Clinical Cancer Research

Mutation Analysis of Cell-Free DNA and Single Circulating Tumor Cells in Metastatic Breast Cancer Patients with High Circulating Tumor Cell Counts

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Abstract

Purpose: The purpose of this study was to directly compare mutation profiles in multiple single circulating tumor cells (CTC) and cell-free DNA (cfDNA) isolated from the same blood samples taken from patients with metastatic breast cancer (MBC). We aimed to determine whether cfDNA would reflect the heterogeneity observed in 40 single CTCs.

Experimental Design: CTCs were enumerated by CELLSEARCH. CTC count was compared with the quantity of matched cfDNA and serum CA15-3 and alkaline phosphatase (ALP) in 112 patients with MBC. In 5 patients with $\geq$100 CTCs, multiple individual EpCAM-positive CTCs were isolated by DEPArray and compared with matched cfDNA and primary tumor tissue by targeted next-generation sequencing (NGS) of about 2,200 mutations in 50 cancer genes.

Results: In the whole cohort, total cfDNA levels and cell counts ($\geq$5 CTCs) were both significantly associated with overall survival, unlike CA15-3 and ALP. NGS analysis of 40 individual EpCAM-positive CTCs from 5 patients with MBC revealed mutational heterogeneity in *PIK3CA*, *TP53*, *ESR1*, and *KRAS* genes between individual CTCs. In all 5 patients, cfDNA profiles provided an accurate reflection of mutations seen in individual CTCs. *ESR1* and *KRAS* gene mutations were absent from primary tumor tissue and therefore likely either reflect a minor subclonal mutation or were acquired with disease progression.

Conclusions: Our results demonstrate that cfDNA reflects persisting EpCAM-positive CTCs in patients with high CTC counts and therefore may enable monitoring of the metastatic burden for clinical decision-making. *Clin Cancer Res; 23(1); 88–96. ©2016 AACR.*

Introduction

Breast cancer is the most common cancer in women and ranks as second in the world in terms of cancer deaths. Recent studies using next-generation sequencing (NGS) have shown that cancer evolves in the patient (1–3), and therapies can induce the evolution of clones of cells that are refractory to the treatment (4, 5). Much research has been devoted to developing bioassays that can help in the selection of therapies as breast cancer evolves, including characterization of circulating tumor cells (CTC) and the tumor-derived fraction of circulating cell-free DNA (cfDNA) termed circulating tumor DNA.

Although a number of commercial platforms are under investigation for isolation and characterization of CTCs without the use of a surface marker, CELLSEARCH (Janssen Diagnostics), which enumerates CTCs of epithelial origin [$CD45^-$, $EpCAM^+$, and cytokeratins (CK) 8, 18, and/or 19 positive cells], is currently the only FDA-approved platform in clinical use. EpCAM-positive CTCs as measured by CELLSEARCH are rarely detected in patients with primary breast cancer and many patients with metastatic disease also have few EpCAM-positive CTCs (0–5 per 7.5-mL blood). When detected, the number of EpCAM-positive CTCs reflects both the effects of systemic therapy (6–8) and prognosis (9). In contrast, circulating tumor DNA is detected in early and late stages in a high proportion of cases (10–12) and can persist for many years after supposedly successful therapy (13), despite no evidence of overt distant metastases. In metastatic breast cancer (MBC), the dynamics of circulating tumor DNA compare favorably to serum CA15-3 and CTC counts determined by CELLSEARCH in reflecting changes in tumor burden (14).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-0825

Translational Relevance

Next-generation sequencing is a key approach for monitoring tumor genomic alterations in circulating cell-free DNA (cfDNA). High circulating tumor cells (CTC) count (≥5 EpCAM-positive CTCs per 7.5-mL blood as measured by CELLSEARCH) is a poor prognostic indicator, but thus far individual CTCs have not been directly compared with matched cfDNA by mutation profiling. However, concurrent analysis of individual CTCs and matched cfDNA has the potential for comprehensive characterization of tumor-derived genetic alterations in blood by "liquid biopsy." In this article, we show that individual CTCs have heterogeneous mutations and that cfDNA isolated from the same blood sample provides an accurate reflection of mutations seen in individual CTCs. We also show that the total cfDNA level, like CTC counts, is an independent prognostic marker in patients with metastatic breast cancer. Overall, our results suggest that cfDNA reflects persisting EpCAM-positive CTCs in patients with high CTC counts and could potentially enable monitoring of the metastatic burden for clinical decision-making when CTCs cannot be obtained.

Metastases are heterogeneous, both between metastatic sites and also between the cells that compose each metastasis. Early metastases are composed of a greater proportion of cells with EMT and "stem-like" characteristics, whereas larger metastases are dominated by more differentiated, heterogeneous patterns of cells, more closely reflecting the heterogeneity seen in primary tumors (15). Taking all these considerations into account, together with the well-established phenomenon of clonal evolution (16, 17), it is evident that a single conventional tissue biopsy will not necessarily provide accurate information regarding appropriate therapy. An additional layer of complexity is suggested by the role of the immune system in patients with cancer, since immunodestruction of persisting micrometastases could mean that the circulating tumor DNA is predominantly a reflection of destroyed cancer cells; whereas persisting CTCs could be envisaged as a more accurate guide to appropriate therapy.

Previous studies have compared mutations in cfDNA to either biopsies of metastases or CTCs (2, 18). We reported emergence of *ESR1* mutations in cfDNA, and in a single patient with a very high CTC count (>3,000 EpCAM-positive CTCs in 7.5-mL blood), the *ESR1* p.D538G mutation was detected in cfDNA and matched CTCs (2). Since cfDNA is in principal easier to obtain than CTCs for molecular analysis, it is essential to determine whether mutations in cfDNA are the same as those seen in individual CTCs, as heterogeneity is frequently observed in metastatic biopsies (19, 20). Here, we compared total cfDNA levels and CELLSEARCH CTC counts with serum CA15-3 and alkaline phosphatase (ALP) levels in patients with MBC. We performed deep amplicon sequencing of 50 genes and compared mutation profiles in cfDNA and individual EpCAM-positive CTCs isolated from the same blood sample in 5 patients with high CTC counts (≥100 CTCs in 7.5-mL blood). Results show that cfDNA mutation profiles reflect mutational heterogeneity seen across individual CTCs and that total cfDNA levels and ≥5 CTCs are significantly associated with overall survival. Circulating tumor DNA is a useful substitute for metastasis biopsy (18). These data suggest that circulating tumor DNA reflects persisting EpCAM-positive CTCs in patients with high CTC counts and could enable monitoring of the metastatic burden but requires confirmation in larger groups of patients.

Material and Methods

Patients and samples

The study protocol was approved by the Riverside Research Ethics Committee (Imperial College Healthcare NHS Trust; REC reference number: 07/Q0401/20) and conducted in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki. All patients gave written informed consent prior to participation. We recruited 112 patients with radiologically confirmed MBC (Table 1). Twenty milliliters of blood was taken into EDTA tubes (BD Biosciences) and processed to plasma for cfDNA (21), and 7.5 mL blood was taken into CellSavepreservative tubes for CTC capture and enumeration with the CELLSEARCH CTC Test (Janssen Diagnostics) as described previously (22). CA15-3 and ALP results were obtained from patient notes.

Extraction and quantitation of DNA

cfDNA was isolated from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer's instructions. Isolation of DNA from lymphocytes and quantitation of total cfDNA was as described previously (21). cfDNA levels (ng/mL) were converted to copies/mL plasma assuming 3.3 pg DNA per haploid genome. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks with matching hematoxylin and eosin (H&E) were retrieved from the histopathology archive and reviewed by a consultant histopathologist. Two separate regions of tumor were then removed from the block using a 1-mm tissue microarray needle. DNA was extracted from the FFPE tissue core using the Qiagen GeneRead Kit according to manufacturer's instructions. CTC isolation by DEPArray, QC to identify those samples with a genomic integrity index (GII) of 3 or 4 suitable for sequencing (20) and *Ampli1* whole-genome amplification were carried out as a service by Silicon Biosystems. Details of the exact numbers of single cells recovered and analyzed for each patient are given in Supplementary Table S1.

Targeted NGS

AmpliSeq reactions were set up using 10 ng WGA DNA, an average of 8 ng cfDNA and 10 ng FFPE tumor tissue DNA. All samples with sufficient DNA (including all individual cell samples) were sequenced using 2 AmpliSeq panels: an in-house 30-amplicon panel, designed using Ion AmpliSeq designer software (https://www.ampliseq.com), covering mutation hotspots in *ESR1*, *PIK3CA*, *TP53*, and *ERBB2* (Supplementary Table S2) and the Custom Cancer Hotspot Panel v2 for *Ampli1* WGA DNA (49 genes, >2,200 COSMIC mutations; Silicon Biosystems, see http://www.siliconbiosystems.com/fee-for-service and Supplementary Table S3). Three genes (*PIK3CA*, *TP53*, and *ERBB2*) were on both panels making a total of 50 genes surveyed for hotspot mutations. Overlapping mutations were

reviewed in Integrated Genomics Viewer (IGV) if they were only detected on one panel. In total, the two panels surveyed about 2,200 COSMIC ID mutations.

Sequencing on a 316 chip using the Ion PGM and data analysis was as described previously (2). In brief, sequencing data were accessed through the Torrent Suite v.4.2.0. Reads were aligned against the human genome (hg19) using Alignment v4.0-r77189 and variants called using the coverageAnalysis v4.0-r77897 and variantCaller v4.0-r76860, respectively. Because of the sensitivity of the in-house panel, for cfDNA and FFPE tumor tissue, variantCaller was configured to call on high stringency, somatic variants with downsampling set to 6,000 and gen_min_coverage set to 6. COSMIC IDs were obtained using COSMIC v71 (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/; last cited on 21st February 2016). Variants in individual CTCs and white blood cells (WBC) were analyzed using a preset high stringency germline threshold, as recommended by the manufacturer, which is optimized for high-frequency variants and minimal false-positive calls. The allelic dropout rate (ADR) and false-positive rate (FPR) was calculated as described by Leung and colleagues (23). All mutations with a quality score below 25 were omitted, and all variants detected in the first or last 10 bases of an amplicon were omitted as likely mispriming events (2, 24). ANNOVAR (25) was used to annotate all private mutations with refGene ID, functional consequence (e.g., nonsynonymous), and functional predictions [using SIFT (26), Polyphen-2 (27), and MutationTester (28)]. All variants detected were also manually confirmed across all samples using the IGV 2.3 (29, 30). The mutant allele frequency (termed AF) was calculated as the proportion of total reads at a site, which contained the variant allele [e.g., if you have 200 reads in total and 8 of them have the variant, then the AF is $(8/200) \times 100 = 4\%$].

Digital droplet PCR

Droplet digital PCR (ddPCR) was used to validate selected mutations in *ESR1* p.Y537C/N/S, p.D538G; *PIK3CA* p.E545K and H1047R; and *KRAS* p.G12D. Each assay was performed in duplicate using a Bio-Rad QX200 ddPCR system as described previously (31). Inventoried assays were used for detection of *PIK3CA* p.E545K (assay numbers dHsaCP2000075 and dHsa2000076) and p.H1047R (assay numbers dHsaCP2000077 and dHsaCP2000078) and *KRAS* p.G12D (assay numbers dHsaCP2000001 and dHsaCP2000002; Bio-Rad Laboratories) according to manufacturers' instructions. For the *ESR1* mutations, a dual-labeled locked nucleic acid (LNA) probe strategy was used (see Supplementary Table S4 for sequences). Thermal cycling conditions for the *ESR1* assays were: 10-minute hold at 95°C, 40 cycles of 95°C for 15 seconds and then 60°C for 60 seconds. Raw fluorescence amplitude was analyzed using the Quantasoft version 1.6.6.0320 software and used to obtain the fractional abundance for a given mutation. This was reported as the allele frequency to be consistent with NGS data. Calculation of the allele fraction was performed as described previously (32, 33). The total number of droplets (with and without DNA) was used to calculate DNA copies/μL, then dividing the number of mutant copies by the number of total DNA copies (mutant plus wild-type), and multiplying by 100 to give the percentage (allele fraction) of mutant DNA copies on the basis of Poisson distribution of positive to empty droplets (33).

Statistical analysis

Correlation analysis of total cfDNA levels, CA15-3, ALP, and CTC counts, was performed using a nonparametric Spearman correlation. *P* values were 2-tailed and considered significant if $P < 0.05$. Survival analysis was performed using multiple Cox regression as described previously (14) with each biomarker as a continuous time-dependent variable (Supplementary Table S5). Briefly, for each variable, a model was constructed using a counting process notation (i.e., start, end, event). In our study, start was taken as the date of the baseline blood sample, and the end was taken as an arbitrary cutoff of 01/12/2015.

Results

This was a prospective study set up to identify patients with high CTC counts ($\geq$100 CTCs per 7.5-mL blood) suitable for isolation of individual CTCs by DEPArray. The threshold of $\geq$100 CTCs per 7.5-ml-blood was based on a previous study by Polzer and colleagues (20), that analyzed individual CTCs from patients with similar high CTC counts but did not compare individual CTCs with matched cfDNA. We recruited 112 patients with MBC to the study (Table 1). Each had undergone multiple lines of treatment. Sixty-one patients were CTC-negative and 51 patients had $\geq$1 CTCs measured by CELLSEARCH (median, 5; range, 1–701). Serum CA15-3 levels were elevated (>30 U/mL; ref. 34) in 72 patients (64%), ALP was elevated (>100 IU/L) in 40 patients (36%), and both were elevated in 31 (28%). Total cfDNA was obtained for all samples (median, 2757 copies/mL of plasma; range, 30–115,724 copies/mL of plasma). The markers were significantly correlated (Spearman correlation analysis; Supplementary Table S6), except cfDNA and CA15-3 ($P = 0.058$). High

Table 1. Clinicopathologic features of 112 patients with MBC

Clinicopathologic feature	Details	*n*
Age, y	≤40	9 (8%)
	>40	103 (92%)
	Median	59
	Range	25–88
ER	Positive	96 (86%)
	Negative	14 (12%)
	Unknown	2 (2%)
PR	Positive	65 (58%)
	Negative	27 (24%)
	Unknown[a]	20 (18%)
HER2	Positive	21 (19%)
	Negative	73 (65%)
	Not tested[b]	18 (16%)
CA15-3	≤30 U/mL	40 (36%)
	>30 U/mL	72 (64%)
	Median	51
	Range	1–3,613
ALP	≤100 IU/L	72 (64%)
	>100 IU/L	40 (36%)
	Median	88
	Range	30—555
CTCs/7.5 mL blood	0	61 (54%)
	1–4	26 (23%)
	≥5	25 (23%)
	Range	0–701

[a]Record of PR testing not available.
[b]Date of diagnosis predates the introduction of HER2 testing in the United Kingdom.

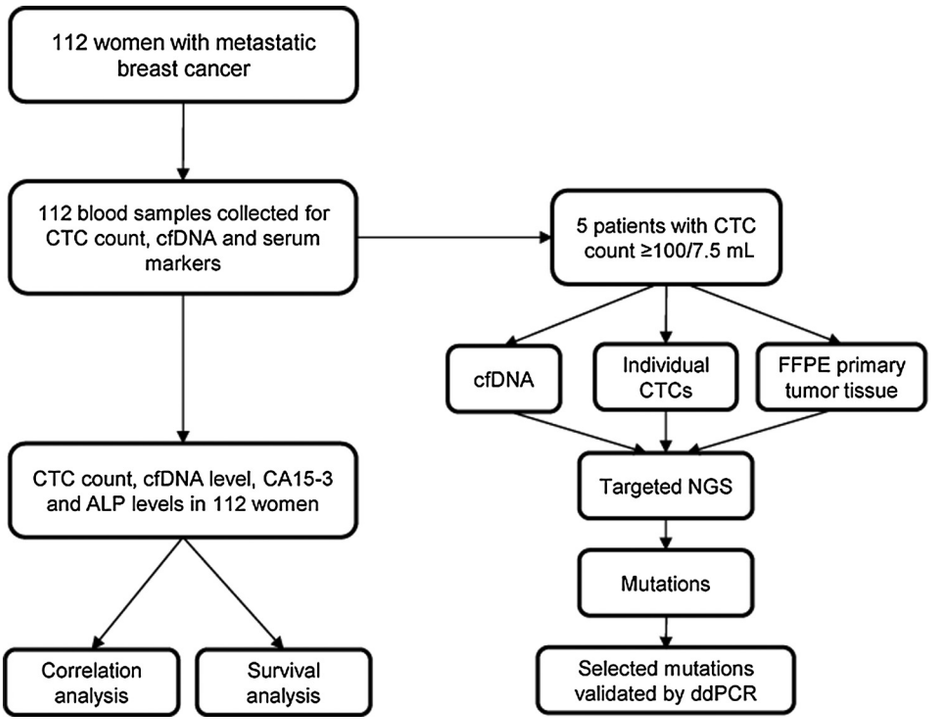


Figure 1.
Study workflow. 112 patients with metastatic breast cancer were recruited to the study. Blood was collected and analyzed for CTC count and total cfDNA levels in all 112 patients. In 5 patients with high CTC count individual CTCs were isolated by DEPArray and subjected to targeted NGS in comparison with matched cfDNA and primary tumor tissue where available.

CTC count [≥5 CTCs; HR, 2.8, 95% confidence interval (CI), 1.4–5.7; $P = 0.005$] and total cfDNA level (HR, 2.2; 95% CI, 1.3–3.6; $P = 0.03$) were significantly associated with poorer overall survival (Supplementary Table S6).

cfDNA and individual CTCs have overlapping mutation profiles

At the time of the study, we had established that adequate cells could be obtained from the DEPArray System for patients with high CTC counts (≥100 CTCs per 7.5-mL blood) based on a previous study (20). We therefore chose 5 patients with high EpCAM-positive CTCs for single CTC isolation by DEPArray: 4 had estrogen receptor (ERα)-positive metastatic disease and one was triple-negative. Clinical details for the 5 patients are given in Supplementary Data 1 and treatment timelines are in Supplementary Fig. S1. FFPE primary tumor tissue was available for 4 patients. Four had serial cfDNA and 1 had serial CELLSEARCH CTC samples (Fig. 1). Individual CTCs were recovered successfully for each patient. In total, 42 individual EpCAM-positive CTCs, 16 individual WBCs as germline and QC controls, 5 CTC pools and 6 WBC pools were isolated by DEPArray. Of these 40 individual CTCs, 15 individual WBCs and all cell pools passed QC checks (genomic integrity index ≥ 3; Supplementary Table S1) for sequencing (20).

All CTC and WBC samples, matching cfDNA and FFPE tumor tissue DNA were analyzed by deep amplicon sequencing (Fig. 1) using 2 panels covering about 2,200 COSMIC mutations in 50 cancer genes (for details of the custom 30-amplicon panel, see Supplementary Table S2, for full details of the *Ampli1* custom cancer hotspot panel, see http://www.siliconbiosystems.com/fee-for-service). A high average read depth was obtained with both panels: 929× for the Custom Cancer Hotspot Panel v2 for *Ampli1* WGA DNA (Silicon Biosystems; ref. 20) and 4,537× for the 30-amplicon custom panel (Supplementary Table S7). The ADR across individual CTCs and WBCs and the FPR for single-nucleotide variants (SNV) were 25.01%, SEM 2.68%, and 0.6%, SEM 0.32%, respectively (Supplementary Table S8), within the range reported previously (23). Mutations were only called in individual CTCs and WBCs if these were present in 2 or more single cells (23). Overall only 3 individual CTCs had no coverage at a specific mutated base (Table 2). In each case, the mutation was called in other CTCs and the matched plasma cfDNA.

Although we examined mutation hotspots in 50 genes across 2 amplicon panels by NGS, mutations were only detected by NGS in 4 genes in *ESR1*, *PIK3CA*, *TP53*, and *KRAS*. Two of these *PIK3CA*, *TP53* were common to both panels. ddPCR was used as validation for *ESR1* p.Y537C/N/S, p.D538G; *PIK3CA* p.E545K and H1047R, and *KRAS* p.G12D when sufficient remaining DNA was available.

Overall, 4 patients had mutations detected in *PIK3CA*, *TP53*, *ESR1*, and *KRAS* genes in cfDNA and individual EpCAM-positive CTCs (Fig. 2; Table 2). Levels of circulating tumor DNA varied. The mean plasma mutant allele frequency ranged from 0.3% to 32% and an increase in levels of circulating tumor DNA generally accompanied an increase in total cfDNA (Fig. 2). Mutational heterogeneity was seen between individual EpCAM-positive CTCs isolated from the same blood sample with the majority of CTCs having 1, 2, or no mutations detected. All mutations detected in individual EpCAM-positive CTCs were detected in the matched cfDNA (Table 2). *PIK3CA* and *TP53* gene mutations were also present in the primary tumor DNA, whereas *ESR1* and *KRAS* mutations were unique to cfDNA and EpCAM-positive individual CTCs (Table 2) suggesting potential clonal evolution. Private mutations of unknown significance were also detected in individual

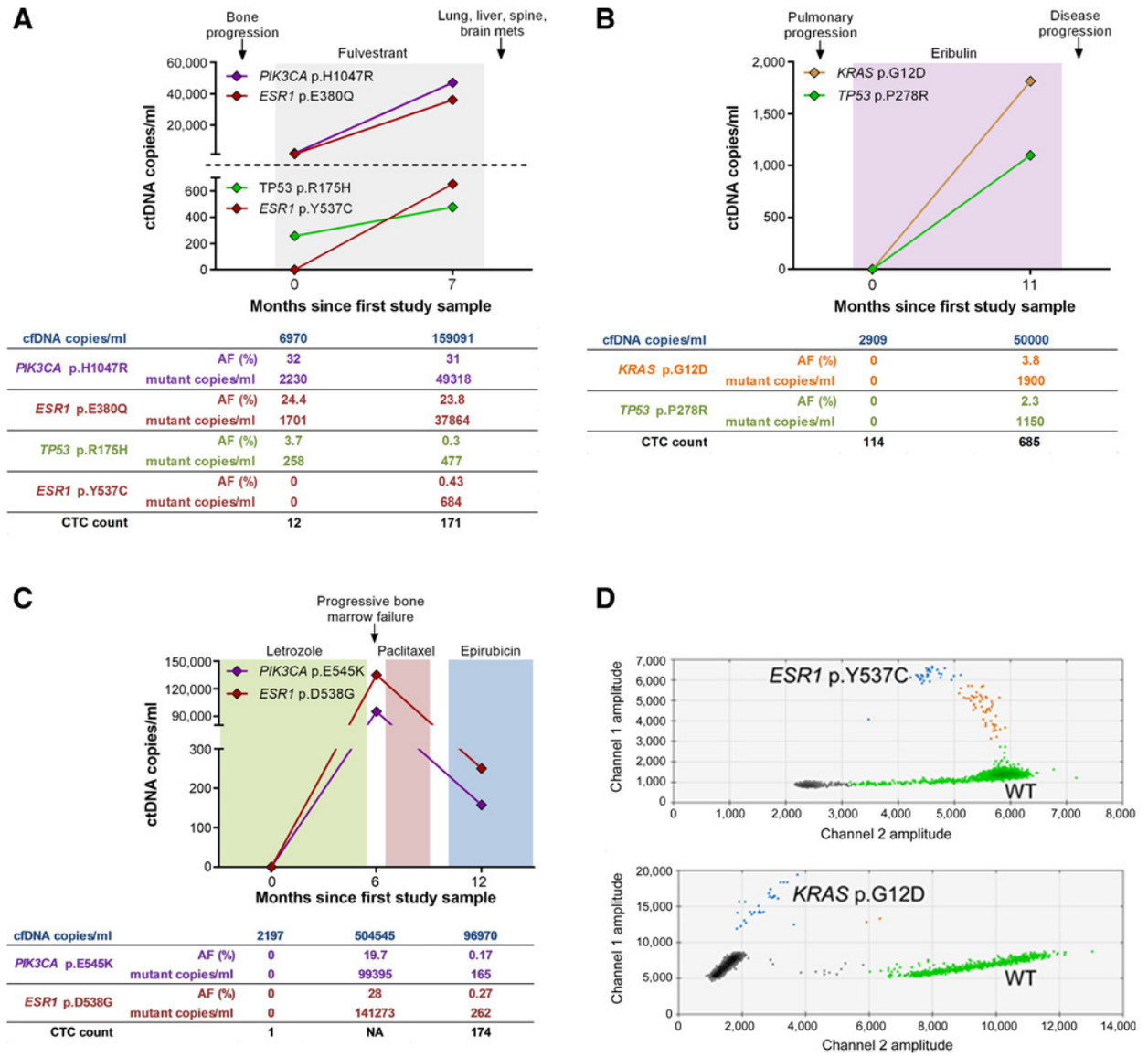
Table 2. Mutations identified in matched FFPE, cfDNA, and individual CTCs

Sample ID	Receptor status		CTC	cfDNA (AF)	Primary tumor region 1 (AF)	Primary tumor region 2 (AF)
CTCM155	$ER^+/PR^+/HER2^-$	C1	PIK3CA p.H1047R; ESR1 p.E380Q	PIK3CA p.H1047R (23.7%); ESR1 p.E380Q (3.9%)	PIK3CA p.H1047R (67.8%)	PIK3CA p.H1047R (72.4%)
		C2	PIK3CA p.H1047R; ESR1 p.E380Q			
		C3	PIK3CA p.H1047R; ESR1 p.E380Q			
		C4	PIK3CA p.H1047R; ESR1 p.E380Q			
		C5	PIK3CA p.H1047R			
		Cpool	PIK3CA p.H1047R; ESR1 p.E380Q			
CTCM138	$ER^+/PR^+/HER2^-$	C1	—/NC	PIK3CA p.H1047R (31%); ESR1 p.E380Q (23.8%); TP53 p.R175H (0.3%); ESR1 Y537C (0.43%)	PIK3CA p.H1047R (14.2%); TP53 p.R175H (1.9%)	PIK3CA p.H1047R (36%); TP53 p.R175H (0.99%)
		C2	PIK3CA p.H1047R; ESR1 p.E380Q			
		C3	PIK3CA p.H1047R; ESR1 p.E380Q			
		C4	—			
		C5	—			
		Cpool	PIK3CA p.H1047R; ESR1 p.E380Q			
CTCM105	$ER^-/PR^-/HER2^-$	C1	KRAS p.G12D	KRAS p.G12D (3.8%); TP53 p.P278R (2.3%)	—	—
		C2	KRAS p.G12D			
		C3	KRAS p.G12D			
		C4	—			
		C5	KRAS p.G12D			
		C6	KRAS p.G12D			
		C7	NC			
		C8	KRAS p.G12D			
		C9	—			
		C10	KRAS p.G12D			
		C11	NC			
		Cpool	KRAS p.G12D			
CTCM292	$ER^+/PR^+/HER2^-$	C1	PIK3CA p.E545K; ESR1 p.D538G	PIK3CA p.E545K (0.17%); ESR1 p.D538G (0.27%)	None available	None available
		C2	PIK3CA p.E545K; ESR1 p.D538G			
CTCM167.1	$ER^+/PR^+/HER2^-$	C1	—	—	—	—
		C2	—			
		C3	—			
		C4	—			
		C5	—			
		Cpool	—			
CTCM167.2		C1	—	—	—	—
		C2	—			
		C3	—			
		C4	—			
		C5	—			
		C6	—			
		C7	—			
		C8	—			
		C9	—			
		C10	—			
		C11	—			
		C12	—			
		Cpool	—			

NOTE: Shown are COSMIC mutations detected in individual CTCs (C1, 2), CTC pools (Cpool) matched plasma cfDNA and two regions of primary tumor. Patient CTCM167 had two serial samples for both CTCs and plasma cfDNA. All data are summarized in Supplementary Tables S10–S13.
Abbreviations: —, no mutation detected; NC, no coverage at this amplicon; —/NC, *PIK3CA* mutation not detected/no coverage at *ESR1* mutation site.

CTCs and WBCs (Supplementary Table S9); however, these were excluded as likely amplification artefacts, as they were not detected in the matched CTC pool, WBC pool, or primary tumor (Supplementary Tables S10–S13).

Patient CTCM155 (ER^+, $HER2^-$) had 2 blood samples taken 11 months apart (Supplementary Fig. S1). Plasma cfDNA was only available for the first sample, as insufficient blood was obtained at the second time point for both cfDNA and CTCs.



cfDNA copies/ml		6970	159091
PIK3CA p.H1047R	AF (%)	32	31
	mutant copies/ml	2230	49318
ESR1 p.E380Q	AF (%)	24.4	23.8
	mutant copies/ml	1701	37864
TP53 p.R175H	AF (%)	3.7	0.3
	mutant copies/ml	258	477
ESR1 p.Y537C	AF (%)	0	0.43
	mutant copies/ml	0	684
CTC count		12	171

cfDNA copies/ml		2909	50000
KRAS p.G12D	AF (%)	0	3.8
	mutant copies/ml	0	1900
TP53 p.P278R	AF (%)	0	2.3
	mutant copies/ml	0	1150
CTC count		114	685

cfDNA copies/ml		2197	504545	96970
PIK3CA p.E545K	AF (%)	0	19.7	0.17
	mutant copies/ml	0	99395	165
ESR1 p.D538G	AF (%)	0	28	0.27
	mutant copies/ml	0	141273	262
CTC count		1	NA	174

Figure 2.

Longitudinal follow up of circulating tumor DNA in 3 patients with high CTC counts. **A–C,** Total cfDNA and circulating tumor DNA levels. (**A**) patient CTCM138; (**B**) patient CTCM105; (**C**) patient CTCM292. Endocrine or cytotoxic therapies are indicated by colored shading. The number of cfDNA copies obtained from the blood sample, mutant allele frequency (AF), number of mutant copies and CTC count is given below each graph. NA = Not available or CellSearch failed. (**D**) Digital PCR detection of low frequency mutations in circulating tumor DNA. ESR1 p.Y537C mutation, patient CTCM138 (top) and KRAS p.G12D mutation, patient CTCM105 (bottom). Green dots represent HEX-labeled wild-type (WT), blue dots represent FAM-labeled mutant DNA and brown dots represent double positive droplets containing WT and mutant DNA. Gray dots represent empty droplets.

EpCAM-positive CTCs were detected in the second sample after progressing on exemestane and everolimus combination therapy. The primary tumor and baseline cfDNA had a *PIK3CA* mutation (p.H1047R, AF 70% and 23%, respectively). A second lower frequency *ESR1* mutation (p.E380Q, AF 3.9%) was also detected in cfDNA, suggesting that either a subclonal origin or this mutation was acquired on treatment. All 5 individual CTCs and the CTC pool had both mutations (Table 2, Supplementary Table S10).

Patients with increasing circulating tumor DNA

Patient CTCM138 (ER^+, $HER2^-$) had a *PIK3CA* (p.H1047R) mutation (AF 25%) and low-frequency subclonal *TP53* (p.R175H) mutation (AF, 1.5%) in primary tumor DNA. She was recruited to the study in 2014 some 10 years after diagnosis of breast cancer and having received multiple lines of endocrine therapy. Both mutations seen in the primary tumor were detected in baseline cfDNA (*PIK3CA:* AF, 32%; *TP53:* AF, 3.7%) as well as an acquired *ESR1* (p.E380Q) mutation (AF, 24.2%). The second

blood sample was taken 7 months later just prior to clinical disease progression. Levels of circulating tumor DNA increased from baseline, the 3 mutations persisted and a second *ESR1* mutation (p.Y537C) was acquired at a low level (AF, 0.43%; Fig. 2A and D; Supplementary Table S11). Five individual CTCs were isolated from the second blood sample, 2 of which had the *PIK3CA* (p.H1047R) and *ESR1* (p.E380Q) mutations but not the lower frequency *ESR1* p.Y537C or *TP53* p.R175H mutations (Table 2), although these could have been missed by sampling 5 individual CTCs.

Patient CTCM105 (triple-negative) was recruited to the study in March 2014 with progressive disease. No mutations were detected across the 50 genes in either the primary tumor or baseline cfDNA sample. A second blood sample was taken 11 months later. Six of 9 individual CTCs isolated from this sample had a *KRAS* (p.G12D) mutation that was also detected in the CTC pool (Fig. 2B and D; Table 2; Supplementary Table S12). Matched cfDNA had the *KRAS* p.G12D mutation (AF, 3.8%; Fig. 2D) and a low-frequency mutation in *TP53* (p.P278R; AF, 2.3%). The low-frequency *TP53* mutation might be missed by sampling 9 CTCs; however, it is also possible that 2 metastatic clones were shedding DNA in to plasma, one reflected in CTCs and the other not. As neither mutation was detected in the FFPE primary tumor tissue, these may either have been acquired on treatment or have arisen from a minor subclone missed by tumor sampling.

Patient CTCM292 (ER^+, $HER2^-$) presented with bone metastases in 2012 and was treated with tamoxifen. She subsequently relapsed and commenced letrozole, which was then switched to chemotherapy with paclitaxel, then epirubicin. Three serial blood samples were taken over a 12=month period commencing upon letrozole. No mutations were detected in the baseline cfDNA sample, but 2 mutations were detected 6 months later while she was on letrozole (*ESR1* p.D538G AF 28% and *PIK3CA* p.E545K 19.7%) and then reduced at 12 months on paclitaxel (AF, 0.27% and 0.17%, respectively; Fig. 2C). Both mutations were also present in individual EpCAM-positive CTCs isolated from the third blood sample (Fig. 2C, Table 2; Supplementary Table S13).

Last, the fifth patient (CTCM167: ER^+, $HER2^-$) was monitored over an 18-month period and had a marked increase in total cfDNA levels and CTC counts at disease progression (Supplementary Fig. S2). No mutations were identified in the 50 genes analyzed in FFPE primary tumor DNA. Moreover, there was no evidence for acquisition of mutations across the 50 genes in either cfDNA or individual CTCs from 2 serial blood samples (Table 2).

Discussion

CTCs are an established prognostic indicator in MBC (7, 9, 35). A recent study has shown that it is possible to interrogate both cfDNA and pools of mutation-bearing CTCs from the blood of patients with MBC (36). Here, we present results of mutations in multiple individual CTCs and matched cfDNA in patients with MBC and high CTC counts. First, in 4 patients, we observed heterogeneity in mutations between individual EpCAM-positive CTCs. In all 4 patients, cfDNA profiles provided an accurate reflection of mutations seen in individual CTCs, and in 2 patients, cfDNA had more mutations than those found in CTCs. *ESR1* and *KRAS* gene mutations were present in individual CTCs and cfDNA but were absent from primary tumor tissue. Therefore, these likely reflect either a minor subclonal mutation or were acquired with disease progression. Of note, the primary tumor, serial cfDNA samples, and multiple individual CTCs from 1 of the 5 patients (CTCM167) had no hotspot mutations detected across the 50 genes. Further analysis, such as whole-exome sequencing or array CGH, is needed to identify mutations and copy number variation in these samples.

Second, in 2 patients, mutations were detected in cfDNA [in *TP53* (p.P278R) and in *TP53* (p.R175H) and *ESR1* (p.Y537C)] that were not seen in individual CTCs or the CTC pool isolated from the same blood sample. As these were low frequency (and likely subclonal) mutations, these may have been missed by sampling small numbers of individual CTCs. It is also possible that these were missed because of allelic distortion/dropout, which had an average frequency of 25.46% across the samples analyzed, similar to a previous single-cell genomics study (23). Of note, in a previous study (2), we also identified an *ESR1* gene mutation unique to cfDNA that was not seen in a matched pool of >3,000 EpCAM-positive CTCs suggesting that 2 or more metastatic clones were shedding DNA in to plasma, one reflected in CTCs, one not.

Third, although we detected private mutations in single CTCs and single WBCs (mutations observed exclusively in single cells; ref. 37), these were excluded as likely polymerase error during WGA (38, 39) or sequencing error as all were absent from matched FFPE tissue, cfDNA, and CTC/WBC pools. Previous studies have suggested "census-based" sequencing of more than one CTC to distinguish private mutations from polymerase errors (38, 39); whereas other studies have compared results with ultra-deep sequencing of the primary and/or metastatic tumor (40) both approaches were performed here.

Although we sequenced about 2,200 COSMIC mutations across 50 genes in 5 patients, mutations were only detected in 4 genes (*PIK3CA*, *ESR1*, *TP53*, and *KRAS*) and only 1 or 2 mutations were detected in the majority of samples. This is consistent with a recent study, which used the same platform and a 50-gene panel (41). *TP53* and *PIK3CA* are the most frequently mutated genes in breast cancer; however, a large number of other genes are less commonly mutated (42). Whole-exome or whole=genome analysis of the primary tumor/metastatic tissue would be required to determine additional mutations not covered by the targeted sequencing approach. However, metastatic tissue is frequently impossible to obtain in this group of patients.

ESR1 mutations have been linked to increased invasive and metastatic behavior (43). The *ESR1* p.D538G mutation leads to ligand-independent activation of ERα and is acquired in patients who have received aromatase inhibitors. The p.E380Q mutation has also been reported, but according to Li and colleagues (44), remains sensitive to antiestrogens. In one patient, we saw evidence of mutational heterogeneity in *ESR1*, with p.E380Q at a higher level than p.Y537C in cfDNA, suggesting heterogeneous clonal responses to treatment, as reported in a recent study (45). The *PIK3CA* p.E545K mutation affects the helical region of the P110α catalytic domain (46), whereas the p.H1047R mutation affects the kinase domain and induces a multipotent genetic program in normally lineage-restricted populations at an early stage of tumor development leading to intratumoral heterogeneity (47).

Some studies suggest that ERα-positive/*PIK3CA*-mutant tumors or ERα-positive cancers with aberrant PI3K signaling are less responsive to antiestrogens than wild-type tumors (48, 49), but other studies have linked these mutations to improved outcome (50, 51), regardless of the type of endocrine therapy used (52). However, *PIK3CA* mutations have been significantly associated with lower rates of pathogenic response to anti-HER2 therapies (53). Of note the patient who had triple-negative breast cancer had different mutations, in *TP53* and *KRAS* in cfDNA and *KRAS* only in individual CTCs, both of which have been implicated in resistance to cytotoxic chemotherapy.

It has been suggested that targeted sequencing of cfDNA could be used for monitoring of patients with cancer during treatment or while in remission and that single CTC analysis may then be needed to guide more appropriate therapy upon relapse (54). Here, we describe the relationship between mutations in multiple single EpCAM-positive CTCs and cfDNA isolated from the same blood sample in patients with high CTC counts ($\geq$100 CTCs per 7.5-mL blood). Results from 5 patients with high CTC counts show that cfDNA sequencing finds more mutations than CTCs as well as mutations not seen in the primary tumor. Our CTC data highlight the mutational heterogeneity of single EpCAM-positive CTCs, supporting other recent studies (19, 20). In 2 of the 4 patients with mutations detected in CTCs, mutations were only detected in about half of the CTCs sequenced (Table 2). Together these results suggest that if EpCAM-positive CTCs were to be used, concurrent analysis of multiple CTCs may be necessary for clinical decision-making, particularly when metastatic biopsy is not possible. In aggregate, our results suggest that cfDNA reflects persisting EpCAM-positive CTCs in patients with high CTC counts and could enable monitoring of the metastatic burden for clinical decision-making. Finally, our results also suggest that total cfDNA levels are also an independent indicator of overall survival ($P = 0.03$), but a confirmatory study is needed in a larger series of patients. Further studies are ongoing to examine the relationship between CTCs and cfDNA using Parsortix filtration (55), an alternative marker independent approach for CTC isolation.

Disclosure of Potential Conflicts of Interest

J.A. Shaw is a consultant/advisory board member for Qiagen. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

Single-cell isolation was performed by Silicon Biosystems as a per fee service. We thank Dr David Moore for reviewing tissue sections, the Imperial and Leicester ECMCs, the Imperial BRC and the Imperial College Tissue Bank for supporting patient recruitment.

Grant Support

This work was supported by a Cancer Research UK Clinical and Translational Research Committee programme award (C14315/A13462X).

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Received March 30, 2016; revised May 13, 2016; accepted June 12, 2016; published OnlineFirst June 22, 2016.

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