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Delayed processing of CPT-collected blood preserves transcriptomic and genomic features of propagated circulating tumor cells

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Abstract

Circulating tumor cells (CTCs) provide a minimally invasive window into metastatic disease but are constrained by the need for rapid specimen processing after blood collection. We evaluated whether a workflow utilizing BD Vacutainer CPT-based blood collection could support delayed processing while preserving molecular features of propagated CTC-derived material. In a pilot cohort of four patients, paired CPT tubes were collected; one tube was processed within 2 hours of phlebotomy, whereas the second was stored at 4°C and processed 24 hours later. Both tubes were then harvested for CTCs, which were propagated *ex vivo* and analyzed by bulk RNA sequencing and whole-exome sequencing. Transcriptomic analyses showed that paired CPT-derived samples demonstrated phenotypes consistent with CTCs, including reduced immune-associated signatures and enrichment of epithelial-mesenchymal transition and KRAS signaling pathways. Direct comparison of paired early- and delayed-processed CPT samples demonstrated strong within-patient gene expression concordance. Whole-exome sequencing further revealed preservation of patient-specific oncogenic alterations and high overlap of detected variants across paired processing conditions. These findings altogether support the feasibility of a CPT-based delayed processing workflow for propagating and studying CTCs, thereby reducing a key logistical barrier to broader CTC research.

Introduction

Circulating tumor cells (CTCs) are rare malignant cells shed from primary or metastatic tumors into the bloodstream and are widely regarded as key intermediaries in the metastatic cascade [1]. Because they can be obtained through minimally invasive blood draws, CTCs are widely viewed as a novel and non-invasive window into metastatic biology, tumor evolution, and treatment response [2, 3]. However, their clinical and research utility has so far been limited by their extreme rarity, phenotypic heterogeneity, and the technical challenges associated with their isolation, propagation, and downstream molecular characterization [4, 5]. These barriers become especially relevant for workflows that aim not only to enumerate CTCs but also to study their genomics and functional significance. Nonetheless, a substantial body of clinical evidence supports the prognostic relevance of CTCs [2]. Furthermore, recent process in ex vivo CTC expansion methods—both as short-term cultures and as CTC-derived-xenografts (CDX) models—has strengthened the case for using CTCs not only as biomarkers but also as viable surrogates for studying metastatic disease [6, 7].

Presently, limitations on the application of CTCs to routine clinical workflows are rooted in a few key bottlenecks. One of the most clearly identifiable of these bottlenecks starts at the moment of phlebotomy, during which these exceedingly rare cells are extracted from their natural environment and subjected to the harsh limitations of the *ex vivo* processing world. Indeed, pre-analytical variability, including but not limited to time-to-processing and method of blood transport, all have clear downstream effects and can factor into cell loss during sample handling. These effects are exponentially greater when dealing with samples with as little as 5 cells/tube. Many CTC studies are therefore forced to utilize sample capture methods that produce a tradeoff between capture efficiency, purity, viability, and

molecular fidelity [1, 8, 9]. These limitations subsequently restrict adoption of CTC processing and research to only specialized centers capable of the fast-processing times currently required for cell viability.

Here, we evaluate a workflow using BD Vacutainer CPT tubes to support delayed processing of blood specimens intended for CTC enrichment and downstream propagation. We tested whether specimens processed after short delay (within 2 hours post collection) and after 24 hours of refrigerated storage retain transcriptomic and genomic features relevant to propagated CTC analysis. Across a pilot cohort of patients with metastatic pancreatic cancer, CPT-derived specimens showed concordant within-patient transcriptomic profiles across collection timepoints and preserved patient-specific genomic alterations after ex vivo expansion. These findings suggest that CPT-based delayed processing has the potential to decouple sample collection from specialized laboratory infrastructure, expanding the accessibility of CTC-based analyses for multicenter studies and broader clinical research settings without loss of key biological signal.

Results

CPT-derived samples yield consistent transcriptomic profiles after delayed processing

BD Vacutainer CPT tubes permit a single centrifugation step that separates red blood cells from the mononuclear cell fraction (which includes white blood cells and circulating tumor cells), while an inert gel barrier minimizes remixing during storage and transport. To test whether this platform could support delayed CTC processing, we performed a pilot study in four patients with metastatic cancer (patients 107, 110, 111, 114). For each patient, two CPT tubes were collected and centrifuged within 2 hours of phlebotomy. One tube was processed immediately for downstream CTC culture (“CPT A”) whereas the second tube was stored at 4°C and processed 24 hours later (“CPT B”) (Fig. 1). A portion of the immediately processed fraction was also used to isolate RNA prior to CTC expansion and served as the matched whole blood (“WBM”). CTC-enriched fractions from CPT A and CPT B were propagated ex vivo using our previously described culture method [6, 10], and expanded cells were harvested for RNA sequencing, whole-exome sequencing (WES) or both depending on material availability.

We first asked the question of whether delayed processing altered transcriptomic profiles of propagated CPT-derived samples. Bulk RNA sequencing of matched WBM, CPT A, and CPT B specimens showed that the two CPT-derived samples from each patient clustered more closely with one another than with the matched WBM sample when evaluated using unbiased hierarchical clustering (Fig. 2A). When we restricted analysis to an epithelial-mesenchymal transition (EMT)-associated gene set, CPT samples again grouped together and apart from WBM (Fig. 2B), suggesting preservation of biologically relevant transcriptional signals despite delayed processing.

One concern of delayed processing of primary cells is potential loss of cell viability. To further assess whether CPT-derived material retained phenotypic features expected of CTCs, we examined expression of KRT7 and CD45. Traditionally, CTCs have been identified based on their CD45-/CK7 + immunophenotype [11]. CPT-derived samples showed higher KRT7 expression and significantly lower CD45 expression than WBM controls (Fig. 2C). Pathway analysis similarly demonstrated relative depletion of innate and adaptive immune system signatures and enrichment of Hallmark pathways related to EMT and KRAS signaling in CPT-derived specimens (Fig. 2D).

Finally, to directly compare the two CPT processing timepoints, we evaluated gene-level concordance between matched CPT A and CPT B samples within each patient. These comparisons showed strong intra-patient concordance in gene expression between samples processed at 2 hours and those processed after 24 hours of refrigerated storage (Fig. 2E), supporting the conclusion that delayed processing within this workflow does not grossly alter the transcriptional landscape of harvested CTCs.

CPT-derived samples retain patient-specific genomic features and clonal heterogeneity

A major rationale for CTC analysis is the possibility of capturing tumor subclones with metastatic relevance. To test whether delayed CPT processing preserved tumor-informative genomic features, we performed WES on CPT-derived material from patient 107 as well as a pooled CPT-derived material from patient 114. Annotation against OncoKB identified distinct patient-specific oncogenic alterations, supporting the biological specificity of the propagated samples rather than convergence toward a non-specific culture-associated profile (Fig. 3A) [12].

We also examined potentially druggable alterations and observed recurrent involvement of pathways related to genome maintenance, Hedgehog signaling, cellular metabolism, and receptor tyrosine kinases (RTKs), several of which were represented across collection conditions (Fig. 3B). To determine whether delayed processing altered detectable mutational heterogeneity, we compared the paired CPT A and CPT B samples from patient 107. These two samples overall shared 97.1% of detected mutations (n = 9292) whereas 1.4% (n = 131) were unique to CPT A and 1.3% (n = 146) were unique to CPT B (Fig. 3C). Although these findings are exploratory, they demonstrate that harvested CTCs from CPT tubes can yield tumor-relevant genomic information while preserving a modest degree of sample-specific heterogeneity, thus suggesting this workflow and subsequent CTCs are suitable for downstream biological interpretation.

Discussion

CTC-based studies are often limited by pre-analytical constraints, particularly the need to process blood specimens rapidly after collection in order to preserve viable cells for downstream molecular or functional assays. In this study, we evaluated a CPT-based workflow intended to stabilize and enrich these rare CTCs, thereby permitting delayed handling (Fig. 1). Across four patients, transcriptomic

profiling of CPT-derived specimens demonstrated retained features consistent with CTC phenotypes, including pathway-level patterns suggestive of reduced immune cell contributions and enrichment of tumor-associated signaling pathways such as EMT and KRAS signaling (Fig. 2). Notably, within-patient comparisons revealed strong concordance at the gene expression level between specimens processed shortly after collection and those processed after 24 hours of refrigerated storage (Fig. 2, 3).

These findings have practical implications for the design of translational CTC studies. Conventional CTC workflows often require rapid transport to an experienced processing laboratory [1]. This requirement has been a major bottleneck for broader translational deployment of CTC-based models, which otherwise hold promise for molecular profiling, functional testing, and personalized therapeutic investigation [5, 13]. By extending the allowable time-to-processing to at least 24 hours while preserving key molecular signals and viability, CPT-enabled delayed processing may help decouple specimen collection from immediate access to specialized CTC infrastructure. This has potential downstream benefits for future study design and equity of access, enabling centralized processing models.

Our transcriptomic analyses further support the biological plausability of the propagated CPT-derived samples. Relative to matched WBM controls, CPT-derived specimens showed reduced immune-associated signatures and increased expression of pathways linked to EMT and KRAS signaling. These observations are consistent with enrichment of tumor-associated CTCs and aligns with prior work implicating EMT-related transcriptional programs in metastasis-associated CTC biology [5, 10]. Importantly, the strong concordance observed between paired CPT A and CPT B samples suggests that the delayed-processing condition preserves these signals at a level sufficient for downstream comparative analyses. Genomic data from this study also provide support for the utility of this approach. WES identified patient-specific oncogenic mutations and highly overlapping mutation profiles across processing timepoints. Although exploratory and limited by sample number, these results suggest that delayed CPT processing can preserve tumor-informative clonal heterogeneity rather than introducing bias into the propagated genomic landscape. This is particularly relevant in the context of prior evidence that biologically important CTC subsets may represent metastasis-competent or tumor-initiating populations [14–17].

This pilot study nonetheless contains several limitations. First, because pre-analytical variability is a recognized determinant of downstream analyses, larger studies will be needed to define the acceptable limits of transport-associated variables such as time, temperature, and handling of CTCs. Second, the number of patients evaluated was small, limiting statistical power and generalizability across tumor types, disease stages, and treatment exposures. Third, our transcriptomic comparisons rely on bulk RNA sequencing, which can be strongly influenced by cell mixtures and mask biologically meaningful subpopulations. Although decreased CD45 expression and immune pathway expression are consistent with a tumor cell enrichment, bulk profiles cannot definitively attribute signals to CTCs versus residual tumor-associated immune cells. Single-cell RNA sequencing would therefore strengthen claims regarding cellular composition and heterogeneity. Fourth, our genomic analyses were limited to a single patient given the nature of this pilot study. Although concordance was high, the impact of delayed

processing on detecting rare subclonal mutations remains uncertain. Finally, while we demonstrate preservation of molecular profiles, the study does not yet establish clinical or functional activity such as associations with metastatic burden, treatment response, or the ability of CPT-derived CTC cultures to recapitulate drug sensitivities observed in patients. Establishing these links will be important to position the CPT workflow not only as a convenient sampling method, but also as a clinically informative platform for translational study.

In summary, this pilot study supports a CPT-based workflow as a promising strategy to reduce the logistical and infrastructure burden of CTC research by allowing delayed processing while retaining transcriptomic consistency, viability, and capturing tumor-informative genomic variations. If validated in larger cohorts, this strategy could broaden access to CTC-based investigations, enabling more scalable multi-center collections, centralized processing pipelines, and more inclusive study recruitment.

Methods

Patient Enrollment

Patients with metastatic solid tumors were recruited from the MedStar Georgetown University Hospital oncology clinics after providing written informed consent in accordance with the Health Insurance Portability and Accountability Act and institutional guidelines approved by the Georgetown University Institutional Review Board (approval ID: 2016 – 0419). Eligible patients were adults aged 18 years or older with histologically confirmed malignancy and clinically evident metastatic disease. Clinical annotation and specimen handling were performed in a de-identified manner. For the present pilot study, samples from four patients (Patients 107, 110, 111, and 114) were included for downstream molecular analyses.

Sample Collection and DNA/RNA collection

Peripheral blood was collected into BD Vacutainer® CPT™ Cell Preparation Tubes. For each enrolled patient, two CPT tubes were obtained at the time of phlebotomy for circulating tumor cell (CTC)-enriched specimen processing. Following blood collection, the two CPT tubes were assigned based on time-to-processing. Tube A was processed within 2 hours of collection. Tube B was stored at 4°C and processed 24 hours after collection. After centrifugation and CPT-based mononuclear/CTC-enriched fraction recovery, cell pellets were collected for expansion of CTCs ex vivo as described previously[6]. RNA and DNA extraction were performed using column-based kits following manufacturer protocols as detailed previously [10]. Briefly, RNA samples were DNase-treated and DNA samples were RNase-treated to remove contaminating nucleic acids, and similar principles were applied here to maximize purity for sequencing-based assays. Extracted RNA and DNA were stored at -80°C until use.

RNA Sequencing and Pathway Analysis

Bulk RNA sequencing was performed on RNA isolated from WBM and CPT-derived samples. All sequencing libraries were prepared with the support of third-party company Psomagen (Rockville, MD). Briefly, sequencing libraries were prepared using a stranded mRNA workflow and sequenced using paired-end 151-bp reads on an Illumina NovaSeq platform. Low-input RNA sequencing parameters were used to accommodate limited starting material from CPT-derived specimens. Raw sequencing reads were quality-checked and processed using a standard RNA-seq workflow. In this workflow, reads were trimmed with Trimmomatic, aligned or quantified against the GRCh38 human reference transcriptome, and imported into R for downstream differential gene analysis using DESeq2. Unsupervised clustering was used to assess transcriptomic similarity across sample groups. Pathway-level analyses were performed using curated gene set collections from MSigDB-derived gne sets. Hallmark and Reactome-based analyses were used to identify biologic programs enriched in CPT samples relative to WBM, with particular attention to epithelial-mesenchymal transition (EMT), KRAS signaling, and immune-related pathways, as these were the principal signals observed in the manuscript results.

Whole Exome Sequencing

Whole Exome Sequencing was performed on DNA isolated from WBM and CPT-derived samples. All sequencing libraries were prepared with the support of third-party company Psomagen (Rockville, MD). Briefly, exome libraries were prepared using a SureSelect V5-post capture workflow and sequenced as paired-end 151-bp reads on an Illumina NovaSeq platform. Bioinformatic processing followed a standard exome workflow, including alignment to the human reference genome via BWA, variant calling for single-nucleotide variants (SNVs) and small insertions/deletion via GATK and SnpEFF, and downstream variant annotation via OpenCravat. To interpret cancer relevance, identified variants were cross-referenced against OncoKB, an FDA-recognized precision oncology database. Detected mutations were compared across CPT samples and categorized as shared or sample-specific based on variant allele frequency.

Abbreviations

CPT
Cell Preparation Tube
CTC
circulating tumor cells
EMT
epithelial-mesenchymal transition
IRB
Institutional Review Board
RTK
receptor tyrosine kinase
WBM
whole blood matched

WES
whole-exome sequencing

Declarations

Ethics approval and consent to participate

This study involved human participants and was approved by the Georgetown University Institutional Review Board under approval ID 2016 – 0419. All participants provided written informed consent prior to sample collection and study participation.

Consent for publication

This manuscript does not contain any personally identifiable data, images, or videos. All clinical annotation and specimen handling was performed in a de-identified manner and in accordance with institutional guidelines and the Health Insurance Portability and Accountability Act.

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Author Contribution

J.X. and S.A. conceived and designed the study. J.X. and S.A. developed the experimental workflow and oversaw sample processing, CTC propagation, and molecular analysis. B.A.W., R.M., R.H., and G.S. contributed to patient recruitment, clinical annotation, biospecimen collection, and interpretation of clinical context. J.X. performed the primary bioinformatic analyses of RNA-sequencing and whole-exome sequencing data, with input from S.S. and S.A. J.X. generated the figures and drafted the initial manuscript. S.S., G.S., B.A.W., R.M., R.H., and S.A. revised and edited the manuscript. S.A. supervised the study and secured funding support. All authors reviewed and approved the final manuscript.

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Data Availability

The datasets generated and analyzed during this study are available in the Gene Expression Omnibus (GEO) repository under accession number GSE333160.

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Figures

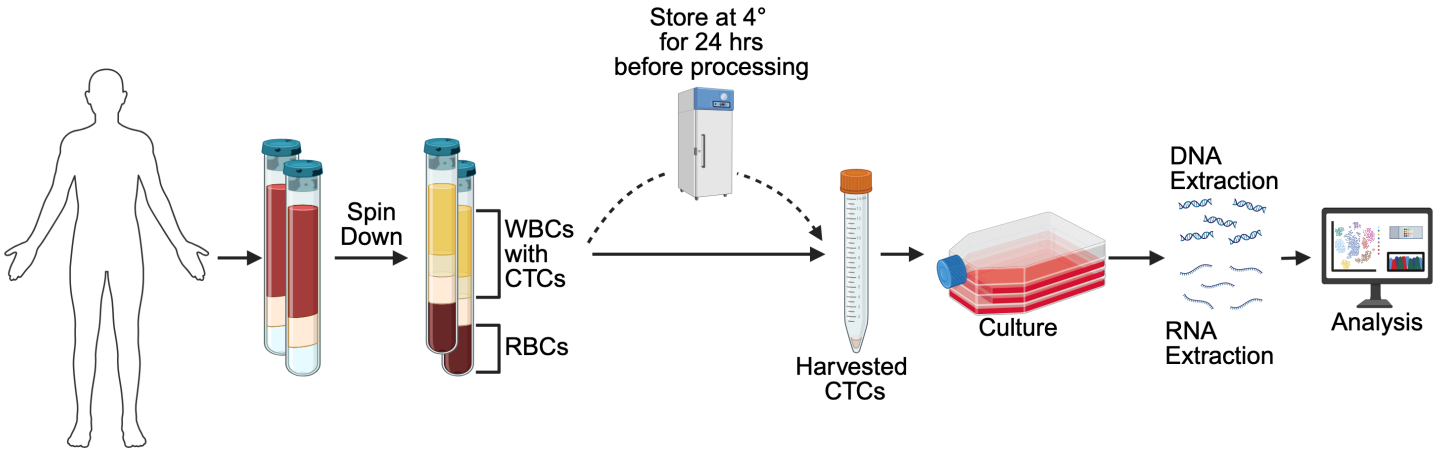


Figure 1

CPT-based workflow for delayed processing of patient blood samples for CTC expansion and molecular profiling. Peripheral blood was collected from patients into BD Vacutainer CPT tubes. For each patient, one tube was processed within 2 hours of collection (CPT A) whereas the second tube was refrigerated

after initial centrifugation and processed 24 hours later (CPT B). A matched WBM fraction was collected from the immediately processed sample. Harvested CTCs were propagated ex vivo and analyzed by RNA sequencing and whole exome sequencing.

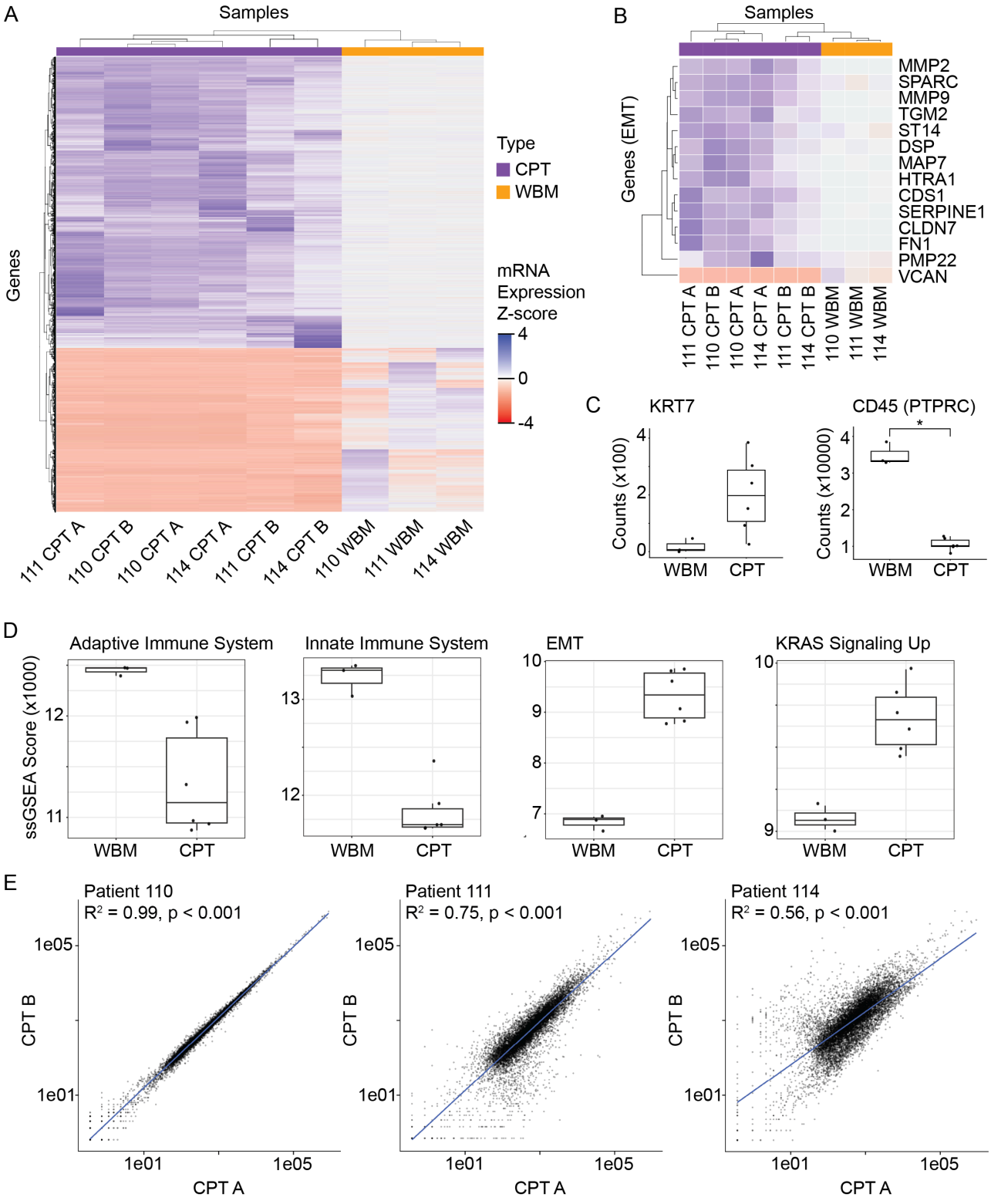


Figure 2

CPT-derived samples preserve transcriptomic features after delayed processing. (A) Unbiased hierarchical clustering of bulk RNA-sequencing data from matched WBM, CPT A, and CPT B samples. (B) Hierarchical clustering using an EMT-associated gene set. (C) Expression of KRT7 and CD45 in WBM and CPT-derived samples. (D) Pathway analysis showing decreased expression of immune-related gene sets and enrichment of EMT and KRAS signaling in CPT-derived samples. (E) Gene-level concordance between paired CPT A and CPT B samples processed within 2 hours and after 24 hours of storage.

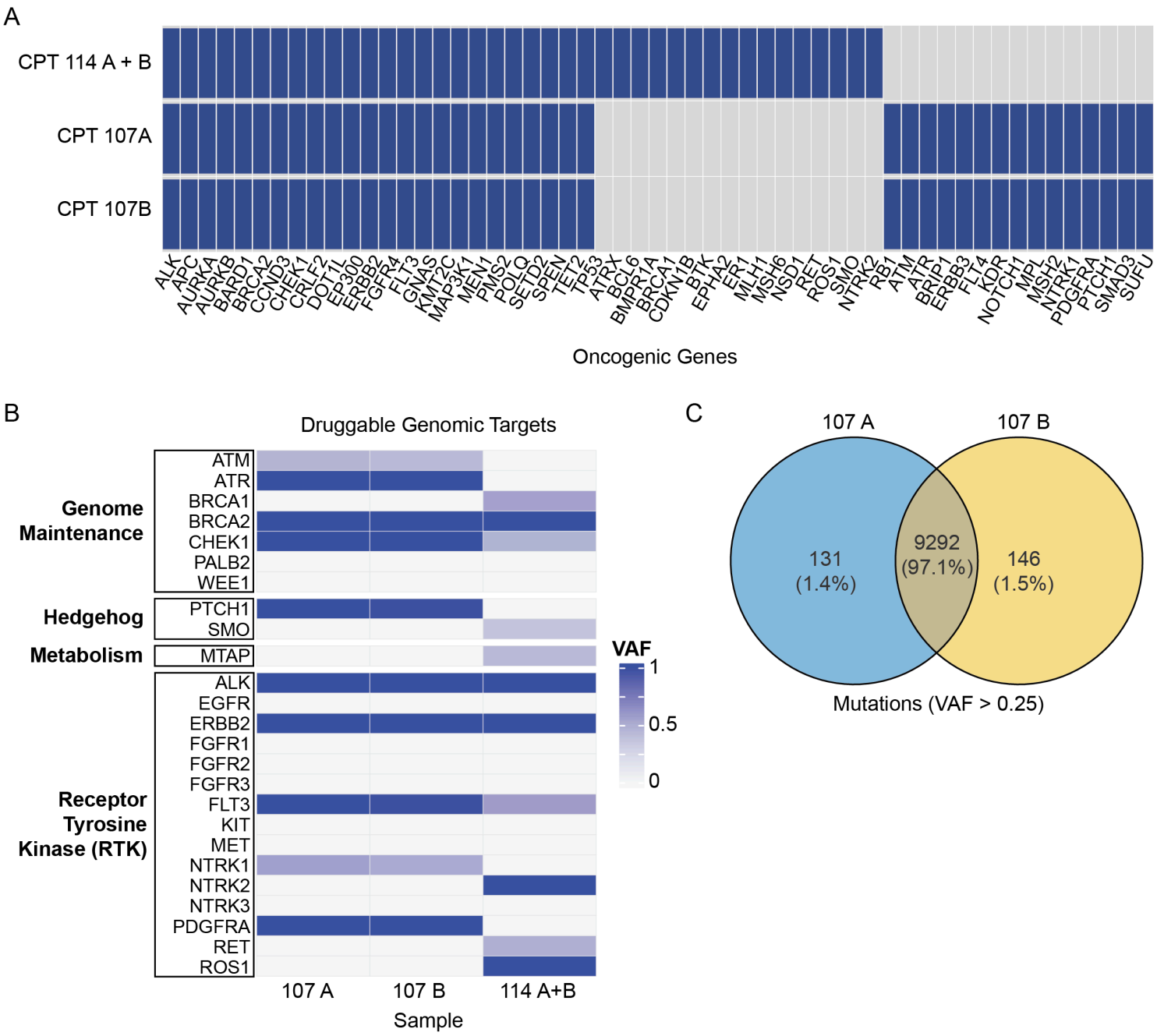


Figure 3

CPT-derived samples retain patient-specific genomic features and clonal heterogeneity. (A) WES of CPT-derived samples from patients 107 and 114 annotated using OncoKB. (B) Genomic profiling of potentially druggable targets across CPT-derived samples, highlighting targetable pathways involving

genome maintenance, Hedgehog signaling, cellular metabolism, and RTKs. (C) Venn diagram comparing mutations identified in Patient 107 CPT A and CPT B samples.