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TITLE: Transferrin Receptor Identifies a Comprehensive pool of Circulating Tumor Cells with unique molecular features from metastatic Prostate Cancer Patients

PRINCIPAL INVESTIGATOR: Paraskevi Giannakakou

CONTRACTING ORGANIZATION: Weill Medical College of Cornell University
New York, NY

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Paraskevi Giannakakou

E-Mail: pag2015@med.cornell.edu

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Weill Medical College of Cornell
University
1300 York Avenue
New York, NY 10065-4805

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Metastatic castration resistant prostate cancer (CRPC) is currently incurable, due to treatment resistance. Elucidation of resistance mechanisms requires frequent tumor sampling to monitor tumor evolution and tailor treatments to the individual. Circulating tumor cells (CTCs) represent a non-invasive, accessible "liquid biopsy" source of tumor cells, allowing for longitudinal molecular disease profiling. Due to limitations with existing EpCAM-based CTC isolation assays we have identified and clinically tested Transferrin Receptor (TfR) as a novel cell-surface antigen that enables capture of all CTCs across the EMT gradient from metastatic patients. Mining large datasets (TCGA, SU2C) revealed **TfR enrichment in metastatic patients,** which significantly correlated with advanced state from localized PC to CRPC to the aggressive neuroendocrine NEPC. RNA-seq analysis indicates that TfR^+-CTCs possess unique expression profile and are enriched in EMT and tumor progression pathways, as compared to $EpCAM^+$- CTCs. Expression of androgen receptor (AR) splice variants (AR-Vs) is known to drive disease progression. We have developed a highly sensitive—down to single cell—digital droplet PCR assay for the quantitation of AR-Vs in patient CTCs. Isolation of TFR^+ vs $EpCAM^+$ CTCs from metastatic patients, revealed significant AR-V **enrichment in TFR^+ CTCs,** while AR-FL expression was similar. When we analyzed **single CTCs** using the same ddPCR assay, we observed even more striking enrichment, with AR-Vs detected in 21% of single TFR^+-CTCs vs 0% in $EpCAM^+$-CTCs. These data support our **hypothesis** that *TfR can identify a comprehensive pool of CTCs (not limited to the epithelial-only phenotypes) and provide an accurate representation of metastatic disease burden*. To test this, we propose to **prospectively** collect peripheral blood from CRPC and NEPC patients to **1**. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes **2.** Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients. In addition, we propose to **3**. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal models.

15. SUBJECT TERMS

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Introduction

Metastatic castration resistant prostate cancer (CRPC) is currently incurable, due to treatment resistance. Elucidation of resistance mechanisms requires frequent tumor sampling to monitor tumor evolution and tailor treatments to the individual. Circulating tumor cells (CTCs) represent a non-invasive, accessible "liquid biopsy" source of tumor cells, allowing for longitudinal molecular disease profiling. Due to limitations with existing EpCAM-based CTC isolation assays we have identified and clinically tested Transferrin Receptor (TfR) as a novel cell-surface antigen that enables capture of all CTCs across the EMT gradient from metastatic patients. Mining large datasets (TCGA, SU2C) revealed **TfR enrichment in metastatic patients,** which significantly correlated with advanced state from localized PC to CRPC to the aggressive neuroendocrine NEPC, as compared to no changes in EpCAM expression. CTC enumeration shows that TfR^+-CTC counts are significantly higher than $EpCAM^+$-CTCs in patients with mCRPC. RNA-seq analysis indicates that TfR^+-CTCs possess unique expression profile and are enriched in EMT and tumor progression pathways, as compared to $EpCAM^+$-CTCs. Expression of androgen receptor (AR) splice variants (AR-Vs) is known to drive disease progression. We have developed a highly sensitive—down to single cell—digital droplet PCR assay for the quantitation of AR-Vs in patient CTCs. Isolation of TFR^+ vs $EpCAM^+$ CTCs from metastatic patients, revealed significant AR-V **enrichment in TFR^+ CTCs,** while AR-FL expression was similar. Taken together these data led us formulate the following hypothesis:

Hypothesis *TfR can identify a comprehensive pool of CTCs (not limited to the epithelial-only phenotypes) and provide an accurate representation of metastatic disease burden*.

To address this hypothesis, we proposed to **prospectively** collect peripheral blood from CRPC and NEPC patients and

Specific Aim 1. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes

Specific Aim 2. Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients.

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids.

2. Keywords: Prostate Cancer, castration resistant prostate cancer (CRPC), neuroendocrine prostate cancer (NEPC), circulating tumor cells (CTCs), Transferrin Receptor (TfR), androgen receptor (AR), AR splice variants (AR-Vs), epithelial cell adhesion molecule (EpCAM)

3. **Accomplishments:**

- **What were the major goals of the project?**

Specific Aim 1. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes

Specific Aim 2. Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients.

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids.

- **What was accomplished under these goals?**

Specific Aim 1. Identify clinically meaningful genes/oncogenic pathways associated with disease progression and/or response to therapy by molecular profiling of TfR+ -CTCs and EpCAM+ -CTCs from CRPC patients and NEPC patients using serial sampling at baseline and progression and correlate with clinical outcomes.

The *working hypothesis* of this aim is that TfR+-CTCs will provide a more accurate representation of metastatic disease burden and include a more comprehensive spectrum of CTCs, whose molecular analysis will be clinically informative. In addition, we hypothesize that TfR+-CTCs will contribute to the diagnosis and molecular phenotyping of NEPC.

In this Aim we have been collecting peripheral blood from patients with metastatic CRPC receiving treatment with AR-targeted therapies (abiraterone/enzalutamide) (IRB0707009283, PI Tagawa) and from patients with NEPC (at Dana Farber, IRB19883, PI: Beltran) and enriching for circulating tumor cells (CTCs) by depleting the contaminating CD45^{+} leukocytes (RosetteSep human CD45 depletion cocktail, STEMCELL™ technologies). CTCs were collected at baseline and at the time of progression to AR-Signaling Inhibitors (ARSI), abiraterone and enzalutamide. After enrichment, TfR^{+} and EpCAM^{+} CTCs were isolated utilizing the automated micromanipulator CellCelector (Automated Lab Solution, Germany). Matching leucocytes (peripheral blood mononuclear cells, PBMCs) were also collected to assess crosstalk between CTCs and the circulating tumor macroenvironment.

CTCs were molecularly profiled by RNA-Sequencing. RNA-Seq raw reads were trimmed and aligned to the human reference genome (hg38).

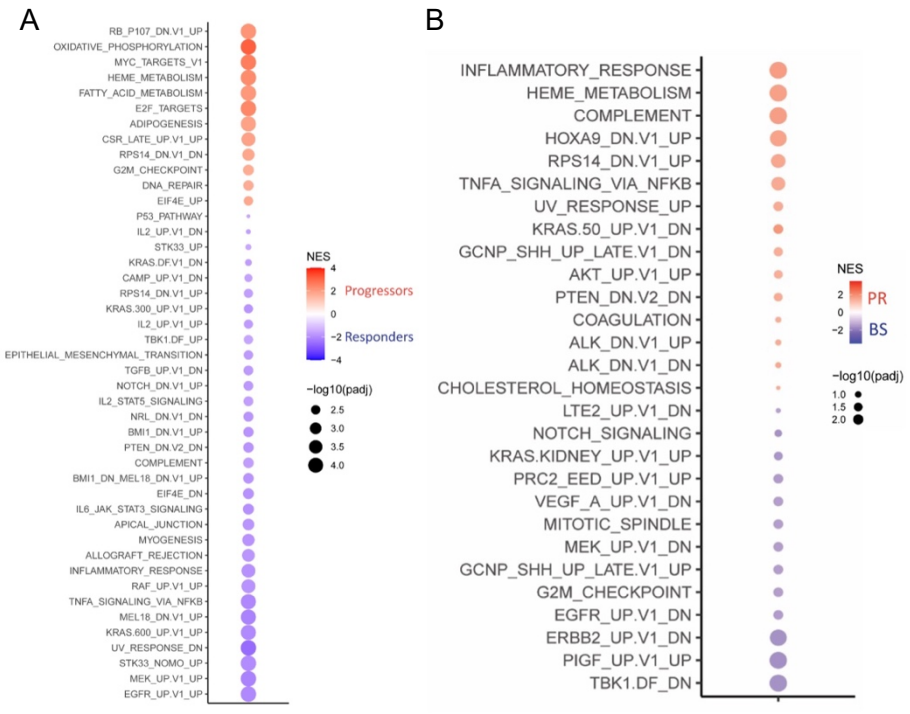

Figure 1. (A) Gene set enrichment analysis (GSEA) of CTCs isolated at baseline from patients with mCRPC who progressed after ARSI treatment (Progressors) and patients who responded (Responders). A total of 12 Hallmark and Oncogenic pathways were specifically enriched in ARSI progressors. (B) GSEA of CTCs isolated at progression (PR) and at baseline (BS). Pathways associated with inflammation and immune response (i.e., complement, TNFα) were significantly enriched upon ARSI progression. Results are ranked based on adjusted p-value and color coded based on normalized enrichment score.

Transcriptomic analysis of the enriched CTCs was performed before treatment initiation (baseline) to identify clinically meaningful genes/molecular pathways associated with intrinsic response/resistance to treatment. Analysis of CTCs at disease progression will be informative of genes/pathways associated with acquired resistance. GSEA analysis at baseline showed enrichment of oncogenic pathways previously associated with resistance to ARSI treatment in prostate cancer, such as RB-loss and E2F activation (E2F targets) (**Figure 1A**). Interestingly, the gene encoding for TfR (TFRC) was found among the leading-edge genes of the E2F targets pathway (data not shown), supporting an association between the expression of TfR, the activation of the RB-E2F axis and intrinsic resistance to ARSI treatment in prostate cancer. Mechanistic studies to explore this relationship are currently undergoing in the lab.

In addition, GSEA analysis of CTCs isolated at ARSI progression showed the significant enrichment of several pathways associated with the inflammatory process and the immune response, as compared to baseline CTCs (**Figure 1B**). Previous results have also shown significant enrichment of TFRC-related pathways enriched at progression (**Figure 2**).

Transcriptomic analysis of the matched TfR^{+} CTCs isolated at baseline and at progression is currently ongoing and will be informative to understand the association between TFRC-related pathways and the pathways associated with resistance to ARSI treatment. Analysis of matching PBMCs will inform on potential crosstalk between TfR^{+} CTCs and the circulating macroenvironment.

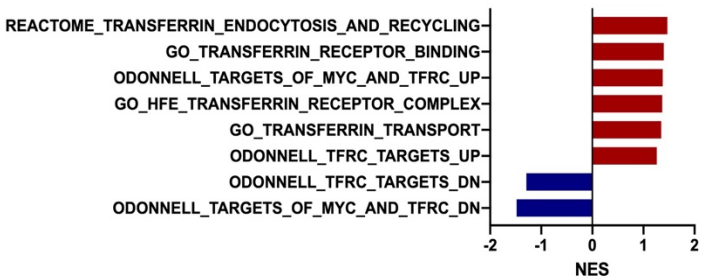

Figure 2 GSEA of CTCs isolated at ARSI progression as compared to baseline. Red bars indicate TFRC-related pathways significantly enriched at progression; blue bar indicate TFRC-related pathways significantly enriched at baseline. NES: normalized enrichment score.

Specific Aim 2. Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR$^+$- and EpCAM$^+$-CTCs from CRPC patients.

In this Aim, we performed and present a single-CTC sensitive digital droplet PCR (ddPCR) assay for the quantitation of the two most common AR-Vs, AR-V7, and AR-v567es, using antigen agnostic CTC enrichment. In a cohort of 29 mCRPC patients, we identify AR-V7 in 66% and AR-v567es in 52% of patients. We next quantified AR-V expression in matching EpCAM$^+$-CTCs and TFR$^+$-CTCs (EpCAM-negative) and identified lower AR-V prevalence in the EpCAM-positive fraction, suggesting that EpCAM-based CTC enrichment likely underestimates AR-V prevalence. Lastly, using single CTC analysis we identify enrichment for AR-v567es in patients with neuroendocrine prostate cancer (NEPC) indicating that AR-v567es may be involved in lineage plasticity, which warrants further mechanistic interrogation.

These results are now published in:

- Gjyrezi, A, Galletti, G, Zhang, J, Worroll D, Sigouros M, Kim S, Cooley V, Ballman KV, Ocean AJ, Shah MA, Scandura JM, Sboner A, Nanus DM, Beltran H, Tagawa ST, Giannakakou P. **Androgen receptor variant shows heterogeneous expression in prostate cancer according to differentiation stage**. *Commun Biol* 4, 785 (2021). https://doi.org/10.1038/s42003-021-02321-9

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids.

In the preliminary data presented above (**Figure 1**), we identified enrichment for RB-loss pathway in CTCs from patients with mCRPC who did not respond to ARSI treatment and who also displayed enrichment in TFRC-pathways. In addition, at disease progression we found enrichment (upregulation) of TFRC-related pathways. To start exploring the functional relationship between TfR and Myc we took advantage of already established GEMs with MYCN overexpression in combination with loss of Rb1, to recapitulate the gene expression clinical associations between RB-loss, treatment resistance and disease progression, presented in Aim 1. Using organoids isolated from these animal models we are currently performing mechanistic studies to determine the role of N-Myc binding at the TFRC promoter and the mechanism by which N-Myc activity leads to upregulation of TFRC and its role in NEPC development.

- **What opportunities for training and professional development has the project provided?**

Drs Giannakakou, Beltran, Tagawa and Rickman are fully committed to furthering the training and professional development of the postdoctoral fellows and students affiliated to this project. Due to the unexpected COVID-19 crisis we had limited opportunities to present our preliminary data. Parts of the data were presented at the scientific conferences and internal research progress meetings mentioned in the following section.

- **How were the results disseminated to communities of interest?**

Prostate Cancer Foundation, Carlsbad, CA (Annual Scientific Retreat, October 2020)

Annual Scientific Retreat

Belfer Basic Research Working Group, New York, NY (May 2021)

Internal research in progress meeting in the Meyer Cancer Center at Weill Cornell Medicine.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we plan to continue our investigations in all 3 Aims. In **Specific Aims 1** and **2** we will expand the molecular profiling of CTCs in additional patients with mCRPC and NEPC, as outlined in the original application. We will isolate pools of TfR+ and EpcAM+ CTCs and perform differential gene expression and pathway analyses to identify genes/pathways that are significantly associated with clinical outcomes. Along

these lines we will perform single CTC analyses to determine the heterogeneity and clinical impact of AR-V expression using our established AR-V ddPCR assay. We have also published a manuscript as part of **Specific Aim 2** (see section 6. Products). In **Specific Aim 3** we will determine if C-Myc and N-Myc are bone fide regulators of TFRC expression and will also determine the impact of TFRC depletion on the landscape of N-Myc binding, N-Myc target genes and associated epigenomic alterations during the transformation from CRPC to NEPC phenotype.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

  Nothing to Report

- **What was the impact on other disciplines?**

  Nothing to Report

- **What was the impact on technology transfer?**

  Nothing to Report

- **What was the impact on society beyond science and technology?**

  Nothing to Report

5. Changes/Problems

Nothing to Report

6. Products

Publications, conference papers, and presentations

Gjyrezi, A, Galletti, G, Zhang, J, Worroll D, Sigouros M, Kim S, Cooley V, Ballman KV, Ocean AJ, Shah MA, Scandura JM, Sboner A, Nanus DM, Beltran H, Tagawa ST, Giannakakou P. **Androgen receptor variant shows heterogeneous expression in prostate cancer according to differentiation stage**. *Commun Biol* 4, 785 (2021). https://doi.org/10.1038/s42003-021-02321-9 (published). Acknowledgement of federal support: yes.

Zhang J, Zimmermann B, Galletti G, Halabi S, Gjyrezi A, Yang Q, Gupta S, Verma A, Sboner A, Anand M, George D, Gregory S, Hong S, Pascual V, Mavragani C, Antonarakis ES, Nanus DM, Tagawa ST, Elemento O,. Armstrong AJ, Giannakakou P. **Transcriptomic profiling of tumor and immune-macroenvironment cells from liquid biopsies identifies the molecular determinants of clinical resistance to Androgen Receptor Signaling Inhibitors in Prostate Cancer** (submitted). Acknowledgement of federal support: yes.

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	Paraskevi Giannakakou
Project Role:	PI
Contribution to Project:	No Change
Name:	Ada Gjyrezi
Project Role:	Lab Manager
Nearest person month worked:	5
Contribution to Project:	Ada was involved in CTC processing and the molecular analysis of the isolated CTCs. In addition, she was responsible of the quantification of AR-variants in the TfR^+ and $EpCAM^+$ CTCs for Aim 2.
Name:	Jiaren Zhang
Project Role:	Research Associate
Nearest person month worked:	6
Contribution to Project:	Jiaren performed the computational analyses that revealed the transcriptomic profiles of the isolated CTCs
Name:	Lucie Van Emmenis
Contribution to Project:	No Change
Name:	Robert Zimmerman
Contribution to Project:	No Change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to Report

- **What other organizations were involved as partners?**

    - **Organization Name:** Dana Farber Cancer Institute

        - **Location of Organization:** Boston, MA

        - **Partner's contribution to the projec**t

            - Subaward