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TITLE: The HGF/c-MET Axis as a Critical Driver of Resistance to Androgen Suppression in Metastatic Castrate-Resistant Prostate Cancer

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14. ABSTRACT In this Physician Research Training Award, the primary goal is to elucidate the role of the HGF/c-MET axis in metastatic castration-resistant prostate cancer. During the current funding period, we have confirmed an inverse relationship between MET expression and AR signaling in prostate cancer cell lines. Our data supports negative regulation of MET by AR signaling, and we found that AR signaling inhibition in AR-positive CRPC models increased MET expression and resulted in susceptibility to ligand (HGF) activation. Likewise, our work over the past year showed that MET inhibition was only effective in blocking cancer phenotypes in cells with MET overexpression. Using multiple AR-positive CRPC models, as detailed in the initial proposal, we found that combined MET inhibition and enzalutamide (AR antagonist) treatment was more efficacious than either inhibitor alone. These data support the concept that the MET pathway may be an key mechanism of resistance in men with CRPC who undergo potent androgen signaling inhibition with abiraterone or enzalutamide.					
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Introduction

In this Physician Research Training Award, the primary goals are both to further my training as a surgeon scientist and to elucidate the role of the HGF/c-MET axis in metastatic castration-resistant prostate cancer. In so doing, the overarching goal is to develop a durable cure of prostate cancer through a deeper understanding of prostate cancer metastasis and mechanisms of therapeutic resistance. The career development component of the award involves training in molecular biology techniques and biomarker discovery, with close mentorship from Drs. Arul Chinnaiyan and Russell Taichman. The primary scientific aims of this grant are to: 1) Define the mechanisms by which the HGF/c-MET axis facilitates proliferation and viability of CRPC cells in vitro, 2) Define the role of the HGF/c-MET axis in supporting resistance to androgen suppression in mCRPC patients with and without next generation anti-androgen therapy, and 3) Identify the mechanisms by which the HGF/c-MET axis emerges as a mediator of acquired resistance to abiraterone therapy.

Keywords

Prostate cancer, metastasis, androgen deprivation therapy, human growth factor, MET, enzalutamide, abiraterone, cabozantinib, circulating tumor cells, disseminated tumor cells

Accomplishments

Major Task 1: *Establish the response to enzalutamide in distinct PCa cell lines and determine the extent to which c-MET inhibition recovers androgen suppression. (months 1-18)*

Substantial progress has been made on this Major Task during the initial year of this grant, and we have recently submitted a manuscript to *Cancer Research* that is built around Aim 1 of the grant. The DOD has been cited as a funding source, and the citation as currently submitted is:

Qiao, Y, Feng, FY, Wang, Y, Cao, X, Han, S., Wilder-Romans, K, Keller, ET, Palapattu, GS, Taichman, RS, Alva, AS, Smith, DC, Tomlins, SA, Chinnaiyan, AM, Tomlins, SA, Morgan, TM: Mechanistic support for combined MET and AR blockade in castration resistant prostate cancer. Cancer Research (In Revision): 2015.

As will be detailed below, we have confirmed an inverse relationship between MET expression and AR signaling in prostate cancer cell lines. Our data supports negative regulation of MET by AR signaling, and we found that AR signaling inhibition in AR-positive CRPC models increased MET expression and resulted in susceptibility to ligand (HGF) activation. Likewise, our work over the past year showed that MET inhibition was only effective in blocking cancer phenotypes in cells with MET overexpression. Using multiple AR-positive CRPC models, as detailed in the initial proposal, we found that combined MET inhibition and enzalutamide (AR antagonist) treatment was more efficacious than either inhibitor alone. These data support the concept that the MET pathway may be a key mechanism of resistance in men with CRPC who undergo potent androgen signaling inhibition with abiraterone or enzalutamide. This suggests potential utility for MET inhibition in select patients with AR therapy resistance and in AR-negative prostate cancer. In addition, our findings to date suggest that combined AR and MET inhibition in CRPC may be more effective than inhibiting these pathways sequentially.

Subtask 1: *Determine cell proliferation, invasion, migration, and expression of AR and HGF/c-MET axis in PCa cell lines*

Subtask 2: *Determine impact of enzalutamide on cell proliferation, invasion, migration, and expression of AR and HGF/c-MET axis in PCa cell lines*

In order to assess the relationship between AR and MET, we performed Western blot analyses and real time PCR to determine their expression in a number of prostate cancer cell lines. These data strongly supported the inverse relationship of AR and MET.

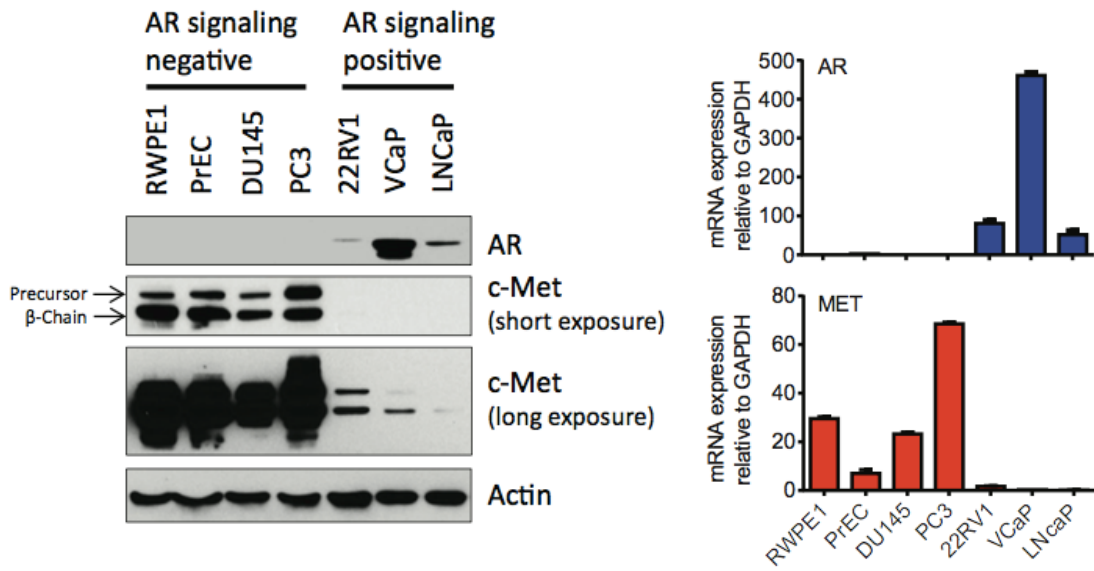


Figure 1: AR and MET expression assessed by Western blot analysis in a series of AR-negative and AR+ prostate cancer cell lines

To further assess MET expression and its correlation with AR expression, we used flow cytometry to sort VCaP cells (AR+/MET^{low}) according to levels of MET expression. We then used real time PCR to compare AR expression in those cells with the highest MET expression with AR expression in those cells with the lowest MET expression. Significant differences in AR expression were present, providing further support for negative regulation of MET by AR signaling.

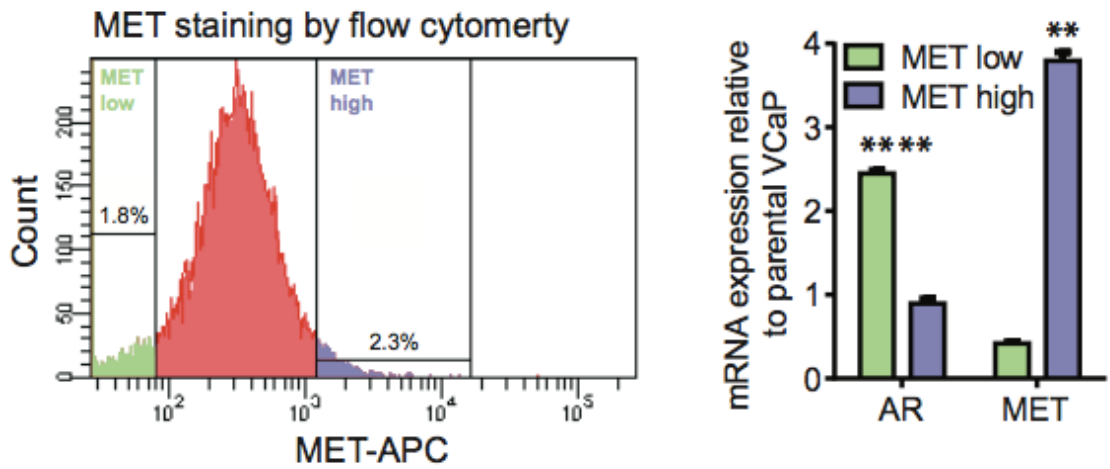


Figure 2: VCaP (AR+/MET-) cells were flow sorted according to the level of MET expression. AR and MET expression was compared by RT-PCR in the MET-low vs. MET-high cells.

Proliferation of LNCaP cells was confirmed to be increased by increasing concentration of DHT and inhibited by enzalutamide.

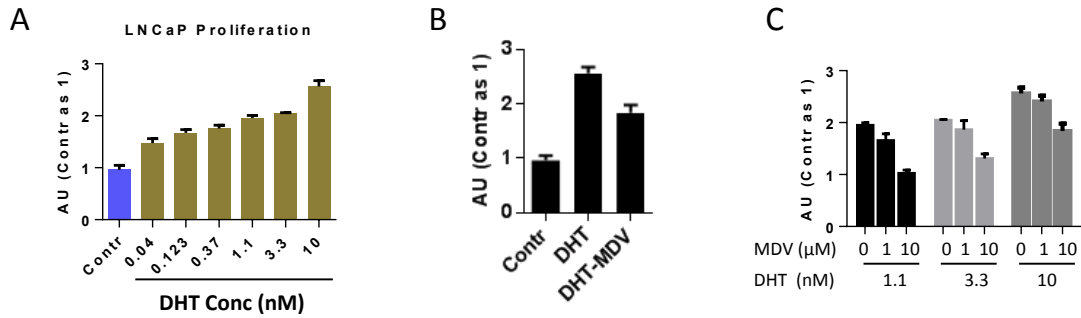


Figure 3: Proliferation of LNCaP cells was assessed with A) varying concentrations of DHT and B) in the setting of DHT + enzalutamide (MDV). C) Enzalutamide inhibited LNCaP proliferation at different doses and in the presence of varying concentrations of DHT.

Proliferation of PC3 (AR-) cells was not impacted by the addition of DHT.

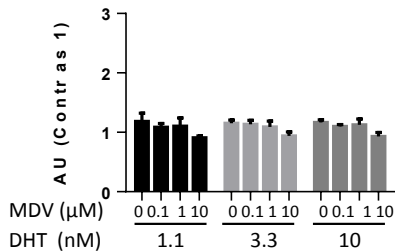


Figure 4: Proliferation of PC3 cells was assessed using varying concentrations of DHT and enzalutamide (MDV), and no significant changes in proliferation were observed.

Additionally, the Figure below shows results of our invasion and migration assays. Both invasion and migration were assessed in the setting of DHT and using DHT + enzalutamide. Invasion and migration of PC3 cells was not impacted by DHT or enzalutamide, as anticipated. LNCaP invasion was increased in the setting of DHT and subsequently inhibited by enzalutamide.

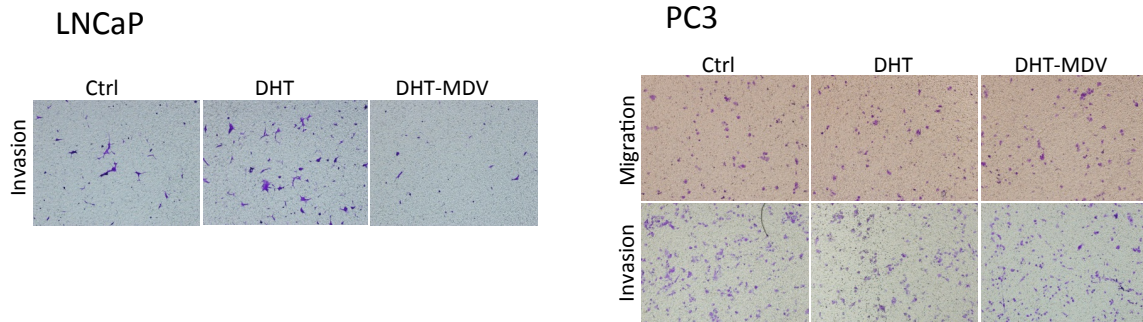


Figure 5: The left panel shows invasion assay results using LNCaP cells and demonstrated the increase in invasiveness associated with DHT, which was subsequently diminished with the addition of enzalutamide (MDV). PC3 cell invasion and migration was unchanged with DHT and with the addition of enzalutamide.

We assessed the impact of enzalutamide on MET expression in VCaP, LNCaP, and LNCaP-AR cells. As shown below, we found that MET expression is increased after exposure to the AR antagonist enzalutamide and when cells are cultured in charcoal stripped medium (to remove AR hormonal ligands). We elected to use the natural androgen dihydrotestosterone (DHT) rather than the synthetic androgen R1881 and determined that DHT decreases MET expression under charcoal stripped conditions in an enzalutamide sensitive manner.

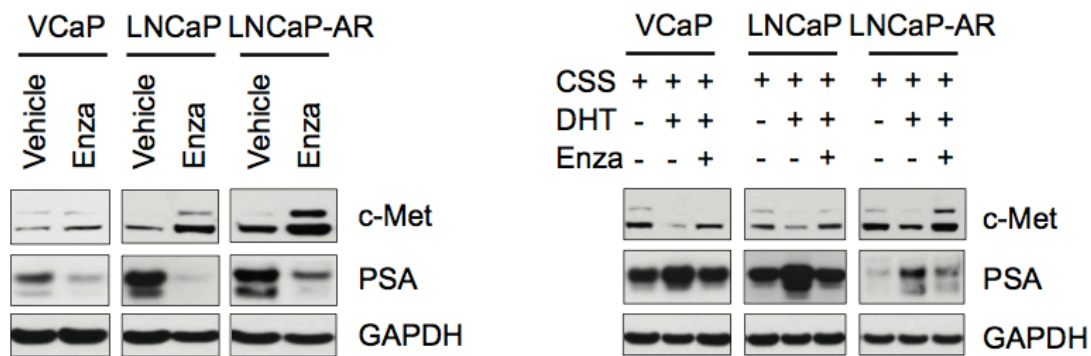


Figure 6 Blockade of androgen signaling increases MET expression *in vitro*. Left: AR-positive cells (VCaP, LNCaP and LNCaP-AR) were treated with 10 μM enzalutamide (Enza), followed by western blot to measure MET and PSA protein levels. Right: Indicated cells were treated with charcoal stripped serum (CSS) for 48 hours prior to stimulation with DHT (10nM) and enzalutamide treatments for another 24 hours. Expression of indicated proteins were assessed by western blot.

Subtask 3: Evaluate impact of *c-MET/HGF* axis inhibition using *c-MET* siRNA, anti-HGF neutralizing antibody, and exogenous HGF in PCa cell lines

In this subtask, we sought to credential MET as a potential therapeutic target in AR-negative prostate cancer. The figure below shows that in the presence of HGF, siRNA mediated MET knockdown in PC3 and DU145 cells (AR-/high MET expression) significantly reduced invasion and migration. However, MET knockdown did not affect cell proliferation in PC3 and DU145 cells. Likewise, in the presence of HGF, levels of both p-MET and p-ERK were substantially reduced after MET knockdown in both PC3 and DU145 cells.

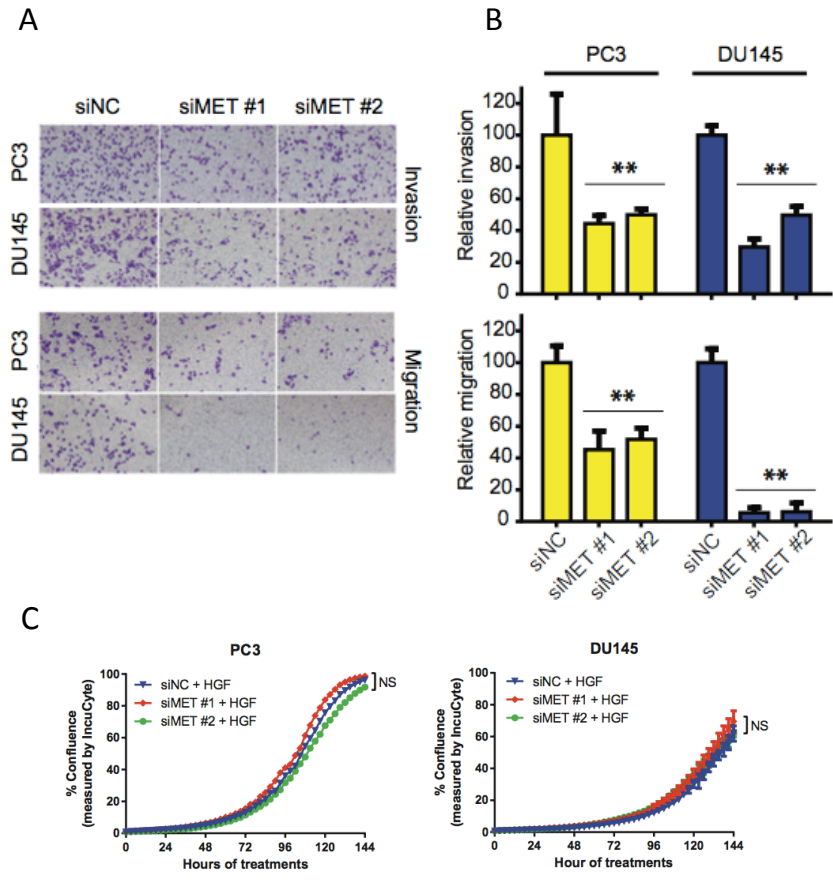


Figure 7: MET/HGF axis promotes invasion in prostate cancer *in vitro*. (A) Knockdown of MET in DU145 and PC3 (AR⁻) prostate cancer cells. Invasion or migration assays were done in the presence of MET ligand HGF for 24 hours. Representative pictures of crystal violet staining are shown in (A), and quantification is shown in (B). (C) Cell proliferation assays were analyzed by IncuCyte for PC3 and DU145 cells with indicated treatments, and results were shown in percentage of confluence.

We further assessed HGF mediated invasion in both DU145 and PC3 cells in response to cabozantinib and found significant inhibition of invasion.

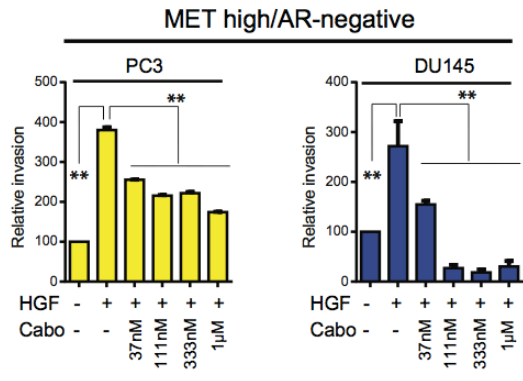


Figure 8: Invasion assays were performed in the presence of HGF and/or various treatment doses of cabozantinib (Cabo) in MET high/AR-negative prostate cancer cells for 24 hours.

Subtask 4: Assess whether *c-MET* siRNA, anti-HGF neutralizing antibody, and/or absence of HGF in the setting of enzalutamide administration recovers the effects of androgen suppression *in vitro*.

We next sought to determine if MET expression results in a phenotype in AR+ CRPC models that is sensitive to MET inhibition. In light of the encouraging results for cabozantinib above, we continued to utilize cabozantinib for inhibition of the HGF/MET signaling pathway. As shown below, MET overexpression promoted invasion in AR⁺ LNCaP cells (in the presence of androgen), which was sensitive to cabozantinib. We then found that p-Met is increased upon HGF stimulation in MET-transfected LNCaP cells cultured in the presence of androgen and showed that exposure to cabozantinib reverses this effect. Importantly, cabozantinib had no effect on VCaP or LNCaP cell invasion under normal culture conditions (androgen present). However, when AR signaling in LNCaP cells was inhibited through the use of charcoal stripped medium, HGF significantly increased invasion in a cabozantinib sensitive manner.

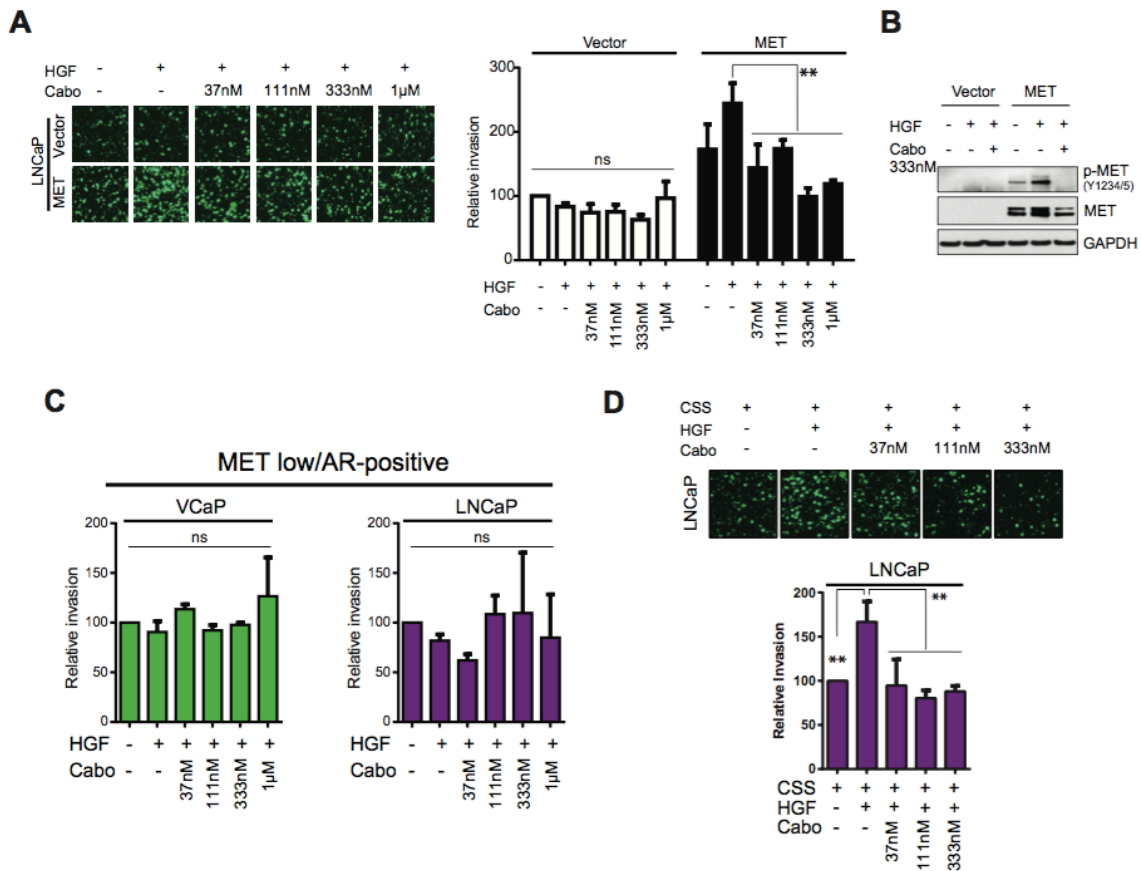


Figure 9: Elevated MET sensitizes AR⁺ prostate cancer to cabozantinib. **(A)** LNCaP (AR⁺) prostate cancer cells were stably transfected with either empty vector or MET, and invasion ability was assessed in the presence of HGF and/or various treatment doses of multityrosine kinase inhibitor cabozantinib (Cabo) as indicated. Left panel is representative pictures of indicated treatment results by fluorescent staining. Right panel is the quantification of invasion relative to vector control. **(B)** Selected treatment outcomes were measured by western blot for phospho-MET and total MET protein levels. **(C)** Invasion assays were performed in the presence of HGF and/or various treatment doses of cabozantinib (Cabo) in MET low/AR-positive prostate cancer cells for 48 hours. **(D)** LNCaP (AR⁺) prostate cancer cells were treated with charcoal stripped serum (CSS) for 48 hours prior to invasion assay under indicated conditions for another 48 hours.

Major Task 2: Isolation and characterization of CTCs and DTCs in patients with mCRPC on conventional ADT or enzalutamide

Major Task 3: Assess interplay between tumor HGF/c-MET pathway activity and response to abiraterone, and identify potential mechanisms of abiraterone resistance

While the key analyses on these two tasks will take place over the last 2 years of this grant, we have begun making progress enrolling patients for blood and bone marrow aspirates. We have obtained a total of 7 bone marrow aspirates from 5 patients. We have obtained 6 blood samples for CTC analyses from 4 patients on abiraterone and 3 blood samples from 2 patients on enzalutamide. We did have to go through a transition process due to the closure of the cabozantinib trial, leading to the revised third aim focusing on abiraterone. We now have the processes in place to continue to accrue patients in order to meet the planned enrollment numbers. Our preliminary data continue to support the ability to measure gene expression in CTCs, and we have evidence from qPCR performed on CTCs supporting the inverse relationship of AR and MET in men with advanced prostate cancer.

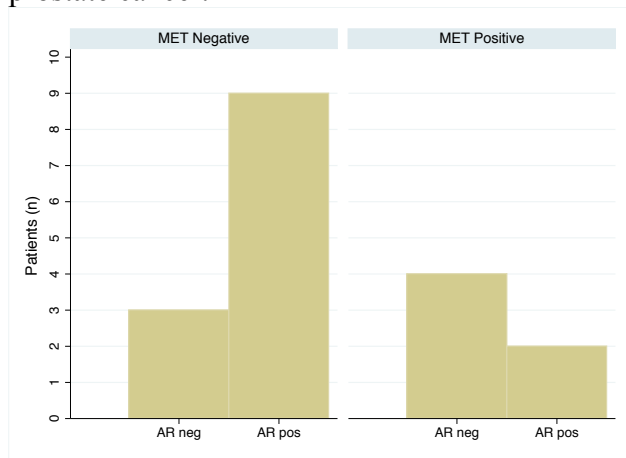


Figure 10: Preliminary data comparing MET and AR positivity from CTCs obtained from men with mCRPC. While not statistically significant, these data indicate a potential trend towards greater AR expression in MET- patients and lower AR expression in MET+ patients

Key Research Accomplishments

Research accomplishments:

- Identification of inverse relationship between AR and MET
- Nomination of MET as a key mechanism of resistance in AR-negative prostate cancer
- Delineating impact of inhibiting MET in AR- prostate cancer
- Support for potential dual targeting of AR and MET in AR+ prostate cancer

Training accomplishments:

- Participation in R01 boot camp program at University of Michigan for training in grant writing
- Attended and presented at 2015 Biomarkers and Diagnostics World Conference
- Continue as instructor/faculty of medical school/graduate courses

Conferences/journal clubs:

- Attend monthly prostate cancer seminars
- Meet with mentors (Drs. Taichman and Chinnaiyan) regularly to discuss research progress/career development
- Continue leadership/active roles in Urology Grand Rounds, GU tumor board, P01 collaborative conferences

Clinical responsibilities

- Continue Urology clinic
- Continue operative schedule

Professional accomplishments: Received NCCN Young Investigator Award and Society of Basic Urologic Research Young Investigator Award.

Impact

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

We have made significant progress to date honing in on a specific cancer promoting pathway, the HGF/MET axis, as a key mechanism of resistance to potent androgen signaling inhibition. We have shown that MET expression is only elevated and drives cancer phenotypes in cell line models of AR- prostate cancer. Importantly, MET activity in AR- prostate cancer appears to be sensitive to the MET inhibitor cabozantinib. Inhibition of AR activity in prostate cancer increases MET expression and renders cells susceptible to HGF stimulation, which can be blocked by inhibiting the MET pathway. From a broader standpoint, this work, our work provides a hypothesis why cabozantinib failed in setting of CRPC, since most of the patients in the pivotal phase 3 trial likely had intact androgen signaling.

2) What was the impact on other disciplines?

Nothing to report

3) What was the impact on technology transfer?

Nothing to report

4) What was the impact on society beyond science and technology?

Nothing to report

Changes/ Problems

1) Changes in approach and reasons for change

Aim 3 was changed during the past reporting period, with approval from the DOD. Due to the recently reported phase 3 trial of cabozantinib in advanced prostate cancer (COMET), which did not meet its primary endpoint, Aim 3 was changed to focus on abiraterone.

2) Actual or anticipated problems or delays and actions or plans to resolve them

Recruitment for bone marrow aspirates has been slower than anticipated. We have addressed this in a number of ways, including pre-screening clinics for eligible patients and expediting the time from consent to the aspirate procedure, making same day appointments possible in many cases. We have also added a \$100 honorarium for patients who enroll in order to increase participation.

3) Changes that had a significant impact on expenditures

Nothing to report

4) Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

IRB approved amendment for \$100 honorarium to patients consenting to bone marrow aspirate procedure.

5) Significant changes in use or care of human subjects

Nothing to report

6) Significant changes in use or care of vertebrate animals.

Nothing to report

7) Significant changes in use of biohazards and/or select agents

Nothing to report

Products

Submitted publications:

Qiao, Y, Feng, FY, Wang, Y, Cao, X, Han, S., Wilder-Romans, K, Keller, ET, Palapattu, GS, Taichman, RS, Alva, AS, Smith, DC, Tomlins, SA, Chinnaiyan, AM, Tomlins, SA, Morgan, TM: Mechanistic support for combined MET and AR blockade in castration resistant prostate cancer. *Cancer Research (In Revision)*: 2015.

Presentations/abstracts:

*Morgan, T.M., Miller, D., Ghani, K., Feng, F., Linsell, S., Gao, Y., Montie, J.: Contemporary use of adjuvant and salvage radiotherapy following radical prostatectomy in the state of Michigan, American Urological Association Annual Meeting, New Orleans, 2015.

*Jones, J.D., Soki, F.N., Roca, H., Thiele, S., Shiozawa, Y., Wang, Y. Morgan, T.M., Hofbauer, L.C., Pienta, K.J., McCauley, L.K.: Alternatively activated monocytes/macrophages support prostate cancer skeletal metastasis, America Society for Investigative Pathology (ASIP) (San Diego, CA), San Diego, CA, 2014.

*Pham, K., Odem-Davis, K., Jeldres, C., Porter, C., Wei, J.T., Morgan, T.M.: Poster #65 Transperineal template guided prostate biopsy in patients initiating active surveillance: A multi-institutional comparison, SUO, 2014.

Kozminski, M., Yoon, H.J., Palanisman, N. Cooney, K., Hussain, M., Alva, A. Morgan, T.M., Nagrath, S.: Application of a graphene oxide based microfluidic device (GO Chip) to prostate cancer circulating tumor cell capture and analysis, AACR, 2015.

*Patnaik, A., Swanson, K., Helenius, K., Thomas, T., Pyzer, A., Csizmadia, V., Signoretti, S., Morgan, T.M., Cantley, L.: Cabozantinib eradicates de novo castrate-resistant PTEN/p53 deficient murine prostate cancer via activation of neutrophil-mediated anti-tumor innate immunity, AACR, 2015.

*Koloff, Z.B., Hamstra, D.A., Wei, J.T., Montgomery, J.S., Tomlins, S.A., Wu, A.J., Morgan, T.M., Palapattu, G.S.: Impact of tertiary Gleason pattern 5 on prostate cancer aggressiveness: Lessons from a contemporary single institution radical prostatectomy series., ASCO GU, 2015.

Lu, L. Singhal, U., Skolarus, T.A., Palapattu, G.S., Montgomery, J.S., Weizer, A.Z., Morgan, T.M., Feng, F.: Prognostic significance of perineural invasion in localized prostate cancer., ASCO GU, 2015.

Zargar-Shoshtari, K., Zargar, H., Fairey, A.S., Mertens, L.S., Dinney, C.P., Mir, M.C., Morgan, T.M., Holzbeierlein, J.M.: A Multi-Institutional Analysis of Outcomes in Patients with Clinically Node Positive Urothelial Bladder Cancer Treated with Induction Chemotherapy and Radical Cystectomy, American Urological Association Annual

Meeting, 2015.

*Magers, M., Zhan, T., Udager, A., Hollenbeck, B., Wei, J.T., Miller, D., Morgan, T.M., Mehra, R.: Anterior prostate cancer: clinicopathologic characterization with biopsy correlation, American Urological Association Annual Meeting, 2015.

*Kozminski, M.A., Tomlins, S., Singhal, U., Lu, L., Skolarus, T.A., Palapattu, G.S., Morgan, T.M.: Defining adverse pathology for lower risk men undergoing radical prostatectomy, American Urological Association Annual Meeting, 2015.

Singhal, U., Lu, L., Skolarus, T., Palapattu, G., Montgomery, J., Weizer, A.Z., Morgan, T.M.: Effect of treatment delay on long-term outcomes in men undergoing radical prostatectomy for prostate cancer., American Urological Association Annual Meeting, 2015.

Montgomery, J., Davenport, M., Kunju, L., Meyer, C., Palapattu, G., Morgan, T.M., Piert, M.: Feasibility and initial results of 18fluorocholine PET/MRI/TRUS

Participants and Other Collaborating Organizations

1) What individuals have worked on the project?

Name	Yugang Wang
Project Role	Research scientist
Person month worked	2
Contribution	Work on cell line experiments and processing of clinical samples
Funding	Departmental start-up funding

Name	Amy Gursky
Project Role	Research coordinator
Person month worked	2
Contribution	Work on patient recruitment, sample acquisition, database tracking
Funding	Myriad Genetics, Inc

Name	Yuanyuan Qiao
Project Role	Post-doc
Person month worked	1
Contribution	Work on cell line experiments
Funding	Chinnaiyan lab, NIH

2) Changes in active other support:

There has been no change in the PI's effort on the current award. The PI has completed the funding period on the SPOR Career Development Award. New active other support is as follows:

5 U54 CA163124-02 (Taichman) 1.80 Calendar Months
 NIH
 Title: Mechanisms of Prostate Cancer Dormancy in the Bone Marrow Niche
 Role: Co-Investigator

Myriad Genetics 0.84 Calendar Months
 Title: Clinical Utility of CCP Score for Prognosis of Renal Cell Carcinoma
 Role: Principal Investigator

NCCN Young Investigator Award 0.60 Calendar Months
 Title: Tissue-based genomics for risk stratification in localized renal cell carcinoma
 Role: Principal Investigator

NIH P01 1.20 Calendar Months
 Title: The Biology of Prostate Cancer Skeletal Metastases
 Role: Co-Investigator