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PIK3CA mutation analysis in circulating tumor cells of patients with hormone receptor positive metastatic breast cancer

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ABSTRACT

In metastatic breast cancer (MBC), blood is a source of circulating tumor cells (CTCs). CTCs may serve as a "real-time liquid biopsy" as they represent metastatic tumor genetics better than primary tumor. *PIK3CA* is one of the most important oncogenes in treatment-unresponsive breast cancers. The aim of this study was to detect *PIK3CA* mutations and hereditary cancer variants in CTCs from MBC patients. Forty-seven blood samples were obtained from 20 MBC patients from at least 1/3 consecutive time points. CTCs were quantified using the CellSearch system and isolated from 11/20 patients with ≥5/7.5 ml CTCs (14/47 blood samples) using the DEPArray system. DNA was extracted and amplified to perform Sanger sequencing on *PIK3CA* gene. Sequencing revealed a pathogenic *PIK3CA* mutation in 2/11 (18 %) cases. Subsequently, we evaluated a 26-target hereditary gene panel by Next Generation Sequencing and identified a concomitant pathogenic mutation in the *TP53* gene in a patient with a *PIK3CA* mutation. No pathogenic germline variants were found. Our data support the conclusion that CTCs analysis may be used to identify mutations in patients to identify those more likely to metastasize.

1. Introduction

Breast cancer (BC) is the leading cause of cancer death in women worldwide. The disease is categorized into subtypes based on gene expression patterns [1–3]. *PIK3CA* is one of the most important oncogenes at the basis of the non-responsiveness to hormonal, anti-HER2 and chemotherapy in BC [4]. Constitutive activation of PI3K mediates the transforming potential of oncogenes and inactivation of tumor suppressors. ER+/HER2- BCs can change the *PIK3CA* status during treatment and PIK3CA activation was observed in 10–40 % cases, usually in ER + resistant tumors. In addition, inactivation of the tumor suppressor gene *PTEN*, detected in 30–50 % of BC patients, results in PI3K pathway activation [5].

Metastatic breast cancer (MBC) involves spreading of cancerous cells from breast to any organ and, to-date, lacks curative options [6]. At the time of the primary diagnosis, most patients have no evidence of metastasis by routine methods. However, invasive BCs shed cells denominated circulating tumor cells (CTCs) into the bloodstream or lymphatic vessels and up to 20 % of the patients develops metastases [6].

Detection of ≥5 CTCs/7.5 ml of whole blood in BC correlates with the metastatic process [7,8]. CTCs can persist for long periods and form metastases in vivo [9]. Previous studies devised protocols for CTCs detection and isolation from the peripheral blood of women with BC, including patients without current evidence of disease [7,10,11]. CTCs isolation is considered a "real-time biopsy", especially in the metastatic setting as they are thought to better represent tumor genetics than primary tumors. Therefore, molecular characterization of CTCs might

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Table 1
Timepoints and number of CTCs detected.
CTCs quantification at 3 timepoints (when available). Patients 1–9: no CTCs or CTCs <5/7.5 ml. All blood samples with CTCs <5/7.5 ml were discarded. CTCs>5/7,5 ml (in bold) were detected in 11/20 patients (14/47 blood samples).

N°	**Basal**	**12 weeks**	**24 weeks**
	nr CTC/7.5 ml	nr CTC/7.5 ml	nr CTC/7.5 ml
patient 1	0	/	1
patient 2	0	/	1
patient 3	0	1	/
patient 4	2	0	2
patient 5	4	2	2
patient 6	0	1	1
patient 7	2	1	2
patient 8	3	/	/
patient 9	0	1	0
patient 10	**13**	0	1
patient 11	**13**	0	**6**
patient 12	0	5	0
patient 13	0	0	**554**
patient 14	**5**	**11**	1
patient 15	4	**14**	3
patient 16	**238**	**1974**	/
patient 17	**109**	/	/
patient 18	**11**	**9**	/
patient 19	**65**	/	/
patient 20	**227**	/	/

provide important insights into mechanisms of metastasis to guide the development of targeted therapies [12].

The aim of this study was to apply a noninvasive “liquid biopsy” from peripheral blood to identify somatic and germline variants in BCs, by using the CellSearch system combined with the DEPArray™ technology. Moreover, we studied and compared the germline versus tumor (CTCs) and metastasis genome in one patient.

2. Materials and methods

2.1. Patients

Twenty MBC patients, recruited at the European Institute of Oncology IRCCS, Milan, Italy, fulfilled the inclusion/exclusion criteria (Supplementary Material S1). Blood samples for CTC enumeration were obtained for at least one of three time points: baseline, 12, and 24 weeks (±2 weeks) after commencing endocrine therapy.

The study was approved by the internal Ethical Committee and informed consents were obtained for all patients.

2.2. CTCs isolation

Seven-and-a-half ml of whole blood drawn into CellSave Preservative Tubes (Menarini Silicon Biosystems) were processed within 96 h of collection. CTCs detection and enumeration were performed using the CellSearch system (Menarini Silicon Biosystems) according to manufacturer’s instructions [13]. CTCs were identified as nucleated cells that lacked CD45 and expressed cytokeratin. The phenotype of tumor cells was EpCAM+, CK+, DAPI+ and CD45^{-}. Results of cell enumeration was expressed as the number of cells per 7.5 mL of blood (Table 1).

CTCs enrichment was performed on the DEPArray™ System (Menarini Silicon Biosystems) [14]: we decided to analyze only those samples with ≥5 CTCs/7.5 mL of blood. We collected CTCs as single cells (CTCs-single) or pools (CTCs-pool, increasing the amount of DNA to be sequenced), as well as a control white blood cell (WBC) sample. Also, we decided to collect CTCs-uncertain, because of the DEPArray operating principles, based on morphology and fluorescence of the selected cells. Indeed, for those cells showing an irregular morphology (often tumor cells have irregular morphology) [12] or weak fluorescence due to CTCs

Table 2
List of samples collected from patients 11/20 at indicated timepoints.

Patient NR	Cell type	SANGER EX 9	SANGER EX 20
Patient 10, basal	#2 CTC	ne	ne
	#7 CTC-U.C.	WT	WT
	#2 WBC	WT	ne
Patient 11, basal	CTC pool	WT	WT
	CTC-U.C. pool	WT	WT
	WBC pool	WT	WT
Patient 11, 6 months	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC-U.C.	WT	WT
	#1 CTC-U.C.	ne	ne
	#1 CTC-U.C.	ne	ne
	#4 WBC	WT	WT
Patient 12, 3 months	#5 CTC	ne	ne
	#4 CTC-U.C.	ne	ne
	#3 WBC	ne	ne
Patient 13, 6 months	#1 CTC	WT	ne
	#1 CTC	WT	WT
	#1 CTC	ne	ne
	#1 CTC	WT	WT
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	WT	WT
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	ne	ne
	#1 CTC	WT	WT
	CTC pool	WT	WT
	CTC pool	WT	ne
	WBC pool	WT	WT
	WBC pool	WT	WT
Patient 14, 3 months	#4 CTC	ne	ne
	#4 CTC-U.C.	WT	WT
	#9 WBC	WT	WT
Patient 15, 3 months	#1 CTC	ne	ne
	#11 CTC-U.C.	ne	ne
	#8 WBC	ne	ne
Patient 16, basal	#16 CTC	WT	ne
	#15 CTC	ne	ne
	#13 CTC	ne	ne
	WBC pool	WT	ne
Patient 16, 3 months	#1 CTC	ne	WT
	#1 CTC	ne	WT
	#1 CTC	ne	ne
	#1 CTC	WT	WT
	#1 CTC	WT	WT
	#1 CTC	WT	WT
	#1 CTC	WT	WT
	#1 CTC	ne	WT
	#1 CTC	WT	WT
	#1 CTC	ne	WT
	#1 CTC	WT	ne
	CTC pool	WT	WT
	CTC pool	WT	WT
	CTC pool	WT	WT
	CTC pool	WT	WT

(*continued on next page*)

Table 2 (*continued*)

Patient NR	Cell type	SANGER EX 9	SANGER EX 20
	CTC pool	WT	WT
	WBC pool	WT	WT
	WBC pool	WT	WT
	WBC pool	WT	WT
Patient 17, basal	#8 CTC	**c. 1636C > A (p. Gln546Lys)**	ne
	#7 CTC-U. C.	ne	ne
	#9 WBC pool	WT	WT
Patient 18, basal	#3 CTC	**c. 1634A > G (p. Glu545Gly)**	WT
	#3 CTC-U. C.	ne	ne
	#7 WBC	ne	ne
Patient 18, 3 months	#1 CTC	**c. 1634A > G (p. Glu545Gly)**	WT
	#1 CTC	ne	WT
	#1 CTC	ne	WT
	#1 CTC-U. C.	**c. 1634A > G (p. Glu545Gly)**	WT
	#1 CTC-U. C.	WT	WT
	#8 WBC	WT	WT
Patient 19, basal	#2 CTC	ne	ne
	#13 CTC-U. C.	WT	WT
	#13 CTC-U. C.	ne	ne
	#12 CTC-U. C.	ne	WT
	#2 WBC	ne	ne
Patient 20, basal	#7 CTC	WT	WT
	#15 CTC-U. C.	WT	WT
	#10 WBC	WT	WT

Legend: WT, wild type; ne, not evaluable; u.c., uncertain classification; WBC, white blood cell. We indicated with "pool" samples with undetermined number of cells.

in transition [8] a clear-cut selection could not be performed by the instrument.

For each sample, we also collected a sample of white blood cells (WBCs) to be used as an internal control and confirm that an eventual CTC mutation would be somatic and not germinal. More specifically, we collected 3 types of cells for each sample: i) CTCs-single or CTCs-pool, ii) CTCs-uncertain and iii) WBCs (Table 2).

2.3. Whole genome amplification and sanger sequencing

Whole genome amplification (WGA) of single cell DNA was performed using the Ampli1™ WGA Kit (Silicon Biosystems). Reactions were carried out in collecting tubes and checked with the Ampli1™ QC Kit. In cases failing the quality control (data not shown), we were able to determine that, when continuing the experimental procedure, a negative QC always corresponds to a complete lack of DNA, either due to the loss of cells, during spinning steps and supernatant removal, or to the suboptimal DNA quality [12].

After WGA, mutation detection was performed by DNA sequencing [15] using the Ampli1™ *PIK3CA* Seq Kit. PCR products were purified with EXO-SAP™ (Thermo Fisher Scientific), amplified using the Big Dye 3.1 Terminator chemistry (Thermo Fisher Scientific), and purified with the Illustra™ Autoseq™ G-50 Terminator removal kit (GE Healthcare). Samples were loaded into the capillary array of a 3500 xL-Dx Genetic Analyzer (Applied Biosystem).

Table 3
List of genes analyzed using Hereditary Cancer Solution (HCS) CE-IVD panel by Sophia Genetics.

GENE NAME	RefSeq
APC	NM_001127511
ATM	NM_000051
BARD1	NM_000465
BRCA1	NM_007294
BRCA2	NM_000059
BRIP1	NM_032043
CDH1	NM_004360
CHEK2	NM_001005735
EPCAM	NM_002354
FAM175A	NM_002354
MLH1	NM_000249
MRE11A	NM_005590
MSH2	NM_000251
MSH6	NM_000179
MUTYH	NM_001048171
NBN	NM_002485
PALB2	NM_024675
PIK3CA	NM_006218
PMS2	NM_000535
PTEN	NM_000314
RAD50	NM_005732
RAD51C	NM_058216
RAD51D	NM_001142571
STK11	NM_000455
TP53	NM_000546
XRCC2	NM_005431

2.4. Library preparation and next generation sequencing (NGS)

Samples from patients with *PIK3CA* mutations were analyzed using the CE-IVD Hereditary Cancer Solution (HCS) (SOPHiA Genetics) as per manufacturer's protocol (version PM_T1_5.1.5_r2en July 2017). Libraries were prepared from 200 ng of gDNA, quantified using the 2100 electrophoresis Bioanalyzer (Agilent technologies) or Qubit 3.0 Fluorometer (Life technologies) and diluted to 4 nM. Following denaturation, a 10 pM library dilution was loaded with 3 % PhiX control (Illumina). Sequencing was performed on the Illumina MiSeq system, using the Illumina MiSeq V2 Standard reagent Kit 2x250 cycles. Results were retrieved and analyzed in the dedicated platform SOPHiA DDM that accurately detects SNVs, Indels and CNVs [16].

DNA from metastatic tissue was analyzed using the HCS CE-IVD probes (SOPHiA Genetics), according to the Solid Tumor Solution protocol (version PM_T1_5.1.13_r1en July 2017) for $10 \leq n \leq 50$ ng of gDNA for library generation and processed as above. Full gene list is shown in Table 3.

2.5. Data analysis

We used the SOPHiA DDM by Sophia Genetics platforms [15] for the analysis of the NGS data.

R studio1.2 with R5.4 packages were used for statistical analysis and graphics.

3. Results

3.1. Detection and characterization of CTCs

In order to develop a "liquid biopsy" from peripheral blood of BC patients aiming to the characterization of a malignancy and its follow-up over time in a minimally invasive way, twenty patients were enrolled into the study; the study workflow is shown in Fig. 1A. A total of 47 blood samples deriving from at least 1/3 time points were collected and tested for the presence of CTCs. We found ≥5 CTCs/7.5 ml of blood in 14/47 (29.8 %) blood samples in 11/20 (55.0 %) patients (Table 1) and used them for the subsequent steps. From 3/11 (27.2 %) patients, CTCs

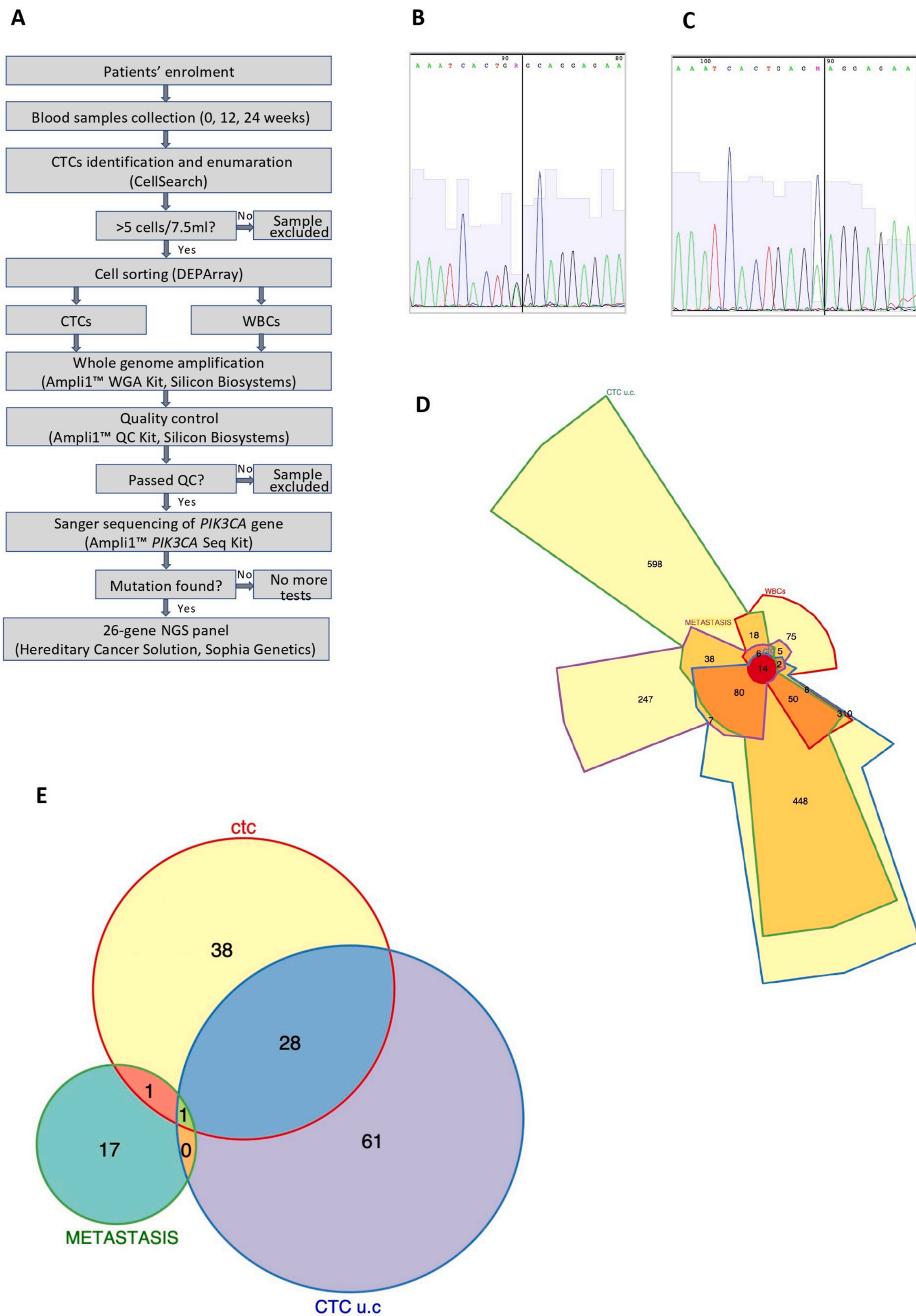

Fig. 1. A – Study workflow; B– *PIK3CA* c.1636 C > A (p.Gln546Lys) in patient 17; C – *PIK3CA* c.1634A > G (p.Glu545Gly) in patient 18, D – Venn diagram of all variants. CTC u.c.: green border; CTC: light blue border; Metastasis: purple border; WBCs: red border. Red circle: 14 non-pathogenic variants common to all samples; light yellow: specific variants for each cell type; yellow: common variants in two different cell types; orange: common variants in three different cell types.
E – Venn diagram of variants classified as class 3, 4, or 5 in CTC, Metastasis and CTC u.c. There is only one common variant for the three cells types. CTC u.c.: blue border; CTC: red border; Metastasis: green border.
CTC, circulating tumor cell; NGS, next generation sequencing; QC, quality control; WBS, white blood cell; WGA, whole genome amplification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Common variants in four sample types from patient 18.

Table 4 - List of common variants in the four samples	
1	chr1:121485241 G > C
2	chr19:27732021 T > C
3	chr2:92151667 A > G
4	chr2:92151689 A > T
5	chr17:59940819 C > T
6	chr1:121485227 A > T
7	chr11:94225920 C > T
8	chr11:108236783 G > T
9	chr5:112175770 G > A
10	chr11:108137775 T > A
11	chr7:6038621 delA
12	chr17:7578712 delTTT
13	chr2:48030458 G > C
14	chr5:112176756 T > A

were isolated at two time points. These data have been summarized in Table 1.

9 blood samples from the 11 selected patients (82 %), contained cells that the DEPArray system could not classify as CTCs based on their morphology and fluorescence (CTCs-u.c.). In two patients, such cells were present at 2 time points. Despite this uncertainty regarding their classification, we subjected also CTCs-u.c. to molecular analysis.

Overall, we DEParray-selected a total of 85 CTC/WBC samples as reported in Table 2.

3.2. Detection of PIK3CA mutations

All CTC samples underwent WGA and a quality control step to ensure sufficient quality of DNA for molecular analysis. To verify the good quality of WGA reaction, we used a quality control kit (QC Ampli1™ Kit). Sixteen out of 85 (19 %) samples failed QC.

Next, we sequenced exons 9 and 20 of the *PIK3CA* gene in the remaining 69 samples using the Ampli1™ *PIK3CA* Seq kit and obtained: i) complete sequences of both exons in 38 samples (55.0 %), ii) complete sequence of either one of the two exons in 14 samples (20.2 %; no evaluable sequences in 7 cases each), and iii) no evaluable sequences in 17 samples (24.6 %) (Table 2). Three out of 69 (5.79 %) samples taken from 2 patients (2/11, 18.18 %) carried pathogenic mutations in *PIK3CA*.

In details, we detected two different mutations in exon 9: p. Gln546Lys in patient 17 (at baseline, Fig. 1B) and p.Glu545Gly in patient 18 (at baseline and at 3-month, Fig. 1C). Interestingly, we also analyzed two CTC-u.c. samples from patient 18 (12 weeks) and found the p.Glu545Gly mutation in one of them.

3.3. NGS analysis

We wished to further characterize tumor genetics in patients with *PIK3CA* mutations by retrieving their primary and metastatic DNA. In order to do so, we decided to use the 26-gene HCS panel (Sophia Genetics) on those patients harboring a *PIK3CA* mutations in our sample. While a FFPE sample from the metastasis, but not from the primary tumor, was available for case 18, there was no stored material for case 17. Therefore, we sequenced all CTC and WBC samples from patient 18 using the HCS panel to confirm the presence of *PIK3CA* p.Glu545Gly and to determine if other gene variants linked to the hereditary BC pathogenesis were present.

NGS data were analyzed by applying the Sophia Genetics DDM somatic and germline pipeline on the metastasis and CTC samples. All variants are listed in Supplementary Table S2. Variants have been analyzed with the use of VarSome Suite (Saphetor), according to reported guidelines [17] and assigned to pathogenicity classes (Supplementary Material 2). We identified 14 non-pathogenic variants common to all samples (WBCs, CTCs, CTCs-u.c. and metastasis) (Table 4) while no common variants of uncertain significance (VUS), likely-pathogenic or pathogenic variants were present. CTC and CTC-u.c. samples shared 28 variants (including the *TP53* p.Arg248Gln mutation) and there were 38 and 61 variants specific to CTCs or CTCs-u.c., respectively. No other common variants were observed between CTCs-u.c. and metastasis. Fig. 1D shows the Venn diagrams of common variants in the analyzed tissues and 1E – common class 3, 4 and 5 variants.

The analysis confirmed the presence of the p.Glu545Gly mutation, both in CTCs and in the metastasis, but not in WBCs (Table 5). We also found a mutation in the *TP53* gene (p.Arg248Gln) in both CTC samples, but not in WBCs or the metastasis from this patient (Table 5).

4. Discussion

Current clinical practice methods for a minimally invasive characterization of malignancies in BC patients rely on biopsy and imaging, which show some drawbacks such as invasiveness and lack of complete information. Liquid biopsy, a potential alternative strategy, may involve cell-free tumor DNA, tumor-derived exosomes or CTCs. There are merits of using each one of these three materials, and there are drawbacks [18]. While cell-free tumor DNA displays mutations characteristic of the primary tumor, it is often difficult to detect due to its extremely low concentration. Tumor derived exosomes carry proteins, lipids, DNA or RNA that represent features of the tumor tissue they originated from, but are challenging for isolation protocols. Therefore, we -focused our analysis on CTCs due to their well-known propensity to form distant metastasis. Moreover, molecular characterization of CTCs already demonstrated that they might serve as surrogates for metastatic disease and as a prognostic tool [18].

Although FDA-approved and widely used, a limit of the CellSearch system for CTCs selection is that of detecting cells of epithelial origin only [19,20]. CTCs may lose their epithelial markers and morphology due to, for example, epithelial-to-mesenchymal transition (EMT). Indeed, for cells showing an irregular morphology [12] or weak fluorescence a clear-cut selection cannot be done [8]. We reasoned that the

Table 5
Mutations in the *PIK3CA* and *TP53* detected in patient 18.

CASE#18	*PIK3CA* mutation status	Genomic position	HGVS coding DNA change	HGVS protein change	Pathogenicity class
WBCs	wt				
CTCs	mut	chr3:178936092A > G	c.1634A > G	p.Glu545Gly	pathogenic
CTCs-u.c.	mut	chr3:178936092A > G	c.1634A > G	p.Glu545Gly	pathogenic
metastasis	mut	chr3:178936092A > G	c.1634A > G	p.Glu545Gly	pathogenic
CASE#18	***TP53* mutation status**	**Genomic position**	**HGVS coding DNA change**	**HGVS protein change**	**Pathogenicity class**
WBCs	wt				
CTCs	mut	chr17:7577538C > T	c.743G > A	p.Arg248Gln	pathogenic
CTCs-u.c.	mut	chr17:7577538C > T	c.743G > A	p.Arg248Gln	pathogenic
metastasis	wt				

Legend: CTCs, circulating tumor cells; CTCs-u.c., circulating tumor cells of uncertain classification; mut, mutated; WBCs, white blood cells; wt, wild type.

collection of CTCs-u.c. may be useful to analyze potentially atypical CTCs. Although, the CTCs-u.c. were collected from most patients, a *PIK3CA* mutation was found in only one single-cell CTC-u.c. sample. The other CTCs-u.c. sample from the same patient had no mutation suggesting that it was not a CTC or that the mutation was not present in all tumor cells. Given the absence of primary tumor material, this issue remained unresolved, while lack of *PIK3CA* mutations in other CTCs-u.c. made it impossible to confirm that they were indeed CTCs.

Cells from primary tumor, metastasis, and CTCs may have diverse and distinct phenotype and immunopathologic characteristics [21–23]. Here, we confirm this finding and show that DNA extracted from CTCs, CTCs-u.c. and metastasis from patient 18 shared the same mutation in the *PIK3CA* gene, whereas the *TP53* mutation was present in CTCs and CTCs-u.c. only.

Parallel or sequential germline and tumor DNA sequencing results in reliable detection of germline variants relevant to therapy selection. Current guidelines recommend sequencing tumor and germline DNA in some cases [15,24]. A recent paper [25] showed that ~8 % of pathogenic germline variants were overlooked in patients in whom only the tumor sample was sequenced, while further 11 % were uncovered only after the onset of a second primary tumor. We applied the procedure described by Polzer et al. for a simultaneous isolation of WBCs and CTCs [12]. The material obtained in this way undergoes identical manipulations allowing for a like-for-like comparisons. WBC DNA was used to search for *PIK3CA* mutations and for HCS analysis.

Amongst our patients, we detected no exon 20 mutations reported to be the most frequent [4,5,26]. Instead, we detected two somatic exon 9 mutations with a described frequency of <1 % in *PIK3CA*-mutated BCs [26].

In this study, the number of CTCs recovered was low but not different than previously reported [10]. A possible explanation is that the CellSearch protocol, optimized for single cells, missed CTC clusters forming in the bloodstream or CTCs that underwent EMT and lost EpCAM expression [27]. Indeed, we collected cells endowed with weak fluorescence but not those negative. Similarly, the rate of 18 % of PIK3CA mutations may seem low, possibly, due to the low number of patients analyzed.

Our data confirm that CTCs can be used to search for tumor-specific mutations and the importance of analyzing multiple cells including those with atypical phenotype and immunophenotype.

Ethics approval and consent to participate

Inclusion of volunteers was conducted according to the Declaration of Helsinki. All volunteers gave written informed consent before any material was taken. The study was approved by the European Institute of Oncology (IEO 151 RE729/1).

Consent for publication

All participants gave written consent to publish.

Availability of data and materials

The dataset variants generated and analyzed during the current study are available in Supplementary Table S2. The pathogenic mutations generated and analyzed during the current study are available in the ClinVar database.

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CRediT authorship contribution statement

EM performed the molecular experiments listed in the manuscript, including NGS, analyzed and interpreted the data, and drafted the manuscript. **CM, MP** and **MCC** performed the CellSearch and DEPArray experiments, analyzed and interpreted the data. **EB** and **LB** drafted and edited the manuscript. **VF** and **LG** performed the bioinformatic analysis, analyzed and interpreted the data. **LZ** and **MTS** conceived and designed the study and reviewed the manuscript. **PGP** reviewed the manuscript. All authors read and approved the final manuscript.

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.

Data availability

Data will be made available on request.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101805.

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