# Phenotypic, Genomic, and Clinical Associations of Circulating Tumor Cells (CTCs) Lacking Epithelial Biomarkers in Metastatic Castration Resistant Prostate Cancer (mCRPC)

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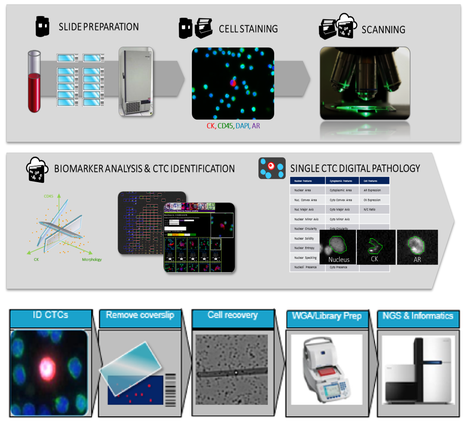
## Background

- Epithelial plasticity (EP) refers to the loss of the epithelial phenotype and replacement with a novel phenotype, including both the epithelial-mesenchymal transition (EMT) and its reverse process, the mesenchymal-epithelial transition (MET)
- EP is a proposed mechanism of immune evasion, drug resistance, apoptotic resistance, and promotion of metastasis
- EP has been extensively explored in cell biological and animal models which confirmed the presence of these tumor cells in circulation.
- Most studies on CTCs in human subjects are based on enrichment of cells expressing EpCAM, which precludes analysis of cells that might be EP.
- Here, the non-enrichment Epic Sciences platform was utilized to identify CTCs in mCRPC patient samples that were phenotypically consistent with EP: CTCs not expressing cytokeratins (CK), but expressing malignant biomarkers such as androgen receptor (AR)

## Methods

221 mCRPC patient blood samples were collected prior to starting Abiraterone (57); Enzalutamide (90), Apalutamide (3), Docetaxel (53) Cabazitaxel (16), and Paclitaxel (2). Patients were monitored for up to 3 years to assess OS outcomes. Samples were processed utilizing the Epic Sciences platform.

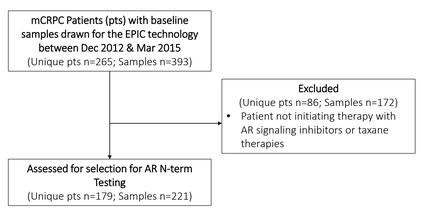



**Schematic of Epic CTC Platform CTC enumeration, morphology, biomarker, and single cell sequencing (CNV) analyses workflow:**

1) Nucleated cells from blood sample placed onto slides and stored in a -80°C biorepository
2) Slides stained with cytokeratin (CK), CD45, DAPI, AR N-term
3) Slides scanned and CTC candidates detected by a multi-parametric digital pathology algorithm
4) Human reader confirmation of CTCs & quantitation of biomarker expression
5) Single CTCs are lysed, whole genome amplified, shotgun libraries constructed, and whole genome sequenced.
6) Data analyzed for copy number variation analysis (CNV).

## Patient Demographics

| Characteristic | No. (%) or Median (range) |
|---|---|
| Number of Baseline Samples (unique patients) | 221 (179) |
| Age, years | 68 (45 - 91) |
| **Primary Treatment** | |
| Prostatectomy | 84 (47%) |
| Radiation | 34 (19%) |
| Brachytherapy | 7 (4%) |
| None | 54 (30%) |
| **Prior Hormone Therapies at Baseline** | |
| 1 - 2 lines | 82 (37%) |
| 3 lines | 50 (23%) |
| ≥ 4 lines | 89 (40%) |
| **Chemotherapy Status at Baseline** | |
| Chemo-naive | 136 (62%) |
| Chemo-exposed | 85 (38%) |
| **Metastatic Sites of Disease at Baseline** | |
| Bone | 194 (88%) |
| Lymph Node | 149 (67%) |
| Visceral Mets | 36 (16%) |
| Other Soft Tissue Only | 2 (1%) |
| **Laboratory Measures** | |
| PSA, ng/mL | 37.7 (0.10 – 3728.2) |
| Hgb, (g/dl) | 12.0 (7.0 – 15.0) |
| ALK, (unit/L) | 110 (25 – 2170) |
| LDH, (unit/L) | 222.5 (123 – 1293) |
| ALB, (g/dl) | 4.2 (3.1 – 4.9) |




- All samples were collected prior to initiation of systemic therapy
- Systemic therapies utilized are standard of care in mCRPC; AR signaling inhibitors (ARSI) abiraterone acetate, apalutamide and enzalutamide, or taxanes docetaxel, cabazitaxel and paclitaxel

## Phenotypic Features of CTCs Lacking Cytokeratins

**A. Representative CK(+)/AR(+) (upper panel) and CK(-)/AR(+) (lower panel) CTC Cell Images**

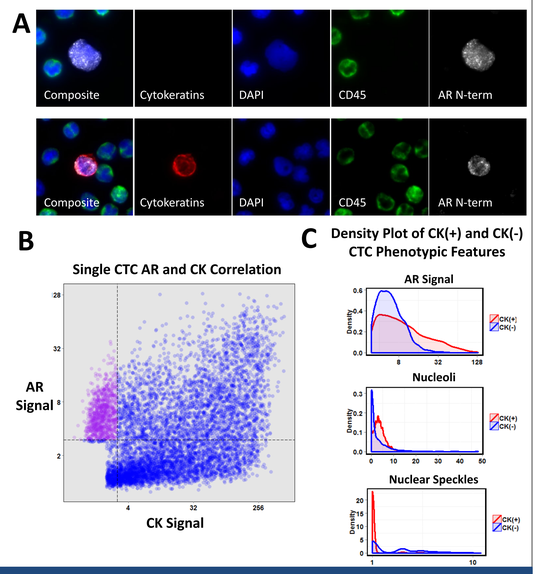
- Cytokeratins (CK) are a family of proteins expressed by epithelial cells
- The canonical definition of a "CTC" includes CK expression, intact nucleus (DAPI) and no CD45 signal
- The EP hypothesis posits that malignant cells can enter the circulation by downregulating epithelial cytoskeletal proteins to become more mesenchymal (EMT)

**B. CTCs with Malignant Biomarkers Can Lack Cytokeratins**

- AR expression (Y axis) and CK expression (X axis) are independent for all CTCs across the cohort
- 935 out of 7573 (12.3%) cells are CK(-)/AR(+), high-lighted in purple.

**C. CK(-) CTCs had trends for less nucleoli, more nuclear speckles, and lower AR expression**

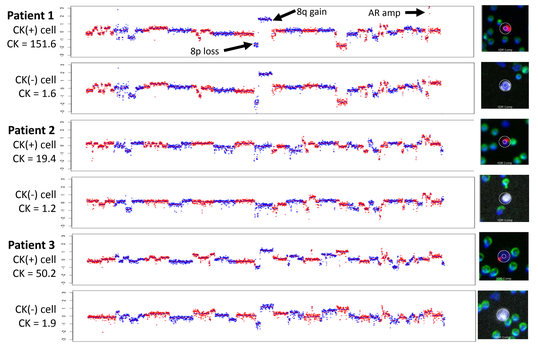
- Density plot showed the distribution of CK(+) CTCs (*n* = 7198, in red) and CK(-) CTCs (*n* = 1509, in blue) over continuous interval of three phenotypic features, AR, nucleoli and Nuclear speckles.




## CTCs Lacking Cytokeratins Have Cancer Genomes

- CK(-) CTCs were picked and individually whole genome sequenced
- Chromosomes are listed from left to right, 1 to Y, relative copy number for 1M bp windows are shown
- CK(-) CTCs frequently have genomes consistent with prostate cancer: AR gene amplification, PTEN loss, Chromosome 8q gain and 8p loss.
- CK(+) and CK(-) CTCs from the same patient may share the same genomic profile

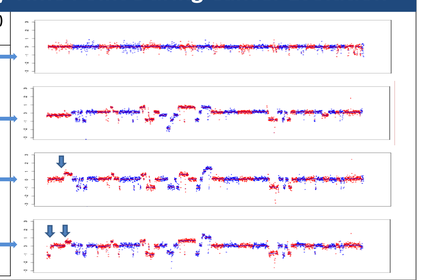
Note: CK signal analytical cutoff = 2.8




## Epithelial Plasticity May Occur at Any Time Point During Cancer Evolution

- 14 CTCs from one patient were single cell sequenced for CNV analysis, with 4 distinct genome profiles.
- CK(+) and CK(-) CTCs were observed in multiple branches of a phylogenetic tree. The absence of CK(+) or CK(-) cells from certain branches may be due to under-sampling.

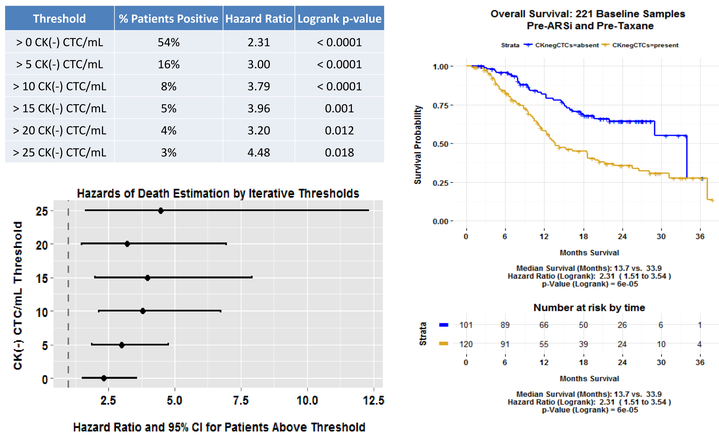
| | *n* of CK(+) CTCs | *n* of CK(-) CTCs |
|---|---|---|
| **Profile 1** | 2 | 0 |
| **Profile 2** | 5 | 1 |
| **Profile 3** | 1 | 3 |
| **Profile 4** | 0 | 2 |



## CK(-) CTCs are Associated with Poor Overall Survival in Univariate and Multi-variate Analysis

**A**

| Threshold | % Patients Positive | Hazard Ratio | Logrank p-value |
|---|---|---|---|
| > 0 CK(-) CTC/mL | 54% | 2.31 | < 0.0001 |
| > 5 CK(-) CTC/mL | 16% | 3.00 | < 0.0001 |
| > 10 CK(-) CTC/mL | 8% | 3.79 | < 0.0001 |
| > 15 CK(-) CTC/mL | 5% | 3.96 | 0.001 |
| > 20 CK(-) CTC/mL | 4% | 3.20 | 0.012 |
| > 25 CK(-) CTC/mL | 3% | 4.48 | 0.018 |




**B**

| Multivariable Cox Proportional Hazard Analysis of Predictors of Overall Survival | | |
|---|---|---|
| **Effect** | **P-value** | **HR (95% CI)** |
| ≥2nd Line Systemic Therapy for mCRPC | 0.00026 | 1.14 (1.19 – 1.76) |
| Visceral Metastases Present Pre-Therapy | 0.0716 | 1.66 (0.956 – 1.87) |
| PSA Pre-Therapy (continuous, log2 + 1) | 0.0516 | 1.10 (0.99 – 1.21) |
| CK(-) CTC Burden Pre-Therapy (continuous, log2 + 1) | 0.000555 | 1.27 (1.10 – 1.45) |

**A. Univariate Analysis**

- The clinical validity of CK(-) CTCs was evaluated using associations to overall survival (OS)
- The relationship between increasing concentration of CK(-) CTCs and OS was evaluated using iterative thresholds
- The presence of any CK(-) CTCs was additionally evaluated with the Kaplan-Meier method

**B. Multi-variate Analysis**

- CK(-) CTC/mL were evaluated as a continuous biomarker with respect to both OS and independence to other established prognostic factors for OS in a Cox PH model

## Conclusions

- CTCs in the peripheral blood of mCRPC patients expressing AR and lacking both blood lineage marker CD45 and epithelial marker CK, have similar genomes to CTCs expressing CK and display gross genomic alterations canonically associated with prostate cancer
- CK(-) CTCs are associated with poor OS and can provide independent and additive prognostic value to established prognostic factors: line of therapy, presence of visceral metastases, and pre-therapy PSA; none of these features strongly associate with the presence of CK(-) CTCs
- The presence of CK(-) CTCs, and the association of these cells with poor OS, are consistent with the Epithelial Plasticity hypothesis
- CK(-) CTCs are a clinically relevant part of a comprehensive portrait of the liquid phase of metastatic disease in prostate cancer. CTC detection technologies that rely on epithelial enrichment (i.e. EpCAM) are likely to under-sample total CTC burden and miss clinically relevant circulating biomarkers

**Support:** MSKCC SPORE in Prostate Cancer (P50 CA92629), the Department of Defense Prostate Cancer Research Program (PC051382), The Prostate Cancer Foundation. Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research, The Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center.