreenCell, France), based on size-exclusion filtration. All blood samples were processed within 3 h of collection [4,6] to assess the presence of CTCs.

2.4. H295R spiking in blood samples

H295R cells for spike-in experiments were harvested by incubation with 2X trypsin for 10 min at 37 °C (Sigma-Aldrich), followed by centrifugation for 5 min at 1300 rpm. Cell concentration was evaluated with a hemocytometer before the spike-in experiment. A total of 500 H295R cells, resuspended in phosphate buffered solution (PBS), were added to a 10 mL-blood sample collected from a healthy volunteer among the authors using Cell-Free DNA BCT® tubes (Streck, USA), and were immediately processed with the Parsortix® technique.

2.5. CTC isolation through ScreenCell® Cyto filtration

Blood samples collected in EDTA tubes were filtered through ScreenCell® Cyto filtration devices (ScreenCell, France), as previously described [3,4]. Briefly, before filtration, 3 mL of blood was diluted to a final volume of 7 mL with a specific dilution buffer for fixed cells (ScreenCell® FC2 dilution buffer, ScreenCell, France). After filtration, an additional 1.6 mL of PBS was passed through the filter to remove red blood cell (RBC) debris. The filter was then disassembled from the filtration module and allowed to air dry. For each patient's blood sample, filtration was performed and analyzed in duplicate filters. CTC enumeration and morphological evaluation were performed after hematoxylin/eosin (H&E) or May-Grünwald-Giemsa staining of each filter, as previously described [4].

CTCs were differentially identified from leukocytes based on the following established morphological criteria: cell size ≥16 μm, nucleo-cytoplasmic ratio ≥50 %, irregular nuclear shape, hyperchromatic nucleus, and basophilic cytoplasm [7]. Blinded cytological analysis was performed by two independent observers (GC and GN) on both the stained filters.

2.6. CTC enrichment using Parsortix® system

CTC enrichment was performed by the Parsortix® Cell Separation System (Angle PLC, Surrey, UK), a microfluidics-based technology for cell separation, which captures CTCs by exploiting size and deformability properties. Briefly, the blood sample was applied to the Parsortix® instrument, which enriched CTCs in 200 μL PBS [8–10].

2.7. SF1 immunofluorescence staining of H295R cells

Steroidogenic factor-1 (SF1) nuclear staining of ACC cells was set up on H295R cells in suspension at serial dilutions ranging from 10^6 to 5×10^2 cells fixed in 0.125 % paraformaldehyde (Sigma-Aldrich) and permeabilized in 0.2 % Triton-X100 in PBS with the addition of PE-conjugated SF1 primary antibody (dilution 1:50, sc-393592 clone A1, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C. Washed cell pellets were resuspended in 13.5 μL of SB115 buffer and analyzed by DEPArray® (Menarini Silicon Biosystems, Milan, Italy).

2.8. Direct immunofluorescence staining of Parsortix®-recovered cells

Parsortix®-enriched cells from the patients' samples, as well as from the H295R spike-in samples, were fixed with paraformaldehyde at 0.125 % and stained with APC conjugated anti-CD45 antibody (dilution 1:10, clone H130, Biolegend, San Diego, CA, USA) for 20 min at 4 °C to detect white blood cells (WBCs). Following a PBS wash, cell pellets were permeabilized with 0.2 % Triton-X100 (Sigma-Aldrich) in PBS with the addition of PE-conjugated SF1 primary antibody (dilution 1:50, sc-393592 clone A1, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C, for the identification of cells of adrenocortical origin. After washing, cell pellets were stained with Hoechst solution (100 μg/mL) in SB115 buffer, washed again, and finally resuspended in 13.5 μL of SB115 buffer (Menarini Silicon Biosystems).

2.9. DEPArray® analysis of stained cells

After cell staining, samples resuspended in SB115 buffer were loaded into the DEPArray® A300K chip and submitted to single-cell sorting by DEPArray® (Menarini Silicon Biosystems, Milan, Italy) according to the manufacturer's instructions. The chip is a single-use, microfluidic cartridge containing an array of individually controllable electrodes, each equipped with embedded sensors. An automated fluorescence microscope scanned the chip to generate an image gallery, and cells were selected according to their morphology and staining patterns as follows: CTCs (Hoechst positive, PE/SF1 positive, and APC/CD45 negative) and

leukocytes (WBC, Hoechst positive, PE/SF1 negative, and APC/CD45 positive). After tumor cell identification, single cells or clusters of cells (CTMs) were moved by dielectrophoresis and recovered into 200 μL SB115 buffer. After 3 washes by centrifuging at 14100×*g* in PBS, the final cell pellet in each sample was resuspended in 1 μL for subsequent Whole Genome Amplification (WGA) and CNA analyses. The recovery efficiency obtained in the H295R spiking experiments was calculated as the percentage of $SF1^{+}/CD45^{-}$ cells identified by DEPArray® compared to the number of H295R cells initially added to the blood sample of the healthy volunteer, after correction for cell loss due to the reduced area analyzed on the DEPArray® chip, which correspond to 66 % of the total loaded volume (according to the DEPArray® A300K DS V2.0 Cartridge technical data sheet) [11,12].

2.10. WGA amplification

WGA reaction was performed on three single CTCs from patient CS172 and on a single H295R cell from the spike-in experiment isolated by the Parsortix®-DEPArray® combined methods using SF1 immunofluorescence detection. Briefly, DNA from single-cell samples was processed with Ampli1®scWGA seq kit according to the manufacturer's instructions (Menarini Silicon Biosytems, Milan, Italy). The procedure is based on ligation-mediated PCR following site-specific DNA digestion by the *Mse*I enzyme. The kit eliminates the need for precipitation steps avoiding DNA loss, and generates a library of fragments of about 0.2–2 kb representing the entire genome. The kit utilizes a mixture of Taq polymerase with a proofreading enzyme, Pwo polymerase, which has been reported to exhibit an error rate more than 10 times lower than that of Taq polymerase.

2.11. WGA quality control

The quality of WGA output products was assessed by the Ampli1®QC kit (Silicon Biosystems) according to the manufacturer's instructions [11]. The kit is a PCR-based assay amplifying two distinct regions of the human genome to produce two amplicons (A and B) of 373 bp and 167 bp, respectively. PCR products A and B were analyzed by capillary electrophoresis on the Agilent 2100 Bionalyzer®. The presence of both amplification products indicates a successful WGA and consequently the suitability of the sample for downstream analysis.

2.12. Low-pass Whole Genome Sequencing EASI genomics

The Ampli1 LowPass Kit for Illumina produces barcoded sequencing libraries suitable for genome-wide copy-number profiling using Ampli1® WGA products as input. Given that the reaction is based on PCR, shorter fragments have higher amplification efficiency and the WGA fragments that are represented in a low-pass library normally have an average length of 200–250 bp. Low-pass Whole Genome Sequencing (WGS) was performed on an Illumina sequencing platform with a sequencing layout of 100bp single-end reads at the Centro Nacional de Análisis Genómico of the Centre for Genomic Regulation (CNAG-CRG, Spain-www.cnag.crg.eu CNAG-CRG) as part of the EASI-Genomics EU-funded infrastructure project (European Advanced infraStructure for Innovative Genomics) coordinated by Prof. Ivo Gut (CNAG-CRG, Spain, www.cnag.eu/news/easi-genomics-eu-funded-project-provide-free-charge-access-sequencing-technologies.

As a result of the sequencing design, longer fragments, even if present in the WGA product, are not captured in the low-pass library analyzed. Indeed, the goal of this study is to demonstrate that our method for isolating CTCs from ACC patients at a single-cell level is suitable for subsequent CNA analysis assessing the genome-wide copy number levels with number of reads counted in genomic windows of a few hundred kb.

Data from each sample were processed with the following bioinformatic tools, in accordance with the Ampl1 kit recommended protocols.

- Raw data were quality checked with FastQC [13] and MultiQC [14].
- The reads were mapped against the human reference genome (GRCh38) using BWA MEM version 0.7.15-r1140 [15].
- Reads were sorted and duplicates removed with SAMtools version 1.12 [16].
- Coverage metrics were calculated and visualized with SAMtools version 1.12, deepTools version 2.0 [17], and karyoploteR version 1.23 [18].
- Control-FREEC version 11.6 [19] was run to perform copy number analysis.

3. Results

3.1. Development of SF1 staining method in Parsortix®-enriched samples combined with DEPArray® isolation of H295R single cells

To validate nuclear labeling with an antibody against SF1 for single cell isolation of ACC cells using DEPArray® technology, optimal conditions for antibody incubation were established employing serial dilutions of paraformaldehyde-fixed and permeabilized-H295R cells. Single cells specifically labeled with a PE-conjugated anti-SF1 antibody were successfully isolated and visualized by the DEPArray® instrument (Fig. 1A).

To assess the validity of the method for detecting cancer cells in the blood of ACC patients, we simulated patient sample by spiking 500 purified H295R cells into 10 mL of blood freshly drawn from a healthy donor. The spiked-blood samples were then processed using the Parsortix® instrument for CTC enrichment. This technology captures CTCs by passing blood samples through a microfluidic cassette with a stepped structure, in which the channel width gradually narrows down to 10 μm. At this point, CTCs and a small number of large WBCs are trapped, due to their size and mechanical stiffness. Parsortix®-recovered cells from the artificial H295R-spiked-blood samples were then fixed and subjected to double immunofluorescence staining. Cells were first incubated with an APC-conjugated anti-CD45 antibody to mark WBCs, followed by Triton-X permeabilization and incubation with a PE-conjugated anti-SF1 antibody to label H295R cells. Stained cells were selectively displayed on the different fluorescence channels of the DEPArray® instrument: the PE signal (green-fluorescence) indicated nuclear SF1 expression in H295R cells, while residual WBCs were shown by the APC/CD45 signal (red-fluorescence) (Fig. 1B). The recovery efficiency obtained in the H295R spiking experiments was 12 %, corresponding to the percentage of $SF1^{+}/CD45^{-}$ cells identified by DEPArray® relative to the number of H295R cells initially added to the blood sample. This is consistent with recovery rate previously reported for DEPArray isolation in spiking experiments using EpCAM surface marker staining [11,12]. The single cells identified were individually collected and stored for CNA analysis.

3.2. Application of the SF1 immunofluorescence staining method to isolated CTCs in ACC patients

The method developed combining cell enrichment via Parsortix® with DEPArray®-based isolation through SF1 and CD45 differential staining was then applied to blood samples obtained from 5 ACC patients, whose main clinical characteristics are listed in Table 1.

Blood samples were collected during active disease (before surgery in 4 out of 5 patients, and 2 months after surgery in 1 stage IV patient) to maximize CTC retrieval. For each patient, blood samples were processed for CTC isolation with the newly developed Parsortix®+DEPArray® method, and for CTC retrieval through filtration by ScreenCell® filters [3,4]. CTCs were retrieved from all patient samples processed using the new workflow (median CTC count [25–75 IQR] = 61[16–74]), and positivity was also confirmed by ScreenCell® filtration in at least one of the filters from 2 independent blood samples collected from each patient

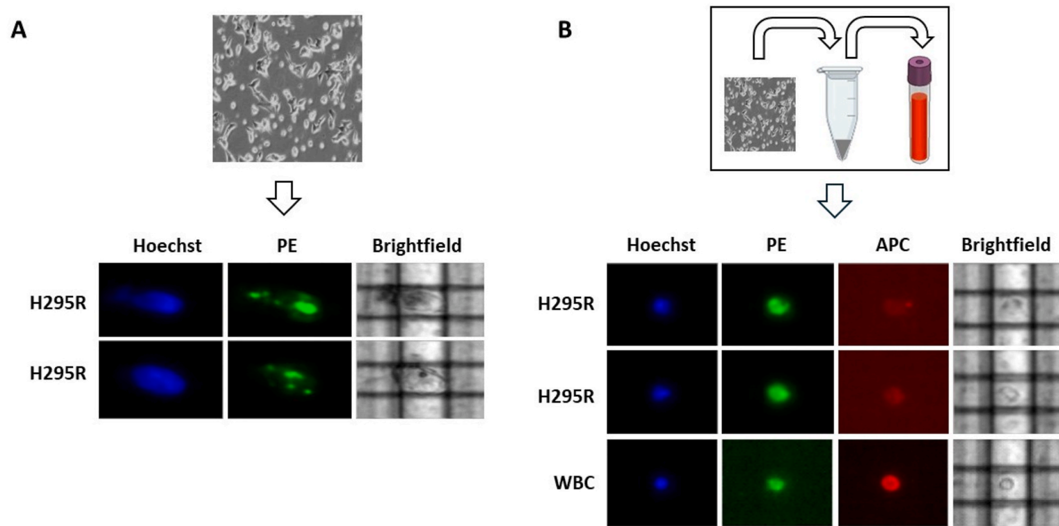

Fig. 1. DEPArray® image gallery of single H295R and white blood cells (WBC) selected by immunofluorescence staining for SF1 and CD45. (A) Representative images of H295R cells showing direct immunofluorescence staining for SF1. Cells are visualized by positive SF1 signal in the PE green channel colocalized with Hoechst blue nuclear staining. (B) DEPArray® images from spike-in experiments after Parsortix® enrichment, showing H295R cells positive for SF1 (green channel) and negative for CD45 (red channel), whereas WBCs are positive for CD45 (red) and negative for SF1 (green). The blue channel indicates nuclear DNA staining with Hoechst. Brightfield images of the selected single cells are shown on the right.

Table 1
Characteristics of ACC patients and CTC counts obtained from blood samples using different techniques.

Patient	Sex	Age	Stage	Size (cm)	Blood Sample	CTC (CTM) by DEPArray® post processing	CTC on paired ScreenCell® filters
CS170	M	46	III	15	PRE	78 (17)	+/+
CS172	F	54	III	13.5	PRE	9 (6)	+/+
CS180	M	51	IV	17	PRE	61 (10)	+
CS194	M	56	IV	15	POST (2 mos)	69	+/−
CS206	F	73	IV	10 *	PRE	22	+/−

CTC evaluation was performed on blood samples collected before (PRE) or after (POST) surgery.
POST (2 mos): blood sample taken at 2 months from surgery. *: tumor size calculated using computed tomography (CT). + : positive ScreenCell® filter after cytological analysis; , -: negative ScreenCell® filter after cytological analysis.

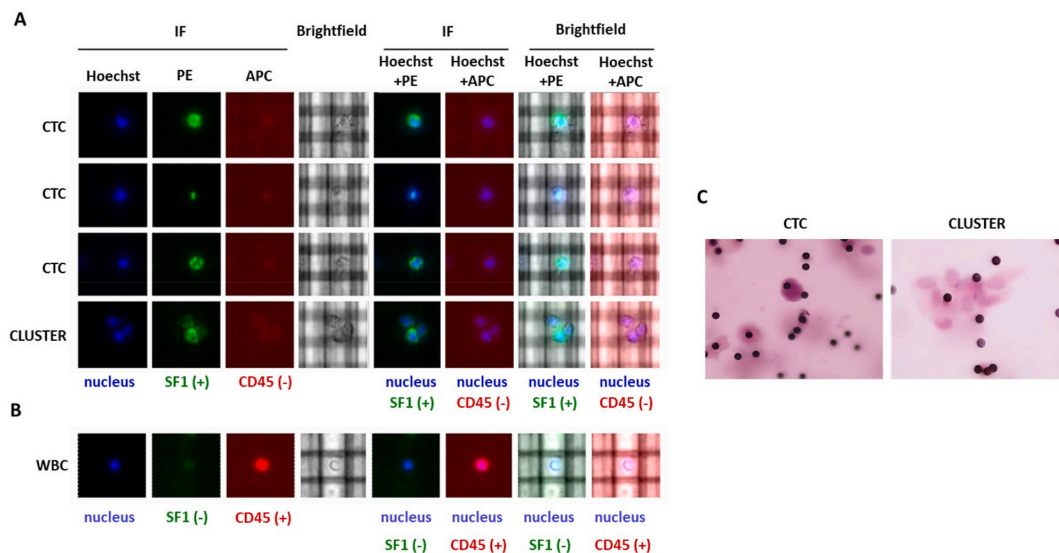

Fig. 2. CTC retrieval from blood samples of ACC patients. Representative DEPArray® images of single CTCs and CTC cluster (A), and of a single WBC (B), obtained after enrichment with the Parsortix® system and double immunofluorescence staining. CTCs of adrenal cortical origin, positive for SF1 and negative for CD45, displaying large size and prominent nuclei (Hoechst blue signal) (A). WBCs are positive for CD45 and negative for SF1 (B). Brightfield images and merged channels for brightfield, PE and APC fluorescence signals are shown. Isolated CTCs and CTC clusters detected in a parallel blood sample using cell filtration followed by H&E staining of cells retained on ScreenCell® filters (C). CTCs are recognizable by the standard criteria reported in the literature [3,7]; black dots represent the scattered filter pores.

(Table 1). CTMs composed of CTCs accompanied by other cells and originating from the primary tumors (previously described in ACC [4] and other aggressive cancers [20]), were identified in 3 ACC patients on SF1 positivity using Parsortix®+DEPArray® analysis (CTM = 10 [6–17]). Fig. 2 shows DEPArray® image profiles of three CTCs, one CTM and one leukocyte isolated from patient CS172, following differential double immunofluorescence staining with anti-SF1 and anti-CD45 antibodies.

Both isolated CTCs and CTC clusters were detected on the basis of morphological criteria, including large size, prominent nuclei, as well as nuclear SF1 positivity and absence of CD45 expression (Fig. 2A), whereas WBCs lacked SF1 expression and were positive for CD45 (Fig. 2B). The presence of CTCs and CTMs was confirmed in matched blood samples processed by ScreenCell® filtration and stained with H&E (Fig. 2C).

Finally, we assessed whether single CTCs isolated using the new workflow could be successfully processed for CNA analysis. Low-pass WGS was conducted after WGA on the Illumina platform by EASI-Genomics European Consortium [https://www.cnag.eu/projects/easi-genomics]. Quality control (QC) analysis of DNA amplified from three CTCs and one H295R single cell, previously isolated and captured by DEPArray® (Fig. 2), passed internal QC. Normalized copy number profiles are aligned by chromosome as shown for three representative single CTCs from an ACC patient and from a representative H295R cell isolated during the spiking experiment (Fig. 3). The expected position of the main candidate genes associated with somatic mutations in ACC are also reported for each chromosome.

The flowchart in Fig. 4 outlines the main steps of the newly developed workflow that enables CTC isolation based on the expression of a nuclear marker.

4. Discussion

In this study, we developed a specific and sensitive method for isolating single CTCs in suspension from the blood of ACC patients.

ACC is an aggressive tumor characterized by marked genetic heterogeneity within the malignant cell population, and such heterogeneity is also expected to be present in cells shed from the primary tumor into the bloodstream. Thus, it is crucial not only to enumerate CTCs, relevant for both diagnosis [3] and prognosis [4] of ACC, but also to define their genetic profile at the single-cell level. Such detailed profiling can provide important insight into minimal residual disease and chemoresistance, thereby improving patient monitoring during follow-up. In particular, CTC postoperative monitoring is increasingly recognized as a minimally invasive predictive tool of recurrence-free survival in solid tumors [23]. Despite recent technical advances in liquid biopsy for single-cell analysis [24], the molecular characterization of CTC at the single-cell level is even more challenging in ACC. This difficulty is partly due to the rarity of this cancer, which limits patient recruitment for research studies. Indeed, in ACC, the number of CTCs recovered from blood samples by filtration methods applied so far [3,4] is lower than other solid tumors, such as lung cancer [20,25] and melanoma [26].

CTC enrichment by size-filtration has several limitations. Firstly, once the large cells are trapped on the filters, further characterization as CTC on the basis of standardized morphometrical criteria [7] is required, followed by specific immunocytochemical staining to confirm adrenal cortical origin [3]. These procedures are technically challenging and may suffer from low reproducibility, potentially leading to underestimation of CTC numbers in the sample. Furthermore, CTC isolation by membrane filtration techniques exhibits relevant drawbacks when combined with downstream molecular analysis, such as gene expression or genetic profiling, because DNA or mRNA extraction is typically performed on bulk samples rather than at the single-cell level. Therefore, single-cell resolution is lost and results are often diluted by contamination from noncancer cells.

To overcome these challenges and enable the genetic profiling of CTCs from ACC patients at the single-cell level, we combined Parsortix® size-based enrichment with single-cell isolation through direct immunofluorescence selective SF1 staining and morphological analysis by DEPArray®. The SF1 immunofluorescence staining protocol was optimized using low numbers of H295R cells spiked into blood samples thereby mimicking the low CTC concentration expected in the blood of ACC patients. In addition, the use of a nuclear antigen like SF1 as a specific marker to select CTCs of adrenal cortical origin takes advantage of the CTC characteristics, including a large nucleus and high nuclear-to-cytoplasmic ratio. Therefore, immunofluorescence staining with nuclear markers is to be preferred to the use of a cytosolic marker for CTC selection. Size-exclusion enrichment methods like Parsortix® combined with single immunofluorescence cell detection with DEPArray® technology have already been employed for CTC isolation in other cancers. Notably, this is the first report of a nuclear marker for CTC immunocytochemical selection being utilized instead of anti-cytokeratin or anti-EPCAM antibodies [27–31]. Potential limitations of nuclear marker

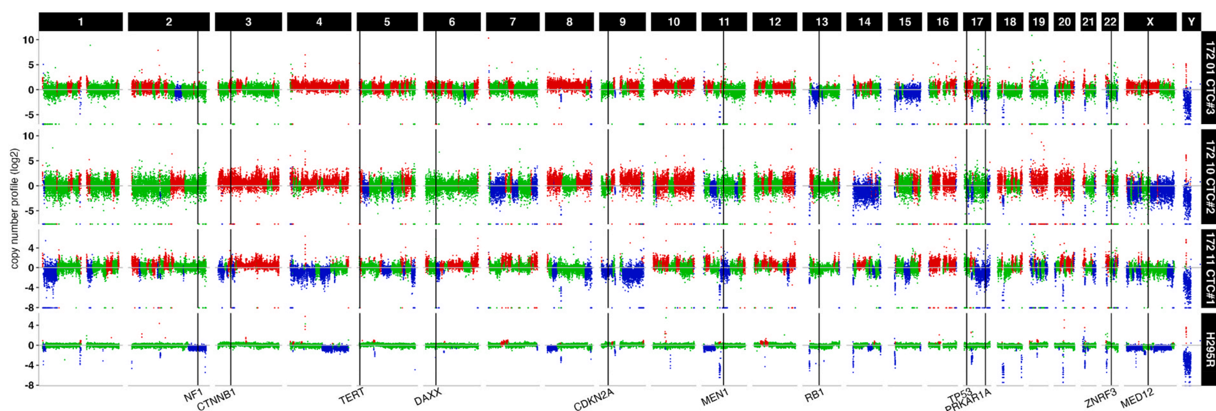

Fig. 3. Low-pass WGS analysis of CTCs and H295R cells isolated using the combined Parsortix®-DEPArray® method with SF1 immunofluorescence staining. Three single CTCs recovered from the blood sample of patient CS172 and one single H295R cell recovered by the spiking experiment after Parsortix® enrichment and DEPArray® isolation were subjected to WGA, and the DNA obtained was sequenced by low-pass WGS analysis performed by EASI-Genomics Consortium. Normalized copy number profiles (log2) for each chromosome are aligned in the three representative isolated CTCs and the representative H295R cell. Copy number gains are indicated in red, losses in blue, and no variation in green. The expected position of the main candidate genes for somatic mutations associated with ACC, as previously described [21,22] are indicated for each chromosome (*NF1, CTNNB1, TERT, DAXX, CDKN2A, MEN1, RB1, TP53, PRKAR1A. ZNRF3, MED12*).

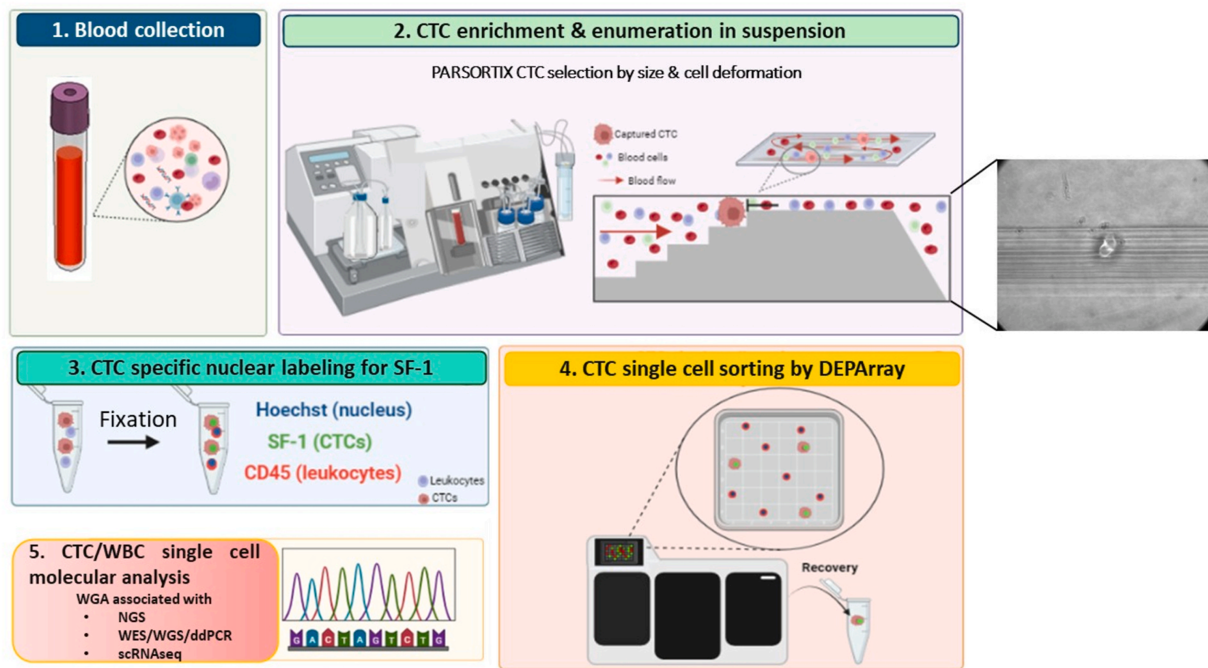

Fig. 4. Flowchart illustrating the combined Parsortix®-DEPArray® workflow using SF1 immunofluorescence staining for the isolation of CTCs of adrenal cortical origin. The procedure consists of five main steps: Step 1, blood collection; Step 2, whole blood sample is processed through the Parsortix® system to enrich cells of larger size (CTCs and leukocytes). The inset shows entrapped cells visualized by optical microscopy; Step 3, Parsortix® enriched samples are fixed and subjected to double immunofluorescence staining with anti-SF1 (to detect CTCs) and anti CD45 (to detect leukocytes) antibodies, followed by nuclear counterstaining with Hoechst; Step 4, single cell morphology and staining are analyzed by DEPArray® across fluorescence channels and selected cells are recovered; Step 5, isolated single cells (CTCs and WBCs) are available for downstream molecular analysis, including Whole Genomic Amplification (WGA), Next Generation Sequencing (NGS), Whole Exome Sequencing (WES), Whole Genomic Sequencing (WGS), and single-cell RNA sequencing (scRNAseq). Figure created with BioRender.

detection, which can reduce recovery efficiency, should be considered. In particular, the additional double step of nuclear and cell membrane permeabilization required by this method, may interfere with the subsequent genomic analysis and requires complex standardization and optimization, according to the tumor type analyzed. However, although only representative and very preliminary, the results of the WGS analysis we performed on the recovered CTCs support the protocol optimization at least for ACC.

To assess the performance of the new method on ACC patient' blood samples, we focused on advanced tumor stages (III and IV) to maximize the likelihood of detecting CTCs, based on our previous findings that metastatic disease is associated with higher CTC counts than localized tumors [3]. CTCs were detected in 100 % of the samples and confirmed by ScreenCell® filtration. Using this novel approach, immunofluorescence-isolated CTCs and WBCs were recovered as single-cells in suspension, enabling subsequent genetic analysis. This is particularly challenging when using filtration methods, because CTCs tend to adhere to the membrane surface and are difficult to recover as single cells by laser-based microdissection procedures. DEPArray®-isolated single cells of adrenal cortical origin (CTCs and H295R) were successfully subjected to WGA and low-pass CNA analysis, as previously described for small cell lung cancer [32]. Correlation between CNA profiles and patient clinical characteristics was not performed, since prognostic molecular analysis was beyond the scope of the present study.

5. Conclusions

We have developed a novel method combining Parsortix® size-exclusion enrichment with DEPArray® single-cell selection of CTCs from ACC patients, utilizing for the first time a nuclear marker antibody (SF1) for specific immunocytochemical identification. This approach provides a potential alternative strategy for the single-cell isolation of CTCs in tumors lacking expression of membrane-bound or cytokeratin markers. Further studies are mandatory to establish standardization and reproducibility of this procedure, also as regards its application to other cancer types.

Ethics statement

This study was designed and conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the local committee (Prot. 2011/0020149, Rif CEAVC Em. 2019-201 November 26, 2019), and all patients provided written informed consent prior to inclusion in the study.

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Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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