

An Accessible and Unique Insight into Metastasis Mutational Content Through Whole-exome Sequencing of Circulating Tumor Cells in Metastatic Prostate Cancer

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Abstract

Background: Genomic analysis of circulating tumor cells (CTCs) could provide a unique and accessible representation of tumor diversity but remains hindered by technical challenges associated with CTC rarity and heterogeneity.

Objective: To evaluate CTCs as surrogate samples for genomic analyses in metastatic castration-resistant prostate cancer (mCRPC).

Design, setting, and participants: Three isolation strategies (filter laser-capture microdissection, self-seeding microwell chips, and fluorescence-activated cell sorting) were developed to capture CTCs with various epithelial and mesenchymal phenotypes and isolate them at the single-cell level. Whole-genome amplification (WGA) and WGA quality control were performed on 179 CTC samples, matched metastasis biopsies, and negative controls from 11 patients. All patients but one were pretreated with enzalutamide or abiraterone. Whole-exome sequencing (WES) of 34 CTC samples, metastasis biopsies, and negative controls were performed for seven patients.

Outcome measurements and statistical analysis: WES of CTCs was rigorously qualified in terms of percentage coverage at 10× depth, allelic dropout, and uncovered regions. Shared somatic mutations between CTCs and matched metastasis biopsies were identified. A customized approach based on determination of mutation rates for CTC samples was developed for identification of CTC-exclusive mutations.

Results and limitations: Shared mutations were mostly detected in epithelial CTCs and were recurrent. For two patients for whom a deeper analysis was performed, a few CTCs were sufficient to represent half to one-third of the mutations in the

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matched metastasis biopsy. CTC-exclusive mutations were identified in both epithelial and nonepithelial CTCs and affected cytoskeleton, invasion, DNA repair, and cancer-driver genes. Some 41% of CTC-exclusive mutations had a predicted deleterious impact on protein function. Phylogenetic relationships between CTCs with distinct phenotypes were evidenced.

Conclusions: CTCs can provide unique insight into metastasis mutational diversity and reveal undiagnosed genomic aberrations in matched metastasis biopsies.

Patient summary: Our results demonstrate the clinical potential of circulating tumor cells to provide insight into metastatic events that could be critical to target using precision medicine.

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1. Introduction

Advances in the development of precision medicine for cancer patients rely on accurate identification of the genomic features underlying a patient's tumor [1]. As the number of genomic targets with matched therapies grows, the current hurdle remains the availability of tumor tissue that can reflect a constantly evolving disease [2–4]. Recognition that metastatic lesions have a discordant genomic fingerprint compared to primary tumor has led to recommendations for invasive biopsies. However, tumor heterogeneity poses the predicament that single-site biopsies may not capture the genomic alterations relevant to targeted therapy [5]. This is especially true in metastatic prostate cancer (mPC), for which bone metastases arising several years after diagnosis and therapeutic interventions are either inaccessible or yield insufficient material for genomic profiling of the disease [6,7]. In addition, biopsies of bone metastases are painful and cannot be repeated multiple times during the disease course, calling for less invasive methods for molecular characterization of mPC and for monitoring disease progression during therapy.

The development of “liquid biopsies” presents new opportunities for noninvasive monitoring of clonal heterogeneity. Circulating tumor cells (CTCs) captured as a “liquid biopsy” are currently regarded as a noninvasive and repeatable source of tumor material that could overcome the sampling challenges for metastatic disease [8–11]. CTCs are likely to arise from distinct metastatic sites, and may better represent tumor heterogeneity in both space and time. Liquid biopsies might allow genomic characterization of mPC and routine monitoring of metastatic spread, drug resistance, and disease relapse during the course of treatment [9–11]. However, it is still unknown whether using liquid biopsies can provide a more complete profile of mPC clonal diversity.

CTCs often exhibit combinations of epithelial and mesenchymal traits, highlighting the role of epithelial-mesenchymal transition (EMT) in the process of intravasation and cancer cell dissemination [12]. Therefore, non-epithelial-based CTC enrichment methods are essential to capture the phenotypic heterogeneity of CTCs in terms of EMT marker expression [13–15]. Reports of single-cell high-dimensional analyses of CTCs are scarce and limited to

EpCAM-positive CTCs [16–18]. The rarity and biological heterogeneity of CTCs have imposed technical challenges to their isolation and analyses at single-cell level, and impacted the success of robust processing of complex and costly downstream methodologies. Once isolated at the single-cell level, CTCs must undergo whole-genome amplification (WGA), a mandatory process to identify CTC somatic variants but prone to amplification bias, polymerase errors, and allelic dropout (ADO) [19].

Our central hypothesis was that the genetic heterogeneity of CTCs assessed at the single-cell level via whole-exome sequencing (WES) reflects their phenotypic heterogeneity in mPC and may help to resolve the clonal relatedness between CTCs and metastasis in mPC. Here, we report for the first time isolation at the single-cell level, qualification, and WES of CTCs harboring various epithelial, mesenchymal, and/or morphological characteristics from 11 patients with metastatic castration-resistant PC (mCRPC). All mCRPC patients were included in the MOSCATO 01 clinical trial evaluating the potential clinical benefit of screening metastasis biopsies via high-throughput genomic analyses to identify actionable alterations in patients with advanced cancer [1]. Somatic mutations shared between CTCs and matched metastatic biopsies, and CTC-exclusive mutations (exclusively identified in CTCs and not in matched biopsies) were comprehensively explored. Our results reveal that CTCs can be used as surrogate samples for mutational analyses of mCRPC and provide a genomic picture of disseminating clones involved in metastasis.

2. Patients and methods

2.1. Patients

Patients with mCRPC were enrolled in the MOSCATO 01 prospective trial (IDRCB 2010-A00841-40; NCT02613962) [1]. The study was authorized by the French national regulation agency ANSM and approved by the ethics committee. Blood was collected into EDTA and CellSave tubes.

2.2. CTC enrichment, detection, and isolation

Isolation by size of epithelial tumor cells (ISET) filtration, immunofluorescence staining, and scanning of filters were performed as previously

reported [20]. CTCs and CD45-positive cells were isolated via laser microdissection. CTCs were detected using CellSearch as previously described [10,20] and isolated using self-seeding microwell chips [21]. Enrichment via RosetteSep was performed according to the manufacturer's protocol (StemCell Technologies, Vancouver, Canada). RosetteSep-enriched CTCs were permeabilized, stained via immunofluorescence, and isolated using fluorescence-activated cell sorting (FACS). The methods are described in the [Supplementary material](#).

2.3. WGA, quality controls, and purification

WGA was performed using an Ampli1 WGA kit version 1 (Menarini Silicon Biosystems, San Diego, CA, USA) and evaluated via a qualitative polymerase chain reaction (qPCR) assay to determine a quality score (QS; [Supplementary material](#)).

2.4. Isolation of genomic DNA from blood and tumor biopsies

DNA from formalin-fixed, paraffin-embedded tumor biopsy specimens and constitutional DNA were purified as described in the [Supplementary material](#).

2.5. WES and bioinformatics analysis

WES and bioinformatics analysis are described in detail in the [Supplementary material](#). Raw data are available on EGA at accession number ega-box-1082.

3. Results

Eleven patients were included in the current analysis ([Table 1](#)). At the time of CTC collection, all patients had metastases from CRPC and all but one had been previously treated with either abiraterone or enzalutamide, while three had been treated with prior docetaxel.

We initially developed a CTC enrichment strategy based on filtration by ISET before a multistep process that includes immunofluorescent staining with epithelial and mesenchymal markers for CTC identification and laser microdissection of individual CTCs ([Fig. 1A](#)). CTCs from five patients (P1–P5) were isolated in this way. We detected very few

epithelial CTCs, especially in two of the patients (P2 and P3), compared to higher CellSearch counts, as confirmed by a recent study [13] ([Supplementary Table 1](#)). In agreement with published results [15,22], ISET revealed hybrid cells, as well as populations of large (nuclei $\geq 16 \mu\text{m}$) and mesenchymal candidate CTCs, of possible interest for further analysis by WES ([Fig. 1B](#)). We developed two other strategies to capture epithelial and other candidate CTCs. In the second strategy, CTCs were enumerated via CellSearch and individually isolated using the self-seeding microwell chips platform [21] ([Fig. 1A](#)). Our third approach involved hematopoietic blood-cell depletion and individual CTC isolation via FACS ([Fig. 1A](#)). These three strategies allowed identification of five phenotypic subtypes of candidate CTCs on the basis of both epithelial and mesenchymal marker expression and/or morphological characteristics ([Fig. 1B](#)). These initial results led us to explore and isolate CTCs in six more mCRPC patients ([Table 1](#) and [Supplementary Table 1](#)). A global workflow was established based on these strategies, WGA and qualification, and WES of CTCs and matched negative controls (CD45⁺ cells; [Fig. 1C](#)). We hypothesized that pools of a limited number of individual CTCs could result in higher-quality WGA and could yield higher sensitivity for variant detection than single CTCs. We isolated 162 pools of two to ten CTCs (each pool containing CTCs of the same phenotype), 17 single CTCs, and matched CD45⁺ cells. The WGA quality was controlled using a qPCR assay, and a QS ≥ 3 was set as the criterion for WES eligibility, which was reached by 87/179 WGA samples (49%; [Supplementary Table 2](#), [Supplementary Fig. 1A and B](#)). As hypothesized, the probability of reaching QS ≥ 3 was higher in pools than in single CTCs ([Fig. 1D](#)). Among the 87 WGA CTC samples with QS ≥ 3 , 31 from seven patients were selected for WES. Two supplementary epithelial CTC pools (P7-E-7 and P7-E-8) from 10 CTCs and a single CTC bearing a hybrid phenotype, all with QS < 3 , were included for patient P7 to address CTC heterogeneity ([Supplementary Table 3](#)). WES for the 34 CTC samples was subjected to rigorous quality control ([Fig. 1E](#), [Supplementary Fig. 1](#)). Of the 34 WES samples, 28 (82%) had

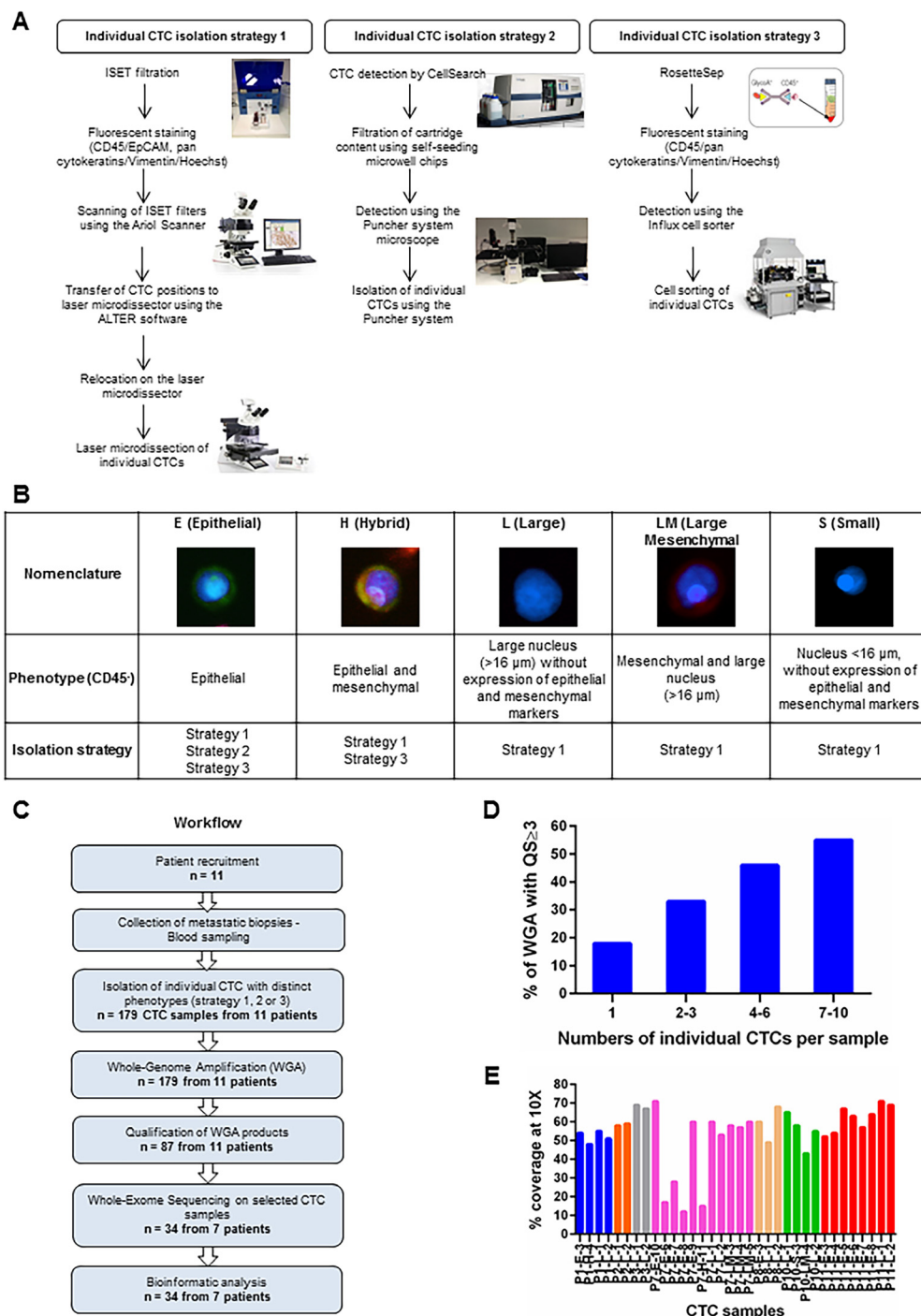
Table 1 – Clinical characteristics of patients with metastatic castration-resistant prostate cancer

Case	Age (yr)	TNM ^a	Metastatic sites	PSA ^b (ng/ml)	Gleason score	TTB (mo)	Biopsy site	Previous treatment			
								CTX	CTN	ENZ	ABI
P1	77	TxNxM1	Bones and LNs	333	7 (4 + 3)	46	LN	No	Yes	No	Yes
P2	77	T1N0M0	Bones and LNs	101	7 (3 + 4)	32	LN	No	Yes	No	Yes
P3	73	T3N0M0	LNs	249	7 (4 + 3)	22	LN	No	Yes	No	Yes
P4	46	TxN0M1	Bones and liver	85	8	14	Liver	No	Yes	No	Yes
P5	68	T3N0M0	LNs	14	9	24	LN	Yes	Yes	No	Yes
P6	65	T3N0M0	LNs	283	8 (4 + 4)	13	LN	No	Yes	No	Yes
P7	60	T3N0M0	Bones and LNs	1715	7 (4 + 3)	26	LN	Yes	Yes	Yes	Yes
P8	66	T3N0M0	Bones and LNs	51	9	2	LN	No	Yes	No	Yes
P9	67	TxN1M1	LNs and bones	27	8 (4 + 4)	12	LN	Yes	Yes	No	No
P10	71	TxNxM1	Bones, liver, and LNs	10	NA	72	Liver	No	Yes	Yes	No
P11	76	T3N0M0	Bones	163	NA	24	Bone	No	Yes	No	Yes

LN = lymph node; PSA = prostate-specific antigen; NA = not available; TTB = time from metastatic prostate cancer diagnosis to biopsy; CTX = chemotherapy; CTN = castration; ENZ = enzalutamide; ABI = abiraterone.

^a At diagnosis.

^b At the time of metastasis biopsy.



<30% ADO and uncovered regions, and 27 (79%) had >50% coverage at 10× depth (10× was the minimum level of coverage considered, but coverage was in practice typically in excess of 10×).

We analyzed mutations shared between CTCs and matched metastasis biopsy samples. Among the 34 CTC samples sequenced, 15 from four patients (P1, P7, P10, P11) shared mutations with the corresponding matched biopsy (Fig. 2A). A total of 136 shared mutations for 65 unique mutations were detected (Supplementary Figs. 2 and 3A). Of these 65, 61 (93.9%) were detected exclusively in epithelial CTCs, while four (6.1%) were detected in CTCs bearing various phenotypical characteristics (Fig. 2B). For patients P7 and P11, for whom a larger number of CTC samples with various characteristics were sequenced, 59% and 33%, respectively, of mutations detected in biopsies were identified in CTCs (Fig. 2C). For patient P7, shared mutations were mostly identified in two CTC pools with high QS (Fig. 2D). For patient P11, 31% of mutations shared with the matched biopsy were present in one pool of five and one single epithelial CTC with QS ≥ 5 (Supplementary Fig. 4). Most of the shared mutations, including those in *GRM8* and *TP53*, were identified in several epithelial CTC samples (Table 2, Fig. 2B, Supplementary Table 4). Overall, 24/53 of shared unique mutations (45%) had a predicted deleterious impact on protein function. Our results demonstrate that WES of a small number of pools or single epithelial CTCs, selected using a rigorous qualification process, can reveal significant mutational similarities to the metastasis biopsy. Shared mutations were recurrent in CTCs, suggesting a possible important role in metastatic spread.

To reliably identify somatic CTC-exclusive mutations, we excluded variants shared between CTCs and matched biopsies and established a customized approach that involves variant classification according to recurrence in CTC samples (Supplementary Fig. 3B). We determined a mutation rate per Mb per CTC sample for criteria of observation in one, two, or three samples, as shown for patient P11 in Fig. 3A. Mutations identified in CTC samples with a mutation rate per Mb of less than that for metastasis biopsies were considered as CTC-exclusive mutations. Among the 240 variants identified in two CTC samples, 31 were known mutations with a predicted deleterious impact on protein function, including variants in cancer genes such as *HSP90AB1* and *KDM5B* (Supplementary Fig. 2, Supplementary Table 5). When variants were required to be observed in three CTC samples, the mutation rate per Mb per sample was less than values for matched metastases (Fig. 3A). Nineteen somatic variants were present in at least three CTC samples, of which ten variants were reported in databases (Table 2). Seven of the 19 (37%) were identified in genes involved in cytoskeleton (eg, *MACF1*) or invasion (eg, *NEDD9*) and impact protein function. For patient P11, 20% of CTC-exclusive variants were identified in at least three CTC samples (Fig. 3B). When we examined the distribution of these 19 exclusive variants according to CTC phenotype, 11 (58%) were detected exclusively in epithelial CTCs, and eight (42%) were present in CTCs harboring various characteristics (Fig. 3C). These data demonstrate the existence of

exclusive mutations in CTCs that were not detected in matched biopsies.

To evaluate the relationship between CTCs with distinct characteristics in patients P7 and P11, we performed hierarchical clustering for shared and CTC-exclusive mutations observed in at least three CTC samples (Fig. 4A and B). The existence of common mutations between CTCs with distinct characteristics supports their phylogenetic relationship but divergent evolution.

4. Discussion

We present the first genomic analysis at the single-cell level of CTCs with various phenotypical characteristics. Our primary aim was to determine the feasibility of noninvasive mutational characterization of mPC patients and to define whether the mutation landscape of CTCs reflected that of matched metastases. We demonstrated that WES of CTCs is feasible by testing different strategies that enabled us to detect CTCs with multiple cellular phenotypes. Our first CTC isolation strategy fell short of identifying a fraction of epithelial CTCs, as recently confirmed [14]. This led us to develop two supplementary enrichment and isolation strategies that were used in conjunction with filtration and laser-capture microdissection to address the challenge of CTC heterogeneity. Our results show the superiority of strategies 2 and 3 for future clinical applications but do not allow us to identify which is the best. We determined a global workflow to generate high-quality libraries for WES in which rigorous qualification of WGA uniformity could reliably predict which CTC pool or single CTC was most likely to yield high-quality WES data. Our results differ from those reported by Lohr et al. [16], who found that ADO and a lack of systematic coverage in WGA products from single EpCAM-positive CTCs necessitated WES of multiple single CTCs ($n = 19$) for variant determination. Although perhaps less pertinent for exploring tumor heterogeneity, the approach presented here, which limited WES to a small number of high-quality CTC samples and integrated CTC pools, is technically more practical and cost-effective for a clinical setting.

We showed that WES for a very small number of pools or single epithelial CTCs yielded significant mutational similarities for the exome of matched metastasis biopsies, including mutations in PC genes such as *GRM8* and *TP53*. Our results indicate that CTC sequencing can provide a reasonable and feasible proxy for metastasis sampling in disseminated cancer. Interestingly, recurrent mutations were systematically identified in both CTCs and matched metastasis biopsies, and sequencing more CTCs did not significantly extend the number of mutations identified. However, a fraction of mutations identified within the matched metastasis biopsy were not detected in CTCs, suggesting that these mutations may not have the ability to disseminate or transit in blood. By analogy, it could be hypothesized that these recurrent mutations found in CTCs and shared by matched metastasis biopsies could play an important role in metastatic

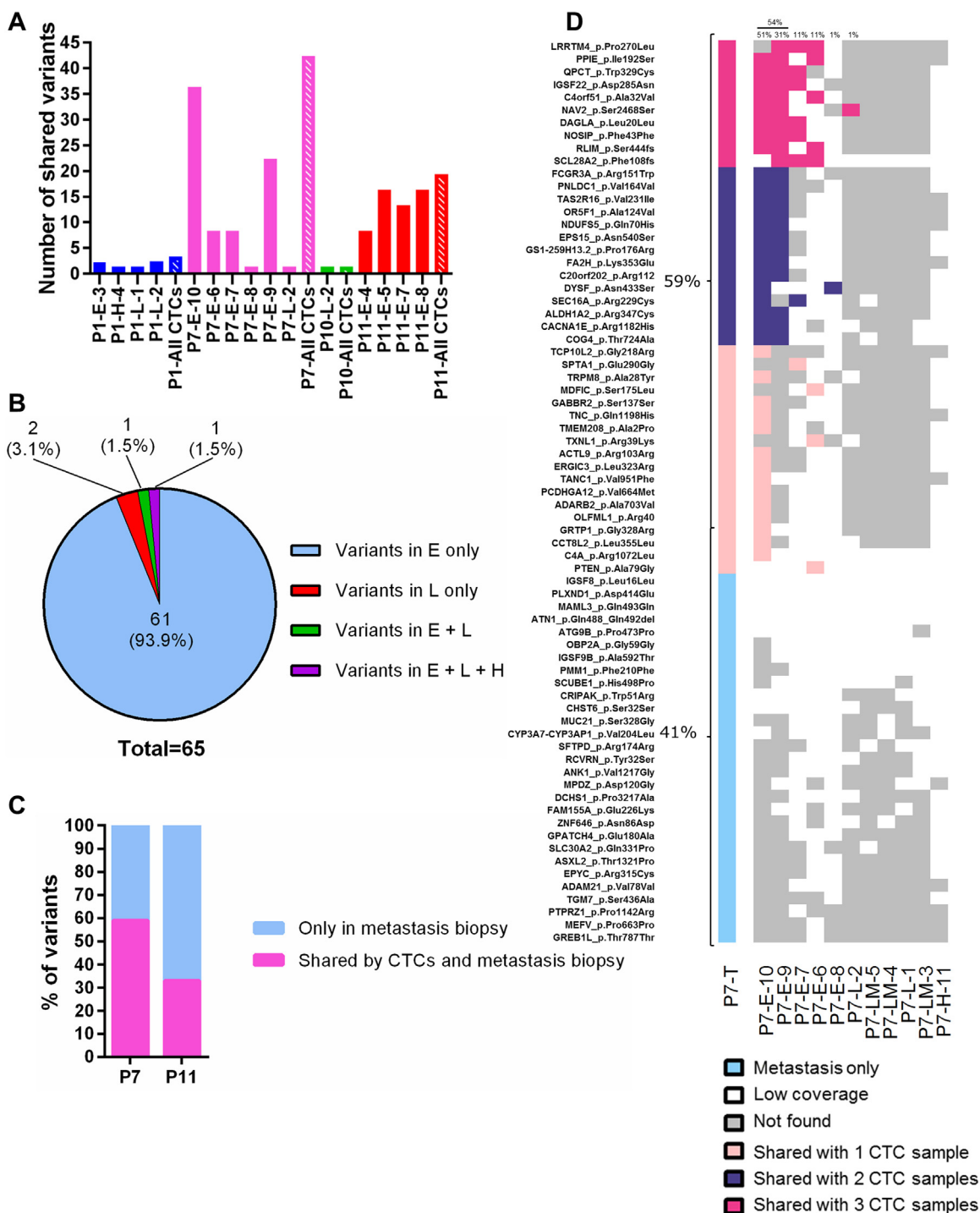


Fig. 2 – Characterization of variants shared by metastasis biopsies and CTCs. (A) Number of variants shared by metastasis biopsies and CTCs. Fifteen CTC samples from four of the seven patients shared a total of 136 variants with matched metastasis biopsy samples, of which 65 were unique. For each patient the sum of variants shared by their metastasis biopsy and CTCs is represented by hatched bars. (B) Distribution of shared unique variants according to CTC phenotype. Sixty-one variants (93.9%) were detected in epithelial CTCs (E), two variants (3.1%) were only detected in large CTCs (L), one variant (1.5%) was detected in both in epithelial and large CTCs (E + L), and one variant (1.5%) was detected in epithelial, large, and hybrid CTCs (E + L + H). (C) Percentage of variants shared by CTCs and metastasis biopsies, or only present in metastasis biopsies, for patients P7 and P11. (D) Heatmap of variants identified in the metastasis biopsy and shared by CTCs in patient P7. Some 41% of variants were exclusively identified in the metastasis biopsy and 59% were shared with CTCs. The P7-E-10 pool (QS = 7) exhibited 51% shared mutations with the matched biopsy. The pools P7-E-10 and P7-E-9 (QS = 6) had almost the same mutations and shared 54% of mutations with the matched biopsy. The positions of variants present in the metastasis biopsy and not covered in CTCs are shown. CTCs = circulating tumor cells; QS = quality score.

progression. Alternatively, we cannot exclude the possibility that mutations identified within a matched metastasis could be missed in CTCs for different technical reasons related to the sensitivity of our approach.

We established a strict pipeline for variant calling and for greater reliability we only considered CTC-exclusive mutations observed in at least three CTC samples. CTC-exclusive mutations included both unknown mutations that warrant

Table 2 – Examples of variants shared between CTCs and matched metastatic biopsies, and CTC-exclusive variants

Gene	Transcript	Database		Somatic single-nucleotide variants		Prediction (Polyphen2 Sift)	Number of CTC samples	PID
		dbSNP	COSMIC	Codon change	AA change			
Variants shared by CTCs and matched metastatic biopsies								
<i>GRM8</i>	NM_000845.2	-	-	c.2207C > T	p.Ala736Val	D, T	4 (P11-E-4 ^a ; P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>TP53</i>	NM_000546.5	-	COSM10656	c.742C > T	p.Arg248Trp	D, D	4 (P11-E-4 ^a ; P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>PTEN</i>	NM_000314.4	-	-	c.236C > G	p.Ala79Gly	B, NA	1 (P7-E-10 ^c)	P7
CTC-exclusive variants observed in at least 3 CTC samples								
<i>PPAPDC1A</i>	NM_001030059.1			c.301_305delATTAA	p.Ile101fs	NA, NA	3 (P7-L-2 ^a ; P7-LM-3 ^a ; P7-LM-5 ^a)	P7
<i>INO80</i>	NM_017553.2			c.2867C > T	p.Pro956Leu	B, T	3 (P7-L-1 ^a ; P7-L-2 ^a ; P7-LM-3 ^a)	P7
<i>C3orf30</i>	NM_152539.2	rs199919487		c.1369G > A	p.Glu457Lys	D, D	4 (P11-L-2 ^a ; P11-E-4 ^c ; P11-E-5 ^b ; P11-E-6 ^b)	P11
<i>ANKRD32</i>	NM_032290.3			c.2992G > A	p.Glu998Lys	B, T	4 (P11-L-2 ^a ; P11-E-3 ^c ; P11-E-5 ^b ; P11-E-7 ^b)	P11
<i>EIF4G3</i>	NM_001198801.1			c.2317C > A	p.Gln773Lys	B, T	3 (P11-L-2 ^a ; P11-E5 ^b ; P11-E-8 ^b)	P11
<i>OTUD4</i>	NM_001102653.1			c.1575A > C	p.Leu525Phe	P, T	3 (P11-L-2 ^a ; P11-E-4 ^c ; P11-E-8 ^b)	P11
<i>ZFYVE1</i>	NM_021260.3		COSM470205	c.488C > A	p.Thr163Lys	B, T	3 (P11-L-2 ^a ; P11-E-3 ^c ; P11-E-6 ^b)	P11
<i>CELA3B</i>	NM_007352.2	rs202129706		c.803C > A	p.Ala268Glu	B, T	5 (P11-E-3 ^c ; P11-E-4 ^c ; P11-E-5 ^b ; P11-E-6 ^b ; P11-E-8 ^b)	P11
<i>PGBD2</i>	NM_170725.2			c.441_442insT	p.Phe149fs	NA, NA	4 (P11-E-4 ^c ; P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>MACF1</i>	NM_012090.5	rs202091916	COSM128604	c.15886A > C	p.Thr5296Pro	D, NA	4 (P11-E-5 ^b ; P11-E-6 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>ABCB11</i>	NM_003742.2	rs200687717		c.1628A > C	p.Asp543Ala	B, T	3 (P11-E-4 ^c ; P11-E-5 ^b ; P11-E-7 ^b)	P11
<i>QPCT</i>	NM_012413.3			c.152C > T	p.Ser51Leu	B, D	3 (P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>FAM161A</i>	NM_001201543.1	rs200331923		c.1573C > A	p.Gln525Lys	B, T	3 (P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>NEDD9</i>	NM_001142393.1			c.1504A > C	p.Thr502Pro	D, T	3 (P11-E-5 ^b ; P11-E-6 ^b ; P11-E-8 ^b)	P11
<i>AKNA</i>	NM_030767.4	rs200970909		c.3287A > C	p.His1096Pro	P, D	3 (P11-E-5 ^b ; P11-E-6 ^b ; P11-E-8 ^b)	P11
<i>C2CD3</i>	NM_001286577.1	rs200719890		c.5233T > G	p.Tyr1745Asp	D, D	3 (P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>IFNGR2</i>	NM_005534.3			c.100C > G	p.Gln34Glu	B, T	3 (P11-E-5 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>LANCL2</i>	NM_018697.3	rs201180232	COSM453253	c.953T > G	p.Val318Gly	D, D	3 (P11-E-6 ^b ; P11-E-7 ^b ; P11-E-8 ^b)	P11
<i>NDUFV2</i>	NM_021074.4	rs72935225		c.401T > C	p.Val134Ala	B, T	3 (P7-E-9 ^c ; P7-E-10 ^c ; P7-H-11 ^c)	P7
CTC = circulating tumor cells; AA = amino acid; PID = patient identity.								
^a Isolated using strategy 1.								
^b Isolated using strategy 2.								
^c Isolated using strategy 3.								

further investigation and known mutations with a predicted deleterious impact on protein function in cancer-driver genes such as *HSP90AB1* and *KDM5B*, as well as in genes that might have a crucial role in the biological specificity (eg, cytoskeletal remodeling) or survival (eg, DNA repair) of CTCs. These CTC-exclusive mutations were mostly present in epithelial CTCs or in both epithelial CTCs and CTCs with other morphological characteristics, a result that indirectly validates the true tumor origin of these cells. Our data suggest that CTCs harboring these exclusive mutations derived from distinct metastatic sites represented minor subclones that were undetectable in metastatic biopsies.

While tumor tissue biopsies remain the gold standard for validating or confirming genetic abnormalities found in CTCs or circulating tumor DNA (ctDNA), it is important to question the representability of a needle or single-site biopsy specimen. As illustrated here via analysis of CTC-exclusive variants, CTCs can be derived from lesions that were not biopsied and may contain a different genetic composition than the tumor material used as a reference.

These results demonstrate for the first time that sequencing of CTCs at the single-cell level can reveal metastasis mutational content and diversity that are otherwise inaccessible. Exome sequencing of cell-free tumor DNA has also

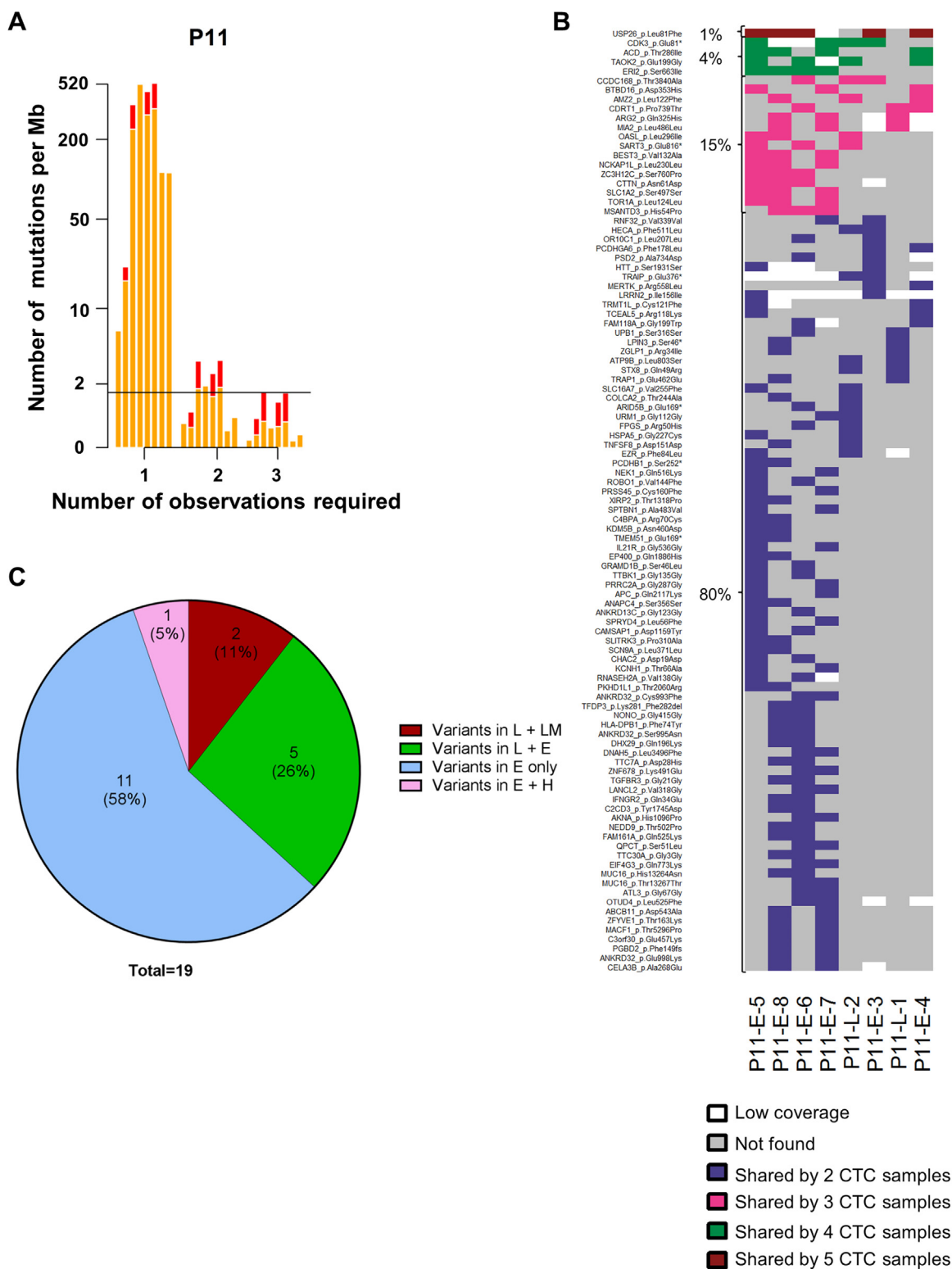


Fig. 3 – Characterization of CTC-exclusive variants. (A) Mutation rate per Mb in CTCs from patient P11 for criteria of variants observed in one, two, or three CTC samples. For the criterion of variants observed in two CTC samples, the mutation rates per Mb per sample were close to the values for the corresponding metastasis, although for some CTC samples, this value was greater, indicating that WGA errors could be conserved. For the criterion of variants observed in three CTC samples, the mutation rates per Mb per sample were less than or equal to the values for matched metastases. (B) Heatmap of CTC-exclusive variants in patient P11. Of 101 CTC variants, 81 (80.2%) were detected in two CTC samples, 15 (14.8%) in three CTC samples, four (4%) in four CTC samples, and one (1%) in five CTC samples. The positions of variants not covered in CTC samples are shown. (C) Distribution of unique CTC-exclusive variants present in at least three CTC samples according to the CTC sample phenotype. Twenty-two unique CTC-exclusive variants were detected in at least three CTC samples; 55% had an exclusively epithelial phenotype, while 45% had different phenotypes. CTCs = circulating tumor cells; WGA = whole-genome amplification.

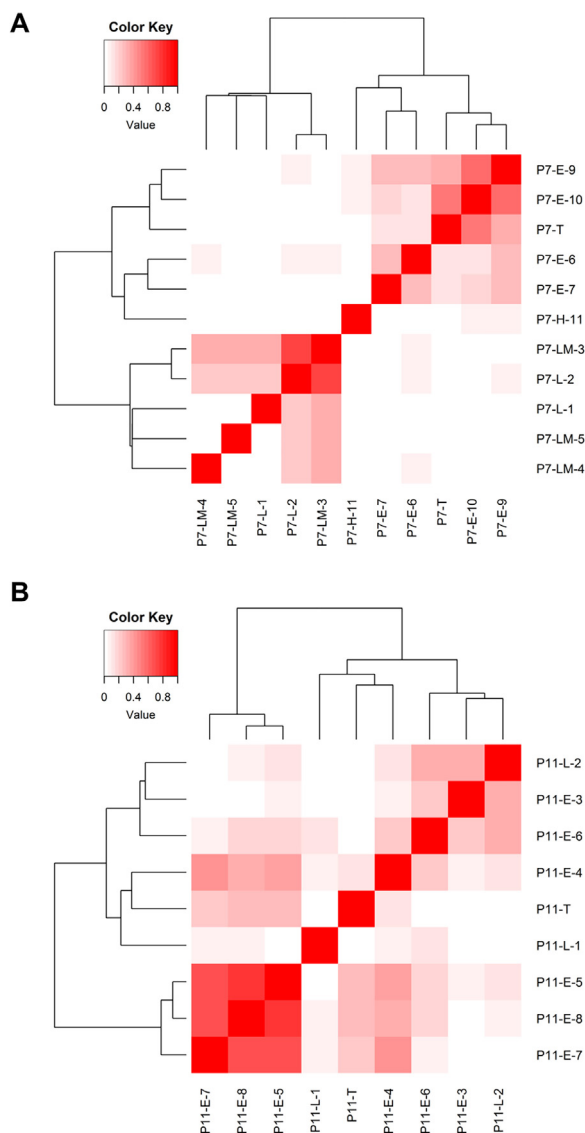


Fig. 4 – Hierarchical clustering of CTCs and matched-metastasis biopsies. (A) Hierarchical clustering of CTC and matched metastasis biopsy samples in patient P7 according to mutational similarities between CTCs and the matched metastasis biopsy, and between different CTC samples for variants observed in at least three samples. The number of shared variants is represented by the red color gradient from dark red (high number of variants) to white (no shared variant). (B) Hierarchical clustering of CTCs and matched metastasis biopsy in patient P11 according to the same representation. CTCs = circulating tumor cells.

demonstrated concordance of mutations with metastasis biopsies [23]. Although ctDNA analyses may indeed offer advantages in terms of simplicity, sequencing of CTCs can provide unique additional and complementary information such as EMT status and cancer stem-cell phenotype [24,25]. The presence of different and multiple genetic alterations can be identified within the same CTC, offering the possibility to explore inter- and intratumor heterogeneity and evolution. It is therefore anticipated that CTCs and ctDNA are complementary in their clinical utility. While tumor tissue biopsies remain the gold standard for confirming genetic abnormalities found in CTCs, it is important to question the representability of a needle or single-site biopsy. Our findings emphasize the potential and clinical utility of detecting such mutations in CTCs via minimally invasive blood draws for

optimal therapy selection, precision medicine, and treatment resistance options. A study quantifying digital pathology features of CTCs recently showed that low CTC phenotypic heterogeneity was associated with better overall survival (OS) among patients treated with androgen receptor signaling inhibitors, whereas high heterogeneity was associated with better OS among patients treated with taxane chemotherapy [26]. Beyond identifying potential targets, WES at the single-cell level could quantify heterogeneity and help in decision-making for mCRPC patients.

5. Conclusions

This pioneering study emphasizes the potential of CTCs to represent metastasis mutational content and tumor

diversity that are otherwise inaccessible. By offering real-time monitoring of a constantly evolving disease and detecting potentially critical mutations via minimally invasive blood draws, CTC sequencing can serve an unmet need for optimal therapy selection and precision medicine.

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Appendix A. Supplementary data

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