Single CTC Characterization Identifies Phenotypic and Genomic Heterogeneity as a Mechanism of Resistance to AR Signaling Directed Therapies (AR Tx) in mCRPC Patients

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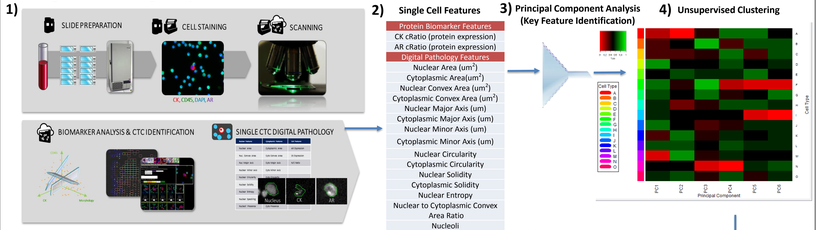
Background

Therapies targeting the androgen receptor (AR) and AR signaling such as Abiraterone Acetate (A) and Enzalutamide (E), and Taxane (T) based chemotherapy, prolong life in castration resistant prostate cancer (CRPC). The optimal sequence to administer them to maximize survival for an individual is unknown. Tumor heterogeneity (diversity) has been proposed as a biomarker of treatment resistance. We studied heterogeneity in CTCs on a cell by cell basis to develop predictive biomarkers of sensitivity for use at decision points in management to better sequence available therapies.

Methods for CTC Detection; Phenotypic, Genomic Characterization, and Heterogeneity Score

221 blood samples from 179 unique patients (pts) were analyzed with the Epic Sciences platform. Analysis included digital pathology of 23 discrete phenotypic cell features inclusive of AR and CK expression, and cellular size and shape measures. 9225 single CTCs were characterized, data standardized, features clustered and categorized into 15 phenotypically distinct CTC subtypes. Individual pt samples were then analyzed for the frequency and heterogeneity (Shannon Index) of CTC subtypes and monitored for clinical endpoints. A subset of CTCs (n=741) were individually sequenced and analyzed for clonality and CNV to assess genomic heterogeneity.



Schematic of Epic CTC Platform CTC enumeration, morphology, and biomarker analyses workflow:

1) Nucleated cells from blood sample placed onto slides and stored in a -80˚C biorepository. Slides are stained with cytokeratin (CK), CD45, DAPI, AR N-term and scanned. CTC candidates are detected by a multi-parametric digital pathology algorithm followed by human reader confirmation of CTCs and quantification of biomarker expression.
2) CTCs are segmented within the DAPI, CK, and AR channels and single cell features are extracted.
3) CTCs undergo Principle Component Analysis (PCA) removing noise and redundant dimensions, and weighing features with more variance.
4) Machine learning clustering algorithms found 15 CTC subtypes from macro trends in high-dimensional biomarkers across all CTCs from all samples in cohort, and assigned each CTC to 1 of 15 subtypes. Heterogeneity is quantified by counting CTCs per "Cell Type" in each sample, then using a standard Shannon Index to quantify CTC phenotypic diversity per patient sample.
5) Single cells are identified, relocated, captured, and sequenced for genomic correlation.

Patient Demographics

Patient Primary Therapy	
Number of Unique Patients	179
Age, years	68 (45 – 91)
Primary Treatment	
Prostatectomy	84 (47%)
Radiation	34 (19%)
Brachytherapy	7 (4%)
None	54 (30%)
Characteristic	**All Samples**
Number of Baseline Samples	221
Age, years	68 (45 – 91)
Prior Hormone Therapies*	
1 - 2 lines	81 (37%)
3 lines	46 (21%)
≥4 lines	94 (42%)
Chemotherapy Status	
Chemo-naive	136 (62%)
Chemo-exposed	85 (38%)
Metastatic Disease	
Bone Only	63 (29%)
Lymph Node (LN) Only**	24 (10%)
Bone & LN	77 (35%)
Bone & Visceral ± LN**	35 (10%)
Laboratory Measures	
PSA, ng/mL	37.7 (0.1 – 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)

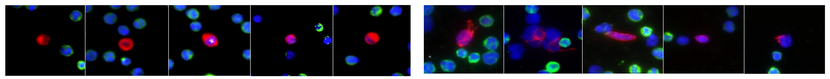
*includes GnRH agonists and antagonists, antiandrogens and next-generation hormonal therapies (abiraterone acetate and enzalutamide) patient was exposed to prior to initiation on the baseline therapy
** includes patients with other soft tissue disease

- Standard of care collection from 221 mCRPC patients at decision points.
- Baseline blood draws collected prior to AR Tx or Taxane Tx.
- Patients monitored for radiographic progression free survival (rPFS) and overall survival (OS).

Patient Line of Therapy

Total Samples	1st Treatment Decision	2nd Treatment Decision	3rd + Treatment Decision
A or E Baseline Blood Draw (n=150)	No Prior A or E (1st Line) n= 64	Previous A or E (2nd Line) n= 36 Previous T (2nd Line) n= 8	Previous A & E (3rd+ Line) n= 5 Previous AR Tx & T (3rd+ Line) n= 37
T Baseline Blood Draw (n=71)	No Prior A or E (1st Line) n=12	Previous A or E (2nd Line) n= 12	Previous A & E (3rd+ Line) n= 10 Previous AR Tx & T (3rd+ Line) n= 37

CTC Heterogeneity is Observed in Patient Samples and Increases by Line of Therapy



Example of CTCs from Low Shannon Index Patient

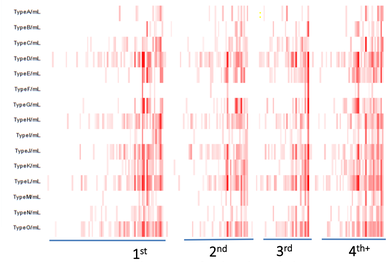
Example of CTCs from High Shannon Index Patient

15 Key Mathematical CTC Phenotypes Identified

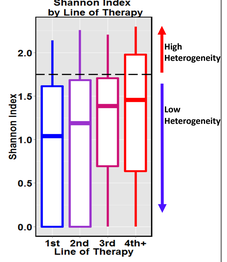
Species	Cells in Cohort
CellTypeA	123
CellTypeB	209
CellTypeC	448
CellTypeD	1448
CellTypeE	701
CellTypeF	144
CellTypeG	867
CellTypeH	659
CellTypeI	266
CellTypeJ	814
CellTypeK	508
CellTypeL	1643
CellTypeM	194
CellTypeN	198
CellTypeO	1003

CTC Subtypes by Patient Sample and Line of Therapy

Sample Burden by CTC Type: None □ Moderate ■ High

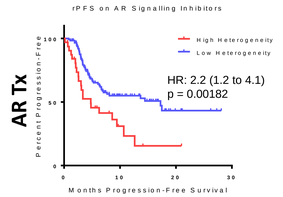


Shannon Index (Diversity Measure)

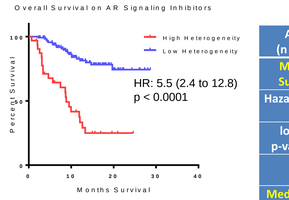


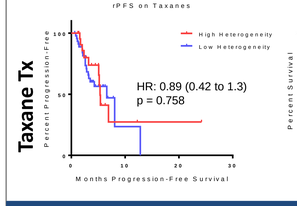
High CTC Phenotypic Heterogeneity Predicts Shorter Survival Times on AR Tx but not Taxane Tx

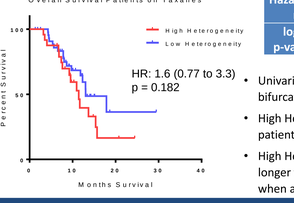
Progression-Free Survival



Overall Survival







Outcomes on **AR Tx** Based on Heterogeneity

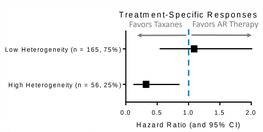
AR Tx (n = 150)	High Heterogeneity	Low Heterogeneity
Median Survival	9 months	not reached
Hazard Ratio: OS	5.51	
logrank p-value OS	< 0.0001	
Median rPFS	5 months	17 months
Hazard Ratio: rPFS	2.2	
logrank: p-value rPFS	0.00182	

- Univariate survival curves are shown for rPFS and OS, bifurcated by Taxanes or AR Tx.
- High Heterogeneity (top 25% Shannon Index scores) patient-exposures have poor outcomes on AR Tx.
- High Heterogeneity patient-exposures to Taxanes survive longer than AR Tx, but the difference is much greater when adjusted for other clinical measures (below).

High CTC Phenotypic Heterogeneity Predicts Better Survival on Taxane over AR Tx in Multivariate Model

Multivariate Cox PH Analysis of Predictors of Overall Survival	
Effect	**p-value**
Heterogeneity (high vs. low)	0.01
3rd line or later (yes vs. no)	0.002
Visceral Mets (yes vs. no)	0.21
PSA pre-therapy (high vs. low)	0.33
LDH pre-therapy (high vs. low)	0.07
Albumin pre-therapy (high vs. low)	0.03
Hgb pre-therapy (high vs. low)	0.05
Alk pre-therapy (high vs. low)	0.06
Treatment (Taxane vs. ARS)	0.08
High Heterogeneity : Therapy Interaction	0.023

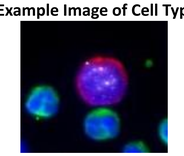
Therapy Interaction: Multivariate Cox PH Model		
	Comparison	**HR**
Heterogeneity & Therapy	High Heterogeneity: Taxane vs AR	**0.323 (0.122-0.857)**
	Low Heterogeneity: Taxane vs AR	1.09 (0.541-2.21)



- High heterogeneity remained independent and additive to all other clinical measures, significant treatment-specific interaction.
- Patient samples with high Heterogeneity have a 68% reduction in the risk of death on Taxanes compared to AR Tx.

Prevalence of a CTC Subtype (Type K) Predicts Poor Outcome on AR Tx and Taxanes

Example Image of Cell Type K

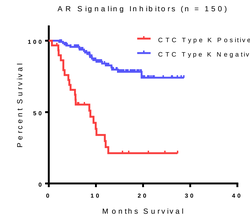


Cell Type K Key Features

Cell Phenotype
- Large Nucleus
- High Nuclear Entropy
- Frequent Nucleoli

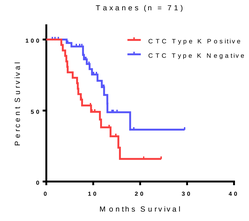
Cell Genotype
- Increased Large Scale Transitions Breakpoints (Genomic Instability)

AR Tx



HR: 6.4 (2.6 to 15.2)
p < 0.0001

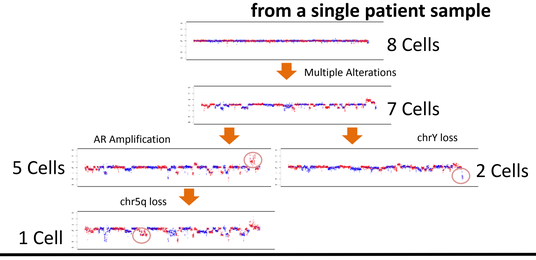
Taxane Tx



HR: 2.3 (1.1 to 4.8)
p = 0.0152

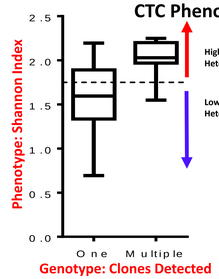
Single CTC Genomics Identifies Genomic Heterogeneity in High Heterogeneity Phenotype Patient Samples

Phylogenetic Tree shows multiple clonal subtypes represented by 23 single CTCs sequenced from a single patient sample



A case study of single CTC genomic sequencing of one patient with high phenotypic heterogeneity. Odd chromosome #'s are red, even are blue. Gains and losses of major regions shown by deviation from center line.

CTC Phenotype Heterogeneity Correlates with CTC Genomic Heterogeneity: Genotype to Phenotype Subset Analysis
Patients= 31 CTCs=741



Genotype (Clonality) to Phenotype	Multiple Genomic Clones (≥2 clone)	Single Genomic Clone (=1 clone)
High Phenotypic Heterogeneity	16	4
Low Phenotypic Heterogeneity	1	10
Odds Ratio (95% CI)	40	
Fisher's Exact	p < 0.0001	

- A sub-cohort of 31 patient samples with CTCs individually sequenced as per case study above. A "clone" is a distinct CNV profile observed by at least two CTCs in a patient sample.

Conclusions

- Single CTC phenotypic and genomic characterizations are feasible and can be used to assess tumor heterogeneity in a patient.
- High phenotypic heterogeneity identifies patients in a cohort with:
  - Increased risk of death on Abiraterone & Enzalutamide but not taxane chemotherapy
  - 40X increased likelihood to have genomic heterogeneity (multiple clones)
- CTC clustering identifies a CTC subtype with:
  - Resistance to both AR and Taxane therapy
  - Increased genomic instability (high LST #)
- A non-invasive liquid biopsy that enables the characterization of individual cells from a patient with metastatic cancer can be used to guide treatment selection. Ongoing validation in progress.

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