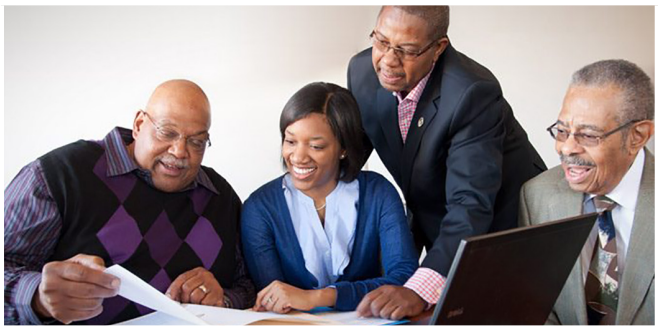
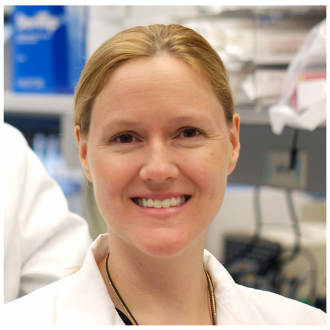
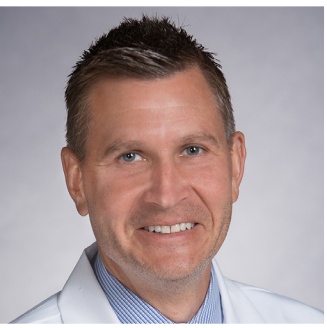
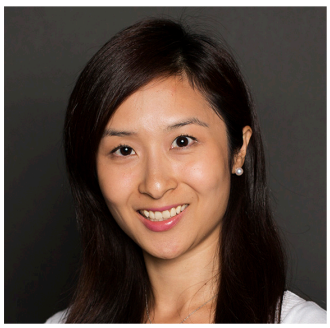
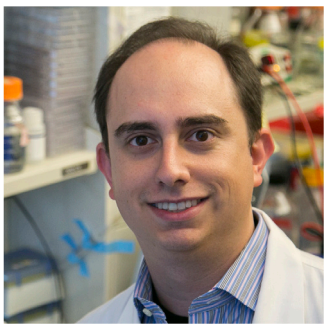
IMPaCT

Innovative Minds in Prostate Cancer Today

Young Investigators Meeting

August 4-5, 2016
Baltimore, Maryland

PROCEEDINGS

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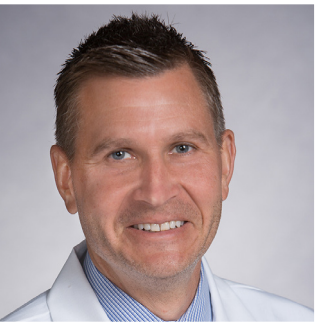
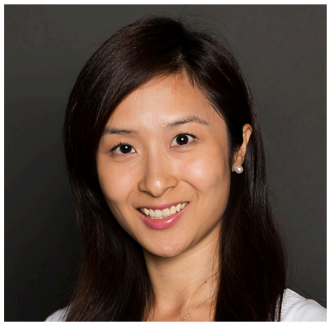
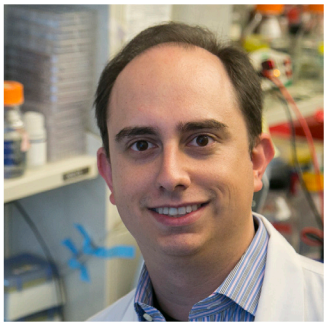
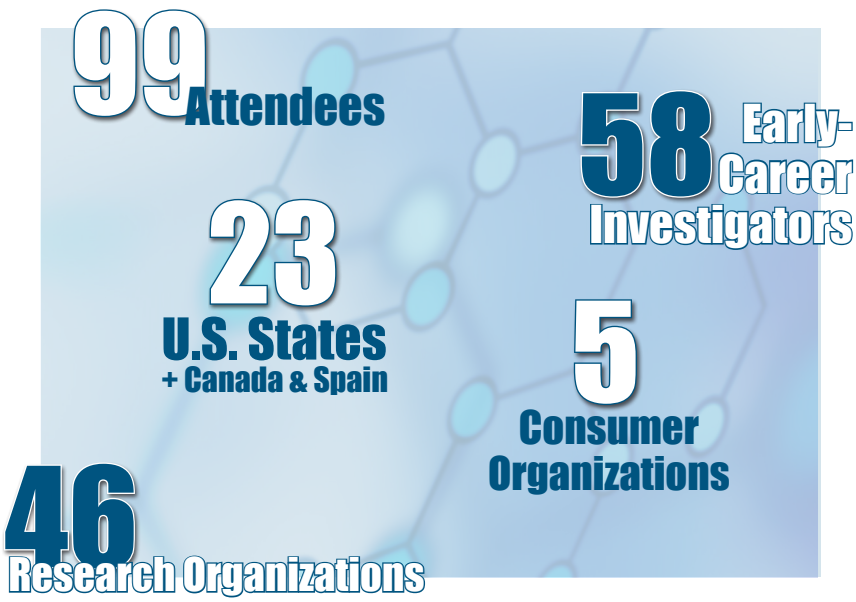




On behalf of the Prostate Cancer Research Program and the Technical Planning Committee, we would like to welcome you to IMPaCT 2016!

Since the Prostate Cancer Research Program's (PCRP) beginning in 1997, the program has been committed to supporting early-career investigators who share our commitment to conquering prostate cancer. IMPaCT 2016 is focused on showcasing the incredible research being done by 58 PCRP-funded early-career investigators with current PCRP Physician Research Training, Idea Development, or Health Disparity Research Awards, as well as keynote presentations by leading prostate cancer experts. Plenary sessions focused on important research topic areas will emphasize the state of the science from invited keynote speakers, ongoing research efforts from PCRP-funded investigators, and active group discussions by meeting attendees. Other special sessions will focus on providing information to assist attendees with furthering their research careers, as well as a look toward the future of cancer research and healthcare. In addition to providing investigators the opportunity to highlight their research, it is our intent that this meeting will provide attendees networking opportunities to share ideas and form new collaborations to facilitate research aimed at addressing critical issues in prostate cancer.

We look forward to a great meeting and hope you all enjoy it as well!



IMPaCT 2016 Planning Committee and PCRP Programmatic Panel

CDMRP Planning Committee

Lymor Barnhard, Ph.D.
PCRP Science Officer,
IMPaCT 2016 Conference Chair

Melissa Cunningham, Ph.D.
PCRP Program Manager

Jessica Mann, Ph.D.
IMPaCT 2016 Conference Coordinator

Bridget McKenzie, M.S.
IMPaCT 2016 Planning Support

Alyssa Moore
IMPaCT 2016 Planning Support

Technical Planning Committee

Adam Dicker, M.D., Ph.D.
Thomas Jefferson University

Peter Choyke, M.D., F.A.C.R.
National Cancer Institute

James Kiefert, Ed.D.
Us TOO International

Natasha Kyprianou, Ph.D.
University of Kentucky

Timothy McDonnell, M.D., Ph.D.
University of Texas M.D. Anderson Cancer Center

Joel Nowak, M.S.W., M.A.
MaleCare, Inc.

Howard R. Soule, Ph.D.
Prostate Cancer Foundation

Donald Tindall, Ph.D.
Formerly Mayo Clinic, Retired

FY16 Programmatic Panel

Adam Dicker, M.D., Ph.D. (Chair)
Thomas Jefferson University

Timothy McDonnell, M.D., Ph.D. (Chair Emeritus)
University of Texas M.D. Anderson Cancer Center

Philip M. Arlen, M.D.
Precision Biologics, Inc.

Tarek Bismar, M.D.
University of Calgary

Peter Choyke, M.D., F.A.C.R.
National Cancer Institute

William Dahut, M.D.
National Cancer Institute

James Kiefert, Ed.D.
Us TOO International

Natasha Kyprianou, Ph.D.
University of Kentucky

Daniel Lin, M.D.
University of Washington

Lorelei Mucci, Sc.D., M.P.H.
Harvard School of Public Health

Joel Nowak, M.S.W., M.A.
MaleCare, Inc.

Kenneth Pienta, M.D.
Johns Hopkins University School of Medicine

David Quinn, M.B.B.S., Ph.D., F.R.A.C.P., F.A.C.P.
University of Southern California

Marianne Sadar, Ph.D. (FY17 Chair-Elect)
University of British Columbia

Westley Sholes, M.P.A.
California Prostate Cancer Coalition

Virgil Simons, M.P.A.
The Prostate Net

Howard Soule, Ph.D.
Prostate Cancer Foundation

M. Albert Thomas, Ph.D.
University of California, Los Angeles

Invited Speakers and Special Guests

IMPaCT 2016 Invited Speakers and Special Guests

Charles Bieberich, Ph.D.
University of Maryland, Baltimore County
Local Prostate Cancer Research Expert

Peter Carroll, M.D., M.P.H.
University of California – San Francisco
Keynote Speaker, Therapy and Survivorship Session

Peter Choyke, M.D., F.A.C.R.*
National Cancer Institute
Keynote Speaker, Imaging Session

Angelo DeMarzo, M.D., Ph.D.
Johns Hopkins University School of Medicine
Local Prostate Cancer Research Expert

Adam Dicker, M.D., Ph.D.*
Thomas Jefferson University
Moment of Silence and Meeting Purpose and Goals Speaker

Andrew Hruszkewycz, M.D. Ph.D.
National Cancer Institute
Local Prostate Cancer Research Expert

Karen Knudsen, Ph.D.
Sidney Kimmel Cancer Center at Thomas Jefferson University
Keynote Speaker, Tumor Biology and Genetics Session

Jerry Lee, Ph.D.
National Cancer Institute
Keynote Speaker, Working Lunch – Looking to the Future

Christopher Logothetis, M.D.
University of Texas M.D. Anderson Cancer Center
Keynote Speaker, Mechanisms of Resistance Session

Ravi Madan, M.D.
National Cancer Institute
Local Prostate Cancer Research Expert

Timothy McDonnell, M.D., Ph.D.*
University of Texas M.D. Anderson Cancer Center
Overview of Day 2 Speaker

Folakemi Odedina, Ph.D.
University of Florida
Keynote Speaker, Health Disparity Session

Elizabeth Platz, Sc.D., M.P.H.
Johns Hopkins Bloomberg School of Public Health
Programmatic Panel Member, Working Lunch – Panel Discussion

Ganesh Raj, M.D. Ph.D.
UT Southwestern Medical Center at Dallas
Peer Review Panel Member, Working Lunch – Panel Discussion

COL Inger Rosner, M.D.
Walter Reed National Military Medical Center
Local Prostate Cancer Research Expert

Howard Scher, M.D.
Memorial Sloan Kettering Cancer Center
Keynote Speaker, Biomarkers Session

Westley Sholes, M.P.A.*
California Prostate Cancer Coalition
Moment of Silence Speaker

Virgil Simons, M.P.A.*
The Prostate Net
Programmatic Panel Member, Working Lunch – Panel Discussion

Shiv Srivastava, Ph.D.
Uniformed Services University of the Health Sciences
Local Prostate Cancer Research Expert

COL Paul Taylor, M.P.A.
ZERO
Peer Review Panel Member, Working Lunch – Panel Discussion

**FY16 PCRP Programmatic Panel Member*

Agenda

Day 1 – August 3	Fitzgerald Ballroom
4:00 – 6:00 p.m.	Meeting Registration and Attendee Poster Set-Up

Day 2 – August 4	Fitzgerald Ballroom
7:00 – 8:00 a.m.	Meeting Registration and Attendee Poster Set-Up
8:00 – 8:45 a.m.	***Opening Session*** *Welcome* – Col. Wanda Salzer, CDMRP Director *Introductions* – Melissa Cunningham, PCRP Program Manager *Moment of Silence* – Adam Dicker *Meeting Purpose and Goals* – Adam Dicker
8:45 – 10:30 a.m.	***Session 1 – Tumor Biology and Genetics*** *Moderator* – Donald Tindall *Keynote* – Karen Knudsen *Targeting DNA Repair-Hormone Crosstalk in Advanced Prostate Cancer* Early Career Investigators – David VanderWeele, Travis Jerde, Yue Wu Moderated Discussion
10:30 – 10:45 a.m.	Break
10:45 a.m. – 12:15 p.m.	***Session 2 – Therapy and Survivorship*** *Moderator* – James Kiefert *Keynote* – Peter Carroll *Prostate Cancer 2016: Technology, Good Sense or Both?* Early Career Investigators – Nicholas Mitsiades, Xin Lu, Travis Young Moderated Discussion
12:30 – 2:00 p.m.	***Working Lunch – Panel Discussion*** *Moderator* – Virgil Simons *Perspectives from PCRP peer and programmatic review panel members to maximize success* Programmatic Panel – Elizabeth Platz, Virgil Simons Peer Review Panel – Ganesh Raj, Paul Taylor
2:00 – 2:15 p.m.	Break
2:15 – 3:45 p.m.	***Session 3 – Mechanisms of Resistance*** *Moderator* – Natasha Kyprianou *Keynote* – Christopher Logothetis *Monitoring the Emergence of Resistance in Prostate Cancer to Inform a Marker-Driven Treatment Strategy* Early Career Investigators – Hung-Ming Lam, Atish Choudhury, Himisha Beltran Moderated Discussion
4:00 – 6:00 p.m.	Poster Session

Agenda (cont.)

Day 3 – August 5	Fitzgerald Ballroom
7:00 – 8:00 a.m.	Registration
8:00 – 8:25 a.m.	***Opening Session*** *Welcome* – Melissa Cunningham *Moment of Silence* – Westley Sholes *Overview of Day 2* – Timothy McDonnell
8:25 – 10:00 a.m.	***Session 4 – Biomarkers*** *Moderator* – Timothy McDonnell *Keynote* – Howard Scher *Prostate Cancer Biomarker Development: Validation and Utility Are All That Really Matter* Early Career Investigators – David Miyamoto, Tamara Lotan Moderated Discussion
10:00 – 10:15 a.m.	Break
10:15 – 11:50 a.m.	***Session 5 – Health Disparity*** *Moderator* – Howard Soule *Keynote* – Folakemi Odedina *Science of Prostate Cancer Health Disparities: Progress, Challenges, and Future Direction* Early Career Investigators – Franklin Huang, Karen Sfanos Moderated Discussion
11:50 a.m. – 1:15 p.m.	***Working Lunch – Looking to the Future*** *Moderator* – Joel Nowak *Keynote* – Jerry S.H. Lee *Advancing Innovation and Convergence in Cancer Research*
1:15 – 2:45 p.m.	***Session 6 – Imaging*** *Moderator/Keynote* – Peter Choyke *Progress and Opportunities in Prostate Cancer Imaging* Early Career Investigators – David Karow, Mekhail Anwar Moderated Discussion
2:50 – 3:00 p.m.	**Closing Session**

Abstracts

High-Grade Prostate Cancer Characterization Using Fractional Order Calculus Diffusion-Weighted MRI

M Abern, B Caldwell, V Macias, W Mar, K Xie, A Kajdacsy-Balla, R Magin, J Zhou, and P Gann

The University of Illinois at Chicago

Background: Accurate detection of aggressive prostate cancer (PCa) using existing clinical prediction tools is a challenge. Prostate MRI is a promising technology for PCa detection and characterization. However, its accuracy has been sub-optimal, especially in the setting of benign prostate inflammation or hyperplasia. We have developed a more sophisticated computational model of diffusion-weighted MRI (FROC-DWI) that produces quantitative information regarding tissue architecture in-vivo. We hypothesize that the use of FROC-DWI in men with clinical suspicion for PCa will differentiate high-grade PCa from indolent PCa and benign prostate pathology and therefore improve biopsy detection of aggressive PCa.

Objectives: Generate a quantitative FROC-DWI signature that differentiates high-grade PCa from low-grade PCa and benign prostate tissues

Methods: A cohort of patients that underwent in-vivo 3T multiparametric MRI (mpMRI) including FROC-DWI prior to radical prostatectomy (RP) was identified. Whole embedded RP sections were digitized at 20X magnification using a digital scanner (Scanscope CS, Aperio Technologies), and all tumor foci were annotated by Gleason pattern by a board-certified genitourinary pathologist (Aperio ImageScope 11.2.0.780, Leica Biosystems). MRI DWI images were acquired at multiple B values (50, 500, 1000, 1500, 2000). On each image, the prostatic capsule and regions of interest were annotated by a board-certified genitourinary radiologist with extensive MRI expertise (Matlab R2015b, MathWorks). The quantitative FROC-DWI parameters B, μ, Δ were calculated on a voxel basis. A zonal scheme was devised for matching the MRI to the RP sections based on the orientation to the urethra (anterior/posterior, left/right, level from base to apex).

Results: Nine cases with whole embedded RP sections and complete mpMRI with DWI collected at the desired B values were identified. We have created an image resolution matching technique for the annotated RP histology to match the voxel size of the mpMRI. Several quantitative models of the FROC parameters will be fit with the presence of Gleason pattern ≥ 4 as the gold standard. This model will be validated prospectively using a new cohort of patients.

Conclusions: Matching in-vivo mpMRI to whole embedded RP sections is a challenging technical process given the vast differences in image resolution and scale. This process is critical for evaluating new imaging modalities.

Impact Statement: Development of a non-invasive quantitative imaging biomarker for high-grade PCa will be useful for improving biopsy yield and grade accuracy, accurately identify men appropriate for surveillance versus curative therapy, and reduce biopsies needed on surveillance of indolent disease.

Real-Time Intraoperative Fluorescent Imaging for Microscopic Residual Disease in Prostate Cancer with a 1-mm Thin Microfabricated Planar Optics-Free Imaging Array

M Anwar[1], E Papageorgiou[2], B Boser[2], and C Park[1]

[1] University of California, San Francisco
[2] University of California, Berkeley

Background: Patients undergoing radical prostatectomy (RP) for prostate cancer continue to suffer from increased cancer recurrence due to the inability to visualize and remove microscopic residual disease (MRD) during surgery. Despite advancements in targeted imaging agents and intraoperative guidance, the imagers themselves remain the limiting factor for intraoperative imaging: the size and bulk from complex optics prevent thorough imaging of the entire prostate bed. We hypothesize that by labeling prostate cancer cells *in vivo* using a systemically injected fluorescently labeled antibody targeting prostate specific membrane antigen (PSMA), a sub-millimeter thin planar fluorescent imaging array "coating" surgical instrumentation can transform the surgical tool itself into an highly sensitive imager, identifying areas for immediate re-resection or focal post-op therapy. Accurate intraoperative assessment of the tumor bed also solves two problems inherent to post-op evaluation: co-registration and sampling error. Here we demonstrate a 1-mm thin planar optics-free microfabricated fluorescent imager for prostate cancer.

Methods: A 1-mm thick, 2.5-mm x 2.5-mm 1024-pixel microfabricated fluorescent imager was designed and fabricated. On-chip micro-collimators (2.4 µm x 6.8 µm) replace traditional optics, deblurring the image, and an integrated filter blocks background light. Spatial resolution and antibody sensitivity was established by imaging a microfabricated stencil pattern (1951 USAF) overlaying fluorescent antibodies. *In vitro* (LNCaP PSMA+, PC3 PSMA-) and *in vivo* mice (22RV1 PSMA+, PC3 PSMA-) models of prostate cancer were tested with the humanized anti-PSMA antibody, J591. A model of MRD was creating using 3D cultures of LNCaP cells in matrigel labeled with J591 and anti-Human qDot 705.

Results: Previously, we described our optics-free sensor design demonstrating sensitivity of <250 photons/µm^2/sec. On-chip microcollimators, increasing spatial resolution by 2X, combined with placing the sensor <500 µm from the cell surface, eliminated the need for optics. Fluorescent antibodies (10,000 antibodies/µm^2) patterned in 125-µm features were successfully imaged. *In vitro* and *in vivo* models of PMSA-overexpressing prostate cancer demonstrate a required sensitivity of 30,000 to 50,000 antibody cells, detectable by our device. A focus of 200 fluorescently labeled cells in 3D culture was successfully imaged.

Conclusions: Leveraging a targeted antibody to prostate cancer, used in human clinical studies, we are developing an ultra-thin planar fluorescent imaging platform for intraoperative visualization of MRD with a form-factor enabling placement within the tumor bed during surgery. Our current prototype is capable of 125-µm, 200-cell resolution using a 1-mm optics-free planar fluorescent imager. Future efforts will further thin the imager and optical filter, with total thickness ~300 um, increasing sensitivity and spatial resolution by 6X.

Impact Statement: MRD occurs in 21%, 29%, 53% of patients with T1, T2, and T3 tumors, respectively, resulting in a 3.7X greater risk of progression. While randomized trials have shown a clinical benefit of empiric treatment post-operative RT for MRD, 60% of patients recur in 4 years. Accurate intraoperative identification of MRD will enable complete resection or ultra-focal post-op RT, improving patient outcomes.

Divergent Clonal Evolution of Castration Resistant Neuroendocrine Prostate Cancer

H Beltran[1], D Prandi[2], JM Mosquera[1], M Benelli[2], L Puca[1], J Cyrta[1], C Marotz[1], E Giannopoulou[1], DM Nanus[1], ST Tagawa[1], E Van Allen[3], O Elemento[1], A Sboner[1], LA Garraway[3], MA Rubin[1], and F Demichelis[1,2]

[1] Weill Cornell Medicine
[2] University of Trento, Trento, Italy
[3] The Broad Institute of MIT and Harvard

Background: The mainstay of therapy for patients with metastatic prostate cancer is hormonal therapy targeting the androgen receptor (AR). Enzalutamide and abiraterone are potent AR-targeted therapies approved for the treatment of men with castration-resistant prostate cancer. Although the use of these agents improves outcomes, most patients ultimately develop treatment resistance. Predictive biomarkers that help distinguish responders from nonresponders before starting the next line of hormonal therapy are needed. A subset of resistant tumors lose AR expression and develop small cell/neuroendocrine features on metastatic biopsy (CRPC-NE), likely reflecting an epithelial plasticity that enables tumor adaptation. Prognosis of CRPC-NE is poor due to late recognition and lack of effective systemic therapies.

Methods: We interrogated 114 metastatic tumors from 81 individuals, including 30 patients with CRPC-NE. Serial or synchronous samples were included to characterize heterogeneity and the transition from prostate adenocarcinoma to CRPC-NE. Whole exome sequencing and analysis of clonality and allele-specific copy number were performed. RNA-seq and quantitative assessment of AR signaling genes together with single-cytosine-resolution DNA methylation analysis were evaluated in the context of genomic changes.

Results: The mutational and copy number landscape of metastatic CRPC-NE and castration resistant prostate adenocarcinoma (CRPC-Adeno) showed significant overlap and did not differ significantly by rate of non-synonymous mutations or copy number burden; polyploidy was frequently detected together with common allelic imbalances. Comparative analysis at the DNA and mRNA level identified significant decrease in AR signaling and enrichment of copy number losses in CRPC-NE (including RB1, TP53, and other tumor suppressors) and focal high level AR amplification in CRPC-Adeno (p=0.0007). There were marked epigenetic differences between the two subgroups including epigenetic dysregulation of neuronal, EMT, and developmental pathways in CRPC-NE. DNA allele specific analysis of multi-sample cases from individual patients supported an evolutionary model most consistent with divergent clonal evolution of CRPC-NE from a CRPC-Adeno precursor. Through integrative analyses, we identified a molecular classifier of CRPC-NE and validated the classifier in independent cohorts (n=683).

Conclusions: This is the largest study to date focused on the molecular landscape of the CRPC-NE resistance phenotype. CRPC-NE is characterized by distinct genomic and epigenomic alterations. A subgroup of CRPC-Adeno demonstrates lower AR signaling and molecular overlap with CRPC-NE. Overall, this study supports the emergence of an alternative, “AR-indifferent” cell state through divergent clonal evolution as a mechanism of treatment resistance in metastatic prostate cancer.

Impact Statement: Identification of patients with advanced prostate cancer that are less likely to respond to subsequent AR-directed therapies improves patient selection for systemic therapies and prognostication. This study provides new insights into mechanisms driving treatment resistance and has implications for early detection of CRPC-NE (potentially non-invasively using ctDNA) and identifies new therapeutic targets.

Loss of Function Mutations in ETS2 Repressor Factor, ERF, Reveal a Balance between Positive and Negative ETS Factors Controlling Prostate Oncogenesis

R Bose, WR Karthaus, J Armenia, W Abida, PJ Iaquinta, J Wongvipat, EV Wasmuth, N Shah, P Sullivan, N Schultz, and CL Sawyers

Memorial Sloan Kettering Cancer Center

Background: Half of prostate cancers are caused by a gene-fusion that enables androgens to drive expression of the normally silent ETS transcription factor ERG in luminal prostate cells. Recent prostate cancer genomic landscape studies have reported rare but recurrent point mutations in the ETS repressor ERF. The objective of this study is to understand the significance of these mutations and to understand how ERF may promote prostate cancer.

Methods: ERF expression was inhibited using CRISPR and shRNA technology and the androgen receptor cistrome and transcriptome was probed via ChIP-seq and RNA-seq respectively, in normal prostate organoids, patient-derived organoids, as well as existing TMPRSS2-ERG positive models.

Results: Here we show these ERF mutations cause decreased protein stability and ERF mutant tumors are mostly exclusive from those with ERG fusions. ERF loss recapitulates the morphologic and phenotypic features of ERG gain in primary mouse prostate tissue, including expansion of the androgen receptor repertoire, and ERF has tumor suppressor activity in the same genetic background of Pten loss that yields oncogenic activity by ERG. Furthermore, in a human prostate cancer model of ERG gain and wild-type ERF, ChIP-seq studies indicate that ERG inhibits the ability of ERF to bind DNA at consensus ETS sites. Consistent with a competition model, ERF loss rescues ERG-positive prostate cancer cells from ERG dependency.

Conclusions: Collectively, these data provide evidence that the oncogenicity of ERG is mediated, in part, by displacement of ERF and raises the larger question of whether other gain-of-function oncogenic transcription factors might also inactivate endogenous tumor suppressors.

Impact Statement: Further work needs to be performed, but ERF may serve as a predictive marker of response to anti-androgen therapy.

Downregulation of MEK5 Sensitizes Human Prostate Cancer Cells to Ionizing Radiation

C Broustas

Columbia University

Background: Tumor cell resistance to ionizing radiation (IR) poses a major obstacle in prostate cancer therapy. Mitogen/extracellular signal-regulated kinase kinase-5 (MEK5) belongs to the family of MAP kinases. It is activated by the upstream kinases MEKK2 and MEKK3. MEK5, in turn, phosphorylates and activates extracellular signal-regulated kinase 5 (ERK5) at Thr218/Tyr220. MEK5/ERK5 pathway plays a pivotal role in tumor initiation and progression, including prostate cancer. MEK5 protein is overexpressed in prostate cancer cells compared with normal cells and MEK5 levels are correlated with prostate cancer metastasis. This study explores the hypothesis that MEK5 is a contributing factor to the response of prostate cancer cells to IR and seeks to elucidate the mechanism by which MEK5 affects radioresistance.

Methods: Castration-resistant DU145, PC3, and PC3MM2, as well as androgen-dependent LNCaP prostate cancer cells were treated with MEK5 short interfering (si) RNA alone or in combination with γ-rays. Clonogenic survival assays, cell cycle analysis, immunofluorescence and immunoblotting were performed to assess cell proliferation, survival, cell cycle progression and DNA damage response.

Results: We examined MEK5/ERK5 pathway activation in response to IR in prostate cancer cells transiently expressing *Luciferase* (control) or *MEK5* siRNAs. Control cells with normal levels of MEK5 showed an increase in phospho-ERK5 levels at 5 and 15 min post-IR, diminishing at later time points. In addition, we discovered that AKT activation after 4 Gy IR was dependent on the presence of MEK5. AKT phosphorylation at Ser473, which is considered a marker of AKT activation, was increased reaching maximal levels at 30 min post-IR. In contrast, when MEK5 was downregulated by MEK5-specific siRNAs, AKT activation was severely impaired. Moreover, MEK5 silencing had an impact on the DNA damage response pathway. Specifically, MEK5 knockdown, combined with IR, resulted in significantly higher phospho-CHK2 (Thr68) levels 30 min after irradiation compared with irradiated cells with endogenous levels of MEK5. Additionally, increased levels of phospho-CHK2 persisted for at least 8 h post-irradiation, whereas the phospho-CHK2 signal returned to near basal levels by 3 h in control cells. On the other hand, CHK1 phosphorylation at Ser345 and activation in response to IR was elevated in MEK5 control cells 30 min post-irradiation compared with MEK5 knockdown cells. Finally, MEK5 depletion by two non-overlapping siRNAs sensitized prostate cancer cells to IR as determined by clonogenic survival assay. Short-term targeting of MEK5 in combination with IR led to approximately 70% reduction in prostate cancer cell proliferation 6 days post-irradiation.

Conclusions: These data indicate that MEK5 knockdown radiosensitizes prostate cancer cells. In response to IR, MEK5 controls activation of AKT, a kinase involved in radioresistance, as well as DNA damage response by regulating activation of CHK1/2 kinases. Ongoing studies focus on determining the contribution of AKT and CHK1/2 kinases and their downstream effectors to MEK5-dependent radioresistance.

Impact Statement: This study focuses on mechanisms of resistance to radiotherapy for patients with localized prostate cancer. Downregulation of MEK5 can selectively radiosensitize prostate tumors, while sparing normal tissue, thus improving survival of cancer patients.

Targeting Cancer Cell Quiescence in Prostate Cancer

LA Buttitta, D Sun, L Kana, Y Jung, K Yumoto, and R Taichman

University of Michigan

Background: Prostate cancer is characterized by the early spreading of a small number of tumor cells to other tissues, termed disseminated tumor cells (DTCs). DTCs in the bone are problematic because a percentage of patients will later develop recurrent cancer with significant bone metastases from these cells, which are often resistant to treatment. Understanding how DTCs reside undetected in the marrow for long periods of time, and finding ways to eliminate or minimize them, is an important issue in prostate cancer research and treatment.

We hypothesize that DTCs enter a state of cellular quiescence or G_0 in the bone marrow, which renders them insensitive to chemotherapies designed to target actively proliferating cancer cells. Our goal is to test whether the disruption of DTC quiescence could reduce tumor burden and improve treatment outcomes by sensitizing quiescent cancer cells to current chemotherapies.

Methods: To address this hypothesis we are using two unique tools: (1) We use novel *in vivo* fluorescent cell cycle reporters to examine the cell cycle state of disseminated prostate cancer cells during disease progression. (2) We use a validated mouse xenograft prostate cancer model that recapitulates key features of prostate cancer, including metastasis, dormancy, and recurrence.

Results: We generated two prostate cancer cell lines carrying *in vivo* fluorescent cell cycle sensors that together label cell cycle transitions including quiescence. We verified that these sensors accurately indicate the cell cycle state of the cells without disrupting their dynamics and that these cell lines respond to signals from the bone marrow, thought to contribute to dormancy. In our xenograft model, we confirmed that these cell lines metastasize to the bone marrow and can be recovered for further analysis using flow cytometry. In the immediate future, we will follow and quantify the cell cycle status of these cancer cells during tumor dormancy and recurrence. We will then use our model to test whether current chemotherapies fail to eliminate or, possibly, even enrich for cancer cell quiescence in the bone. In the longer term, we will disrupt genes that promote cancer cell quiescence to test whether this sensitizes prostate cancer cells to chemotherapies to limit tumor recurrence.

Conclusions: We used in vivo fluorescent cell cycle reporters to monitor the cell cycle dynamics of prostate cancer cells during disease progression. We have confirmed that signals thought to contribute to tumor dormancy in the bone marrow may do so, in part, by inhibiting prostate cancer cell cycle progression and promoting cellular quiescence.

Impact Statement: A problem with many current cancer treatments is a failure to eliminate quiescent or nonproliferating cancer cells. This is because most chemotherapies target actively dividing cells, leaving behind quiescent cells – thought to seed new, often chemo-resistant cancers at a later date. Thus, there is a need for new approaches that can identify and, either, target the quiescent cancer cells or somehow disrupt their quiescence to eliminate them in combination with current chemotherapies. We are pursuing a research direction that tackles this unmet need by doing precisely the opposite of most current cancer research approaches – *finding ways to monitor and disrupt the quiescence of cancer cells.* This directly addresses the challenge of determining mechanisms of chemotherapy resistance in metastatic prostate cancer.

Metabolism of Saturated Fatty Acids Accelerates Src-mediated Prostate Tumor Progression

H Cai[1], S Kim[1], X Yang[1], Q Li[1], M Wu[1], L Costyn[1], Z Beharry[2], A Bielawska[3], and MG Bartlett[1]

[1] University of Georgia
[2] Florida Gulf Coast University
[3] Medical University of South Carolina

Background: Numerous epidemiological studies suggest that the dietary saturated fatty acids (FAs), myristic acid (MA) and palmitic acid (PA), significantly increase the risk of prostate cancer-specific mortality among patients diagnosed with localized disease. However, the molecular mechanisms explaining these effects of dietary fatty acids in promoting prostate cancer progression remain unknown.

The expression and activity of Src family kinases (SFKs) are frequently increased in advanced prostate cancer. Particularly, overexpression of Src kinase promotes castration resistance in prostate cancer. Src kinase is modified by myristoylation solely at the N-terminus, which determines its kinase activity and oncogenic potential.

Objectives: **(**1) To examine if metabolism of exogenous saturated FAs contributes to an increase of Src activity and its mediated tumor progression; (2) To offer a mechanism of the correlation between exogenous FAs in a high-fat diet with cancer progression; (3) To provide evidence that diet is a potential intervention for reducing the risk of advanced prostate cancer.

Methods: Src-mediated prostate cancer models were previously established to recapitulate activated Src *in vivo*. Host mice carrying Src-induced prostate tumors were fed different fat diets. Additionally, a panel of exogenous saturated and unsaturated FAs was examined for their regulation on Src-mediated signaling *in vitro*. To examine the metabolism of FAs, acyl-CoAs and ceramide biosynthesis were examined by LC-MS/MS and lipidomics analysis. Myristoylation of Src kinase was detected by the Click chemistry.

Results: We demonstrate that exogenous FAs from a high-fat diet accelerate constitutively active Src kinase or c-Src+AR-induced prostate tumor progression *in vivo*. Particularly, exogenous MA or PA predominantly promotes Src-mediated oncogenic signaling. Next, metabolism of exogenous MA and PA significantly elevated the levels of myristoyl- and palmitoyl-CoA and changed the composition of ceramides, elevation of long-chain C16:0 ceramide in particular. Targeting C16:0 ceramide synthesis by knockdown of Ceramide synthase 6 significantly inhibited Src-mediated oncogenic signaling. Additionally, metabolism of exogenous MA increased biosynthesis of myristoylated Src kinase. The combination of these dual effects increased the partition of Src kinase toward detergent-resistant membranes. Finally, Src kinase-mediated dietary FAs accelerated prostate tumor growth. Targeting myristoylation of Src kinase, which is required for its association at the cellular membrane, blocked high-fat diet-accelerated tumorigenesis *in vivo*.

Conclusions: Metabolism of dietary saturated FAs accelerates Src-mediated tumor progression by two complementary mechanisms including alteration of ceramide compositions and elevation of Src kinase myristoylation. Our study suggests that targeting myristoylation of Src kinase is a potential therapeutic approach in inhibiting high-fat diet-accelerated and Src-mediated prostate tumor progression.

Impact Statement: This work provides a mechanistic understanding of how diet choice can be used as a strategy in modulating tumor progression of cancer patients, and a therapeutic approach for inhibiting cancer progression. Our results suggest that, for prostate cancer survivors, a diet low in saturated fat may be beneficial in reducing the risk of Src-mediated aggressive prostate cancer.

Targeting Hedgehog Protein Biosynthesis with Small Molecules; A Search for Inhibitors and Unanticipated Discovery of Anti-Androgen Activators

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Background: Despite earlier detection and improved therapies, prostate cancer (PCa) remains the second leading cause of cancer-related death among men in the US. Our goal is to discover a novel class of molecules that block biosynthesis of hedgehog proteins, potent cell/cell signaling factors that stimulate PCa progression. Integral to this effort is an optical assay of hedgehog biosynthesis suitable for high throughput screening.

Methods: I will present the development of a novel hedgehog biosynthesis assay that uses FRET (Förster resonance energy transfer). Reactions can be measured accurately in multi-well plates to sift large libraries of small molecules at high throughput for potential inhibitors.

Results: Our studies show successful application of the FRET assay to hedgehog inhibitor discovery with the identification of covalent and noncovalent antagonists. In addition, I will describe how the FRET assay was used to study off-target interactions of hedgehog proteins with two PCa anti-androgens. Rather than inhibit, interactions of the anti-androgens with hedgehog leads to biosynthesis activation.

Conclusions: The unique chemistry involved in hedgehog protein biosynthesis is not only targetable for inhibition by small molecules, but sufficiently promiscuous to participate in the metabolism of anti-androgens. We hypothesize that the off-target hedgehog/anti-androgen interaction may represent a chemoresistance pathway in advanced PCa.

Impact Statement: Our work provides starting points for next-generation PCa therapies, while giving insight into the pharmacodynamics of existing PCa drugs.

A Novel Role of BMI1 in Androgen Receptor Pathway

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Background: Each year, over 240,000 American men are diagnosed with prostate cancer (PCa). B lymphoma Mo-MLV insertion region 1 homolog (BMI1) has been shown to be associated with metastatic prostate cancer by cDNA microarray analyses and tissue microarray analysis. BMI1 is an epigenetic component of a Polycomb Repressive Complex 1 (PRC1), maintaining gene repression. We have demonstrated that BMI1 promotes prostate cancer progression by repressing multiple tumor suppressors. However, its precise role in castration-resistant prostate cancer (CRPC) remains unclear.

Objectives: Our preliminary data strongly suggest that BMI1 is a master regulator of CRPC progression. Our objective is to determine how BMI interacts with epigenetic complexes and with androgen receptor (AR) to regulate tumor suppressor gene expression. We aim to identify novel binding partners and regulators of oncogene expression, which will lead to a better understanding of AR signaling and dysfunction. Specifically, we will identify how BMI1 and PRC1 proteins mediate their oncogenic functions by recruiting AR and distinct binding partners to promote castration-resistance of PCa. Furthermore, we will evaluate the therapeutic efficacy of targeting BMI1 and of combinational targeting of BMI1 and AR in castration-resistant prostate cancer.

Methods: By immunoprecipitation and immunoblot analysis, we discovered that BMI1 protects AR from MDM2-mediated ubiquitination and degradation. And this BMI1 novel function is independent of PRC1 complex. By tissue microarray analysis (TMA), we observed that BMI1 is upregulated in CRPC and NEPC, compared to non-CRPC patient samples. Importantly, BMI1 is dysregulated in hormone depletion therapy-treated PCa patients, and BMI1 levels are positively correlated with AR and prostate-specific antigen. The cell proliferation assays demonstrated that PTC209, a newly discovered BMI1 inhibitor, could inhibit PCa cell growth, and achieve the synergistic effect with enzalutamide in AR+ cells.

Conclusions and Impact Statement: Our study demonstrated a novel BMI1 function in regulating AR protein stability and AR pathway, and this function is independent of PRC1 complex. Elucidating the precise role of BMI1 and identifying novel protein interactions in prostate cancer progression and castration-resistant prostate cancer will have a significant impact, not only in the field of prostate cancer, but also in understanding epigenetic regulation in other cancer types. In addition, our study will further the understanding of AR dysfunction in prostate cancer. Furthermore, preclinical testing of BMI1-targeted therapeutics on prostate cancer will provide a platform for future treatment of advanced PCa patients.

Cellular Basis for Androgen Deprivation Induced Acceleration of Sarcopenia

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Background: Acceleration of sarcopenia, the age-related loss of skeletal muscle, is a side effect of androgen deprivation therapy (ADT) for elderly prostate cancer patients. Although androgen signaling is a known regulator of skeletal muscle, the cellular basis for ADT-induced acceleration of sarcopenia is unknown.

Objective: Resident stem cells of skeletal muscle (satellite cells [SCs]) are an essential source of progenitors for the growth and regeneration of skeletal muscle whose numbers and function decline with age. Therefore, in this study we examined the hypothesis that androgen deprivation-induced SC activation is associated with SC-derived progenitor contributions to the maintenance of young androgen-deprived skeletal muscle.

Methods: (1) To examine androgen deprivation-induced SC activation skeletal muscles from young castrated and sham mice were processed for immunofluorescence analysis of SC fate markers. (2) To study the effects of androgen deprivation on SC function and skeletal muscle regenerative capacity young castrated and sham skeletal muscles were subjected to experimental regenerative paradigms. (3) To examine SC-derived cell contributions to androgen-deprived skeletal muscle maintenance $Pax7^{CreER/+}$; $ROSA26^{mTmG/+}$ (P7mTmG) mice to track SC-derived cell fate, and $Pax7^{CreER/+}$; $ROSA26^{DTA/+}$ (P7DTA) mice to deplete SC-derived cells were used.

Results: Castration-mediated androgen deprivation does lead to SC activation; however, SC function and skeletal muscle regenerative capacity are preserved. Although SC regenerative function is preserved, androgen deprivation does induce SC-dependent maintenance of young skeletal muscle. The dependence of young androgen-deprived skeletal muscles on SCs was demonstrated by an increase in SC-derived cell fusion within skeletal muscle fibers, and heightened fibrosis, atrophy, and functional decline of androgen deprived skeletal muscles upon SC depletion.

Conclusions: The maintenance of young androgen-deprived skeletal muscles relies on SC-derived cellular contributions.

Impact Statement: The goal of this line of research is to gain insight into the cellular mechanisms responsible for the loss of skeletal muscle integrity in elderly prostate cancer patients undergoing ADT. Considering the well-described age-related decline in SCs, the results in this study highlight the need to devise strategies that promote SC maintenance during aging to attenuate or reverse the acceleration in sarcopenia ADT-treated elderly prostate cancer patients experience.

NEK6 Mediates Castration Resistance in Prostate Cancer in vivo

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Background: Studies of tissue from patient with metastatic castration-resistant prostate cancer (CRPC) suggest tumors can become resistant either through persistent activation of the AR pathway, or by progression to a state described as androgen pathway independent prostate cancer (APIPC). Tumor samples from patients with CRPC show increased levels of tyrosine phosphorylation and alterations in serine/threonine phosphorylation as compared to treatment naïve prostate cancer, but there is limited evidence for activating kinase point mutations in CRPC, suggesting that kinase pathways are activated by other (structural genetic, epigenetic, microenvironmental) mechanisms. Kinases conferring resistance are obvious druggable targets for potential therapeutic intervention.

Methods: Since kinase signaling pathways whose activation could lead to castration resistance in patients with prostate cancer have not been comprehensively catalogued, we have performed an in vivo functional genomic screen to identify novel pathways that may be involved. Specifically, we introduced a lentivirally delivered ORF library encompassing 601 kinases and other oncogenes into androgen-dependent prostate epithelial cells (LHSR-AR cells) to identify kinases that permit these cells to form tumors under deplete androgen conditions.

Results: In addition to re-identifying known mediators of castration resistance, we discovered that overexpression of the Never In Mitosis A (NIMA) related kinase 6 (NEK6) yielded androgen-independent tumors. NEK6 is overexpressed in a subset of human prostate cancers, and suppressing its expression restores sensitivity to castration in xenograft tumors. NEK6-mediated castration-resistant tumors are predominantly squamous in histology and do not show evidence of AR signaling. Gene expression profiling of post-castration tumors overexpressing NEK6 demonstrated elements of cytoskeletal, differentiation, and immune signaling, and NEK6 maintains the expression of many genes otherwise decreased with castration, including genes involved in interferon signaling. Combined phosphoproteome and interactome analysis reveals CSNK1A1, YES1, and FOXJ2 to be novel NEK6 substrates. Phosphomimetic forms of these proteins recapitulate elements of the NEK6 gene expression signature; in particular, the phosphomimetic form of CSNK1A1 strongly recapitulates a NEK6-mediated signature of squamous differentiation.

Conclusions: These studies reveal NEK6 signaling as a mechanism of resistance in androgen pathway independent prostate cancer (APIPC). Analysis of gene expression changes induced by phosphomimetic forms of newly identified substrates CSNK1A1, YES1, and FOXJ2 revealed that these proteins likely mediate at least some of the immune and cytoskeletal signaling downstream of NEK6 and thus demonstrate a novel role for these pathways in castration-resistant tumor formation.

Impact Statement: Molecular mechanisms underlying transition to androgen pathway independence are not fully characterized, but we hypothesize that APIPC represents a diversity of phenotypes and dependencies; a comprehensive understanding of these mechanisms would identify potential therapeutic targets for intervention, and would also allow for biomarker discovery to stratify patients for likelihood of response to targeted therapies. Thus, the signaling pathways described here warrant further study in this patient population.

Microenvironment-Sensitive Multimodal Contrast Agent for Prostate Cancer Diagnosis

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Background: Treatment and monitoring of prostate cancer (PCa) progression depends on the quantification of dynamic changes in molecular and cellular processes. Measurement techniques currently in use (e.g., biopsy) are invasive and provide limited spatial and temporal sampling. Improved molecular imaging techniques, that are both quantitative and sensitive, and that can detect processes deep within the human body, are required to monitor these changes, which may be predictors of treatment outcomes. The primary objective of this work is to develop a microenvironment-responsive multimodal contrast agent for non-invasive, 3D quantification of PCa aggressiveness through quantification of matrix metalloprotease (MMP) activity in tumors.

Methods: Proteolytic processes are important for cancer growth, progression, and metastasis. In this work, magnetic nanoparticles (MNP), which have the potential to be utilized as MRI contrast agents, were modified with two types of peptides: one that was selectively cleaved by MMP activity and a second that targeted highly metastatic PC3M prostate cancer cells. Cell lines expressing and not expressing MMP extracellularly were used for *in vitro* analysis of the particles, while an orthotopic metastatic prostate cancer model will be used in the pilot animal work. Primary tumor size and proteolytic activity will be followed by MRI and fluorescence imaging, respectively, and compared to *ex vivo* measurements.

Results: To date, we have synthesized the proposed MMP-responsive imaging agent and have conducted extensive *in vitro* evaluation of its performance. Development of a clinically viable nanomedicine for cancer imaging requires the optimization of particle size, surface properties, and microenvironment responsiveness. We have developed techniques to reduce the polydispersity of MNP from the typical value of 20% to less than 5%, which is considered to be monodisperse. Non-specific adsorption of the MNP was reduced using a polymer coat that minimized uptake by macrophages and normal cells. Furthermore, the interaction of MNP with target cells was enhanced by conjugating the F3- and RGD-peptides, which have been used to target tumors. Other PC3M targeting peptides have also been identified using phage libraries. Association of targeted-MNP with PC3M cells was demonstrated compared to non-targeted MNP. When exposed to proteolytic activity, the MNP responded by the release of a fluorescent probe at rates proportional to enzymatic activity.

Conclusions: We have demonstrated the ability to target particles to PCa cells and to also elicit a response to proteolytic activity. Additionally, both MRI and fluorescence imaging studies have demonstrated our ability to quantify the MNP concentration and the proteolytic activity. A final set of studies will be conducted using a rodent model to demonstrate quantification of *in vivo* proteolytic activity.

Impact Statement: The *1975-2007 SEER Cancer Statistics Review* reported that more than 2.2 million men alive in the United States have a history of prostate cancer. Resulting direct costs for prostate cancer was estimated at $4.61 billion for 1996. The cost of “watchful waiting” for 2-year period is itself $24,809/patient – without therapy; active treatments cost about $59,286/patient. Accurate diagnosis of PC aggressiveness would enable more effective treatment and would alleviate both economic and social burdens, and could enable concomitant staging and monitoring of tumor.

Hypoxic Prostate Cancer Cells Promote Pre-Metastatic Niche Preparation through RAB5A-Mediated Exosomes Secretion

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Background: Metastasis is the main cause of death in prostate cancer (PCA) patients. Recent studies have suggested that the primary tumor could remodel the microenvironment of distant organs by promoting the formation of metastasis-supportive conditions, termed as 'pre-metastatic niches' (PMN), prior to the tumor cell dissemination. PMN could determine metastatic success; however, we still have limited knowledge about PMN role in PCA metastasis. Recently, we reported that hypoxic PCA cells secrete nano-sized vesicles, called "exosomes," which promoted the invasiveness of naïve PCA cells by inducing epithelial-to-mesenchymal transition (EMT). Hypoxic PCA exosomes were loaded with unique factors (cytokines, proteinases, etc.) that are also known to play a central role in PMN preparation. In the present study, we characterized the role of hypoxic PCA exosomes in PMN preparation.

Methods: Human PCA PC3 cells were cultured under hypoxic (1% O_2) or normoxic (21% O_2) condition for 48 hours, and exosomes were isolated from the conditioned media by either ultracentrifugation or precipitation (ExoQuick kit) method. Isolated exosomes were characterized for size and concentration by nanoparticle tracking analyses (NTA). Proteins loaded in $Exo^{Normoxic}$ and $Exo^{Hypoxic}$ were analyzed by Western blotting. To assess the RAB5A GTPase role in exosome biogenesis, RAB5A expression was knocked down using specific siRNA. To understand exosomes' role in PMN preparation *in vivo*, $Exo^{Hypoxic}$ or $Exo^{Normoxic}$ (10 µg each) were injected intraperitoneally (IP) in male athymic nude mice (N=5 mice) on alternate days for 4 weeks. At the end, mice were intravenously injected MMPsense 750 dye to image for matrix metalloproteinase (MMP) activity. Thereafter, organs were collected and analyzed for PMN biomarkers.

Results: NTA analyses showed that PC3 cells secreted higher concentration of exosomes under hypoxia compared to normoxia; and $Exo^{Hypoxic}$ were smaller in size compared to $Exo^{Normoxic}$. $Exo^{Hypoxic}$ also showed higher levels of tetraspanins (CD63 and CD9), heat-shock protein 70, MMP2, hypoxia-inducible factor 2α, β-actin, vascular endothelial growth factor and tumor necrosis factor α. Importantly, PC3 cells showed higher RAB5A expression under hypoxic condition, and RAB5A knock-down strongly reduced the exosomes secretion by these cells. *In vivo*, higher MMPs activity was observed in mice injected with $Exo^{Hypoxic}$ compared to $Exo^{Normoxic}$. Furthermore, *ex vivo* imaging showed that $Exo^{Hypoxic}$ treatment significantly increased the MMPs activity in specific organs (liver, kidneys, spleen, and lymph nodes). Importantly, PC3 cells metastasize to these organs when injected orthotopically in the prostate of nude mice. Furthermore, $Exo^{Hypoxic}$ treatment significantly increased the expression of various PMN-associated biomarkers (fibronectin, collagen IV, MMP9, and MMP2) in these organs.

Conclusions: $Exo^{Hypoxic}$ play an important role in PMN preparation mainly through increasing MMP activity at specific organ sites. Also, RAB5A expression is important for exosomes biogenesis in hypoxic PCA cells.

Impact Statement: Currently, there is no cure or preventive strategy available against PCA metastasis as the role of PMN remains unknown. Our studies will fill this critical gap and help in devising better preventive and therapeutic strategies against metastatic PCA.

The Hippo Pathway Effector, YAP, Regulates Motility, Invasion and Castration-Resistant Growth of Prostate Cancer Cells

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Background: Prostate cancer is the most common malignancy and the second leading cause of cancer-related mortality among men in the United States. Although androgen-deprivation therapy (medical or surgical castration) is highly effective for advanced prostate cancer, the majority of patients eventually develop resistance and progress to castration-resistant prostate cancer (CRPC). Unfortunately, most cases of CRPC are currently incurable. The cause of castration resistance is still not completely known. It is expected that understanding the molecular mechanisms and identifying molecular pathways underlying the acquisition of castration-resistance in prostate cancer are critical for the design of therapeutic strategies and may lead to the discovery of novel targets.

Recent genetic mouse models and studies with cancer patients firmly demonstrated the critical roles of Hippo signaling in cancer development. Yes-associated protein, YAP, is an effector of the Hippo tumor suppressor pathway. The oncoprotein YAP has been implicated in promoting several types of tumor formation, such as liver and skin tumorigenesis and rhabdomyosarcoma. The functional significance of Hippo-YAP signaling in prostate cancer has remained elusive.

Methods: Cell culture, xenograft, and genetic (knockout and Tet-inducible transgenic) animal models were used to determine the role of Hippo-YAP pathway in the prostate.

Results: We first showed that enhanced expression of YAP was able to transform immortalized prostate epithelial cells and promoted migration and invasion in both immortalized and cancerous prostate cells. We found that YAP mRNA was upregulated in androgen-insensitive prostate cancer cells (LNCaP-C81 and LNCaP-C4-2) when compared to the androgen-sensitive LNCaP cells. YAP knockdown greatly reduced the migratory and invasive rates of LNCaP-C4-2 cells, while only moderately affecting proliferation and anchorage-independent growth. Importantly, ectopic expression of YAP was sufficient to promote LNCaP cells from androgen-sensitive to androgen-insensitive *in vitro* and YAP conferred castration resistance *in vivo*. Accordingly, under androgen-deprivation conditions, YAP knockdown largely blocked cell division in LNCaP-C4-2 cells. Mechanistically, we found the ERK-RSK signaling was downstream of YAP for cell survival, migration, and invasion in androgen-insensitive cells. Finally, immunohistochemistry showed significant upregulation and hyperactivation of YAP in castration-resistant tumors compared to hormonal-responsive prostate tumors. Deletion of MST1/2 (core tumor suppressors in the Hippo pathway) or YAP did not affect the prostate development. YAP activation or MST1/2 inactivation was not sufficient to promote prostate tumorigenesis.

Conclusions: Our results identify YAP as a novel regulator in prostate cancer cell motility, invasion, and castration-resistant growth, providing an alternative mechanism underlying the development of castration-resistance of prostate tumor cells.

Impact Statement: Our studies identify YAP as a potential therapeutic target for metastatic castration-resistant prostate cancer.

A Targeted Mass Spectrometry Approach to Identify Activated Kinases in Prostate Cancer

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Background: Prostate cancer is the second-leading cause of cancer death for men in the United States. Although relatively indolent compared to other epithelial malignancies, prostate cancer is lethal when untamed metastatic castration-resistant dissemination occurs. Mounting evidence suggests that non-mutated, activated kinases are key players in metastatic CRPC. We have previously identified a strong correlation between increased global tyrosine phosphorylation and prostate cancer progression both in a mouse model of prostate cancer and in human clinical samples. We identified several activated kinases using unbiased quantitative phosphopeptide proteomic analysis by tandem mass spectrometry of human metastatic CRPC tissues. Several of these identified kinases are direct targets of current FDA-approved kinase inhibitors, opening the door to investigate these kinases as viable therapeutic options. Due to the overwhelming evidence for kinase activation in metastatic CRPC tissues, our objective is to utilize a targeted mass spectrometry (MS) approach for the evaluation of current druggable kinases in human CRPC biopsies that will ultimately guide personalized therapy decisions.

Methods: A large panel of approximately 100 phosphopeptides was synthesized and then analyzed on a high-resolution Q-Exactive MS to measure the sensitivity of detection and elution time. Information for each phosphopeptide was used to serve as internal standards and surrogates for kinase activity in preclinical and clinical tissues.

Results: To date, we have analyzed 100+ phosphopeptide standards via targeted MS. Serial dilutions were evaluated to determine the linearity of the assay over several orders of magnitude as well as sensitivity. We were able to establish high sensitivity into the attomole (10^{-18} moles) range for a majority of the phosphopeptides in a non-mammalian matrix of consisting of plant lysate.

Conclusions: We have established a list of phosphopeptides that will serve as a predictive biomarker panel to carry forward for preclinical proof of concept and clinical testing. Future work will first establish the detection of driver kinases in cell lines with known activating mutations of kinases such as BRAF V600E in melanoma or colorectal cancer cell lines or EGFR mutations in non-small cell lung cancer cell lines. Once established, we will proceed to evaluate several prostate cancer cell lines that have no known driver kinase mutations and measure kinase activity for predicted kinase inhibitor therapies followed by subsequent validation studies.

Impact Statement: Advanced predictive tools and effective therapies are necessary to improve the clinical care of patients with metastatic CRPC. The development of a targeted mass spectrometry approach to measure a large panel of druggable, activated kinases in clinical tissues will represent a new clinical paradigm supporting phospho-kinase profiling to determine optimal treatment combinations in patients with metastatic CRPC.

Prostate Cancer Outcomes in VA Hospitals

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Background: Obesity is associated with both tumor biology and a delayed diagnosis of prostate cancer. *Haemodilution of the PSA level can lead* to a delay in diagnosis, which may be a factor in the more adverse outcomes seen in obese men. Other studies have proposed that advanced disease seen in African-American men could be explained by a greater proportion of obesity in this subset of men. Similarly, studies have found that among men with low-risk disease, obese African-American men have a higher risk of recurrence compared to obese white men. The Veterans Health Administration (VHA) is a population that, in previous studies, has shown no racial differences in prostate cancer mortality. As a government-operated institution that provides care regardless of ability to pay, analysis of VHA data will offer unique insights into the impact of socioeconomic status on clinical outcomes.

Methods: We used a retrospective cohort design using data from the VHA national medical care datasets. The VA Cohort includes data on all men diagnosed at any VA hospital across the United States between 1997 and 2009. Analysis was conducted on102,000 prostate cancer patients and data from the VA Central Cancer Registry. Main outcomes assessed are mortality (all-cause and prostate cancer specific) and high-risk disease at time of diagnosis. Mortality status was identified from vital status data. High-risk disease at diagnosis is defined as stage 3 or greater, or Gleason 4+3 or greater. Time to death (all-cause and prostate cancer-specific) was modeled with Cox proportional hazards models, reporting HRs and corresponding 95% CI. Predictors of high-risk disease at diagnosis were assessed in logistic regression models. BMI was categorized as normal, overweight, obese I, obese II, obese III. Additional covariates included race, age at diagnosis, stage at diagnosis, Gleason grade, days to initial treatment.

Results: The mean age at diagnosis is 64. 26.2% of study population are African-American, 34.4% are obese. 11% are in obese class II and III. 33.6% of African-Americans are obese and 34.6% of Whites are obese in the study population. No racial differences were found for mortality (all-cause and prostate cancer-specific) or diagnosis of high-risk disease. In full models, BMI was not significantly associated with all cause or prostate cancer specific mortality. Men with a BMI category of Obese class III at time of diagnosis were significantly more likely to be diagnosed with high-risk disease (OR=1.2).

Conclusions: Similar to other studies using VHA data, no racial differences are shown in the prostate cancer outcomes assessed. However, dissimilar from many clinical (non-VHA) studies, obesity is not significantly associated with prostate cancer outcomes other than at the highest obesity level, obese class III.

Impact Statement: Our study team is cleaning additional data on diagnoses and medications associated with other co-morbidities that might influence the association between obesity and prostate cancer outcomes.

Measuring Glucocorticoid Receptor Expression in Enzalutamide Resistant Prostate Cancer with PET

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Background: Enzalutamide is an androgen receptor (AR) antagonist and standard of care therapy for the treatment of castration-resistant prostate cancer. Tumor responses to enzalutamide are observed only in ~60% of patients for 12 to 16 months. Several resistance mechanisms have been defined, and the major priority for the field is identifying which resistance mechanism predominates in clinical disease. One resistance mechanism is the upregulation of glucocorticoid receptor (GR), which can transcribe AR target genes, but is not inhibited by enzalutamide. To probe for how common this mechanism is in patients, we have developed radioligands to GR to measure its expression in castration resistant prostate cancer.

Methods: A library of ~10 radiolabeled steroidal and non-steroidal GR ligands has been synthesized. Their respective potencies for GR and related nuclear hormone receptors have been assessed in vitro. In vivo microPET/CT imaging studies have been conducted in normal and tumor-bearing mice with controls to assess specific binding to GR in vivo.

Results: All radioligands that were developed have low nanomolar affinity for GR, and 100-fold less affinity for other nuclear hormone receptors in the family. ^{18}F-labeled steroidal radioligands show GR-specific binding in vivo that can be competed with excess mifepristone (RU486). There is also considerable liver binding that may be GR-specific. Non-steroidal ligands to GR have not yet been radiolabeled; however, a tritiated molecule was evaluated in animals and showed specific binding in several normal tissues. There was significantly less uptake of the ligand in liver, which may suggest that it is not bound by chaperone proteins in serum.

Conclusions: Several promising radioligands to GR have been synthesized and show evidence of specific binding in vivo. The radiolabeling of non-steroidal ligands for PET is still ongoing, but the initial evaluation of in vivo biodistribution in mice with stable isotopic labeling suggests that it may have more desirable properties compared to ^{18}F-steroids.

Impact Statement: Antagonists to GR are human ready and currently being evaluated in patients with castration resistant prostate cancer concurrently receiving enzalutamide to pre-empt this resistance mechanism. Having a biomarker like a radioligand presents the opportunity to more systematically define which patients harbor this resistance mechanism and will most likely benefit from a GR antagonist. Moreover, serial imaging pre- and post-GR antagonist administration will likely be an effective assay in patients to demonstrate complete GR blockade in the tumor, as well as to assess off target pharmacological effects.

Low CD38 Defines and Maintains a Progenitor-Like State and Predicts Poor Outcome in Prostate Cancer

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Background: We set out to determine cell types and molecular mechanisms associated with castration resistance and tumorigenesis. We hypothesized that progenitor-like cells may contribute to castration resistance and may reveal new mechanisms associated with survival and proliferation in the absence of androgen. CD38 is an ectoenzyme that consumes the cellular metabolite Nicotinamide adenine dinucleotide (NAD) and is expressed in human prostate.

Methods: Gene expression analysis was performed in castrated vs. intact mouse prostate epithelial cells. Immunohistochemistry was performed on human prostate tissues from hormonally intact and androgen-deprived men and in tissue microarrays representing varying stages of disease progression followed by correlation between stages using Spearman's rank correlation coefficient. Cox-regression analysis was performed in the Memorial Sloan Kettering dataset to assess the prognostic potential of CD38 mRNA levels in prostatectomy specimens. Fluorescence-activated cell sorting was performed to stratify and isolate cells based on differential expression of CD38. Organoid-forming progenitor assays measured growth of distinct cell populations. Overexpression of CD38 in prostate cells was achieved through lentiviral transduction followed by metabolic and transcriptional profiling.

Results: We determined that CD38 mRNA is reduced in mouse prostate luminal cells following castration and confirmed that CD38 protein is reduced in human prostate tissues from men treated with androgen-deprivation therapy. In tissue microarrays, we found CD38 expression was inversely correlated with disease progression, with the lowest expression in the highest Gleason tumors. Low CD38 mRNA in radical prostatectomy specimens is prognostic for biochemical recurrence and metastasis. Low expression of cell-surface CD38 enriches for organoid-forming progenitor-type luminal cells from benign human prostate and prostate cancer cells. Finally, CD38 overexpression reduces cellular levels of NAD, reduces proliferation, and enhances expression of cell-cycle inhibitors/tumor-suppressor genes in prostate cancer cells.

Conclusions: We conclude that CD38 both defines and maintains a progenitor-like state in human prostate. Loss of CD38 may enhance castration-resistant growth and survival, most likely by elevating pools of NAD.

Impact Statement: These findings suggest a potential role for low CD38/high NAD in castration resistance and tumorigenesis and will lead to future studies to assess targeting NAD synthesis or alternate pathways downstream of CD38-loss in prostate cancer. Low expression of CD38 may be useful in combination with other biomarkers to predict poor outcome and guide treatment decisions in the future.

Insights into the Role of Metastasis-associated Proteases and Peptides in Prostate Cancer Pain

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Background: Tumor-induced pain and sensory neuropathy significantly impact quality of life of cancer patients during treatment and survivorship. Increased life expectancies mandate more effective pain therapies. The development of a molecular understanding of cancer pain is crucial. Tumor-derived peptides are one of the sources for cancer pain. Cancer cells and associated stromal cells secrete a complex repertoire of factors that stimulate tumor growth and metastasis. Among them are peptides that have been shown to sensitize or directly excite primary afferent neurons. Here, we used quantitative and activity-based mass spectrometry technologies to identify these peptide products and elucidate the roles of proteases in peptide generation and peptide clearance. This study is a crucial step towards the development of mechanism-based therapies for cancer pain**.**

Methods: We used state-of-the-art quantitative and activity-based mass spectrometry approaches to compare the secreted peptidome and proteome of metastatic and non-metastatic prostate cancer cell lines. Using our PALeO-methodology (proteinase activity labeling employing ^{18}O), we identified the subset of peptides that were actively being produced by proteases. Peptides and proteins (particularly proteases) were identified by nanoLC-MS/MS on Orbitrap mass analyzers and subsequently by algorithmic matching of acquired fragmentation mass patterns with protein database entries or by *de novo* sequencing. Peptides with potential neuroactivity were synthesized in-house using automated peptide synthesis. We validated individual protease-peptide processes *in vitro* using synthetic peptides and recombinant proteases. Kinetics of the reactions were monitored by our PALeO-methodology.

Results: We defined the complex ensemble of peptides and proteases that malignant prostate cancer cells release into their microenvironment. Using our PALeO-technique, we monitored the kinetics of proteolytic processing of individual neuroactive peptides by proteases *in vitro*. We determined previously unknown reaction intermediates during the proteolytic processing of neuroactive peptides mediated by members of the M13-metallo-peptidases. Surprisingly, we also discovered that these cancer-associated proteases are capable of reversing proteolysis and religate cleavage products.

Conclusions: Detailed kinetic and molecular analyses of cancer-associated proteolytic processes by mass spectrometry-based approaches yielded unprecedented insights into the enzymatic mechanisms governing the abundance of neuroactive peptides secreted by prostate cancer cells.

Impact Statement: Our observations provided a deeper understanding of the molecular cross talk of metastatic peptide signaling pathways and the peripheral nervous system. We discovered unique opportunities to manipulate protease that generate pain-inducing peptides. Continuation of this research may ultimately lead to novel cancer pain therapeutics.

HSD3B1 and Resistance to Androgen Deprivation Therapy in Prostate Cancer

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Background: HSD3B1(1245A>C) has been mechanistically linked to castration-resistant prostate cancer by encoding an altered enzyme that augments dihydrotestosterone synthesis. We hypothesized that men inheriting the HSD3B1(1245C) allele would exhibit resistance to androgen deprivation therapy (ADT).

Methods: We determined the HSD3B1 genotype retrospectively in men treated with ADT for post-prostatectomy biochemical failure and correlated genotype with long-term clinical outcomes. Patients who received postoperative adjuvant or salvage radiotherapy were eligible, provided they had residual active disease as reflected by continued increase in their PSA after treatment. We analyzed progression-free survival (PFS; primary endpoint), distant metastasis-free survival (DMFS), and overall survival (OS) according to HSD3B1 genotype. Multivariable analyses were performed to assess the independent predictive value of HSD3B1 genotype on outcomes. Results were externally validated in two additional cohorts, including a second post-prostatectomy biochemical failure cohort as well as a metastatic cohort.

Results: The study included 443 patients: 118 in the primary cohort, 137 in the post-prostatectomy validation cohort, and 188 in the metastatic validation cohort. In the primary study cohort, median PFS diminished as a function of the number of variant alleles inherited: 6.6 years in homozygous wild-type men (95% CI, 3.8 to not reached); 4.1 years in heterozygotes (95% CI, 3.0 to 5.5); and 2.5 years in homozygous variant men (95% CI, 0.7 to not reached); P=0.011. Median DMFS likewise decreased according to the number of variant alleles inherited: 9.1 years (95% CI, 7.4 to not reached); 6.8 years (95% CI, 4.3 to 7.4); and 3.6 years (95% CI, 1.0 to 7.3), respectively; P=0.014. Finally, OS diminished with the number of variant alleles inherited: 5-year and 10-year OS 82% (95% CI, 69 to 94) and 55% (95% CI, 35 to 75) in homozygous wild-type men; 74% (95% CI, 62 to 85) and 35% (95% CI, 21 to 49) in heterozygotes; and 58% (95% CI, 30 to 86) and 0% in homozygous variant men; P=0.0064. On multivariable analysis, the hazard ratio (HR) for progression was 1.6 for men with at least one variant allele (95% CI, 1.0 to 2.7; P=0.074), which compared favorably with Gleason score (HR 1.3 for Gleason score 8-10 vs. 6-7; 95% CI 0.8 to 2.0; P=0.31), though neither factor reached statistical significance with the small sample size. The impact of homozygous variant genotype on metastasis (HR 2.8; 95% CI, 1.1 to 6.7; P=0.025) and death (HR 3.5; 95% CI 1.3 to 9.5; P=0.013) was maintained on multivariable analysis. Findings in the external cohorts independently validated the impact of HSD3B1(1245C) on outcomes, including survival.

Conclusions: Inheritance of the HSD3B1(1245C) allele that enhances dihydrotestosterone synthesis predicts innate resistance to ADT in prostate cancer.

Impact Statement: Our findings nominate HSD3B1 as a powerful genetic biomarker capable of distinguishing men who are a priori likely to fare favorably with androgen deprivation therapy from those who harbor disease liable to behave more aggressively and who therefore may warrant early escalated therapy. Future studies should stratify by HSD3B1 genotype in light of the profound differences in outcomes according to the number of variant alleles present.

Exome Sequencing of African-American Prostate Cancer

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Background: African-American men have the highest incidence and mortality from prostate cancer. Whether there is a biological basis for this disparity is unclear. To date, African-American men have been underrepresented in large sequencing studies of prostate cancer. We hypothesized that differences in mutational events in prostate cancers in African-American men may in part account for prostate cancer disparities. We also hypothesized that the power to discover new cancer genes might increase through inclusion of diverse ancestral backgrounds in large-scale cancer genome studies. To test these hypotheses we performed whole exome sequencing of localized prostate cancers and matched normal tissue from a cohort of African-American men to determine the somatic genomic alterations in these tumors.

Methods: We performed whole exome sequencing on primary prostate cancers from intermediate-risk and high-risk tumors. We performed genomic analysis for genes that were significantly mutated and determined copy number alterations. Based on these data, we also developed a targeted hybrid capture bait set that sequenced the exons of selected prostate cancer genes and intronic regions and performed targeted sequencing and analysis on an extension/validation cohort of primary prostate cancer from African-American men.

Results: We found several genes that were significantly mutated in primary prostate cancer in African-American men. These genes included *SPOP* and *FOXA1* as well as a novel cancer gene, *ERF*, an ETS transcriptional repressor, which has not been previously described as recurrently mutated in primary prostate cancer. Loss-of-function mutations in *ERF* and functional assays provide evidence that *ERF* is a potential tumor suppressor in prostate cancer. Mutations in *ERF* are also found in metastatic castration-resistant prostate cancer samples.

Conclusions: Inclusion of diverse populations in cancer genome studies has the potential to increase the discovery of new cancer genes. Exome sequencing of a large cohort of primary prostate cancer in African-American men identifies loss-of-function mutations in an ETS repressor.

Impact Statement: The potential impact of this work on research in prostate cancer is that it highlights that patients with different ancestral backgrounds may have differences in tumor genetics that could eventually impact therapy decisions.

Novel Pharmacological Blockade of Apurinic/Apyrimidinic Endonuclease 1 Redox Activity Downregulates Survivin Expression and Arrests Prostate Cancer Cell Proliferation

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Backgrounds: Prostate cancers exhibit numerous gene and protein expression alterations that result in enhanced cell proliferation, expanded cell migration, and increased cell survival capability. Survivin affects all three of these effects, and is highly induced during prostate cancer progression. Induction of survivin is critical to the ability of prostate cancer cells to avoid chemotherapeutic intervention. Survivin has been an elusive drug target, and subpar inhibitors of this protein have failed to show benefit in prostate cancer patients. Targeting the regulation of survivin holds promise, but little is known regarding how survivin expression is regulated in prostate cancer cells. Apurinic/apyrimidinic endonuclease 1 (APE1)-Redox Factor 1 (APE1/Ref-1) is upregulated in human prostate cancer and the redox activity of this protein has recently been found to be essential in activating oncogenic transcription factors including NF-κB and STAT3. Since survivin expression is driven by these transcriptional activators, we sought to determine if APE1/Ref-1 redox function regulates prostate cancer cell proliferation and survival, and expression or activity of survivin. Additionally, we sought to determine if novel, selective redox function inhibitors reduced survivin expression and proliferation in vitro and in vivo models of prostate cancer.

Methods: Survivin and APE1/Ref-1 expression was assessed in LNCaP, PC3 and C4-2 human prostate cancer cell lines and noncancerous human prostate cell line E7 by immunoblotting. APE1/Ref-1 and survivin expression were assessed in human prostate cancers by immunofluorescence (IF). Effects of APE1/Ref-1 inhibition were determined using siRNA knockdown and treatment with the redox function-specific inhibitors APEX3330 and APX2009, and cell growth was determined by methylene blue assay and bromodeoxyuridine (BrdU) incorporation; cell survival/death was assessed by caspase 3 cleavage (immunoblotting) and TUNEL assay (IF). Survivin expression was correlated to cell proliferation and cell death. Cell cycle analysis was determined by propidium iodide incorporation and flow cytometry in the presence and absence of inhibitors. Finally, in vivo effects of redox function inhibition were determined by flank grafts of C4-2 cells (6 weeks), followed by 7 days of in vivo drug treatment with APEX3330, harvesting of the tumor, and assessment of survivin expression (IF and immunoblotting) and BrdU incorporation (IF).

Results: Prostate cancer cells demonstrated substantially increased survivin and APE1/Ref-1 expression compared to that of the noncancerous cell line. APE1/Ref-1 and survivin were upregulated and 79% co-localized in human prostate cancer specimens compared to age-matched noncancerous controls. siRNA knockdown of APE1/Ref-1 and treatment with E3330 and APX2009 significantly reduced survivin expression and proliferation of prostate cancer in vivo and induced cell cycle arrest in vitro.

Conclusions: These data indicate that APE1/Ref-1 is a key regulator of survivin expression and prostate cancer proliferation and survival in vivo.

Impact Statement: We have defined a cellular signaling mechanism for how cells induce a critical cell proliferation and survival pathway and resist cell death. This work also introduces the novel APE1/Ref-1 redox function inhibitors APEX3330 and APEX2009 to the prostate field for use in future clinical trials in chemotherapy-resistant prostate cancers.

Restriction Spectrum Imaging MRI Distinguishes Prostate Cancer Tumor Grade

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Background: Current multiparametric magnetic resonance imaging (MRI) for prostate cancer is confounded by variable sensitivity and specificity, which curtails its clinical utility. Restriction spectrum imaging (RSI-MRI) is an advanced diffusion imaging technique that shows improved conspicuity and differentiation of solid tumors compared to traditional diffusion weighted imaging. RSI-MRI can differentiate hindered from restricted diffusion, thought to correspond to the extracellular and intracellular water compartments, respectively. Prior reports show that the quantitative signal derived from RSI-MRI, the cellularity index, is associated with aggressive PCa as measured by Gleason grade (GG), and that RSI-MRI improves sensitivity to the detection of extraprostatic extension of prostate cancer. Here we evaluated the reliability of RSI-MRI to predict variance with GG at the voxel-level within clinically demarcated PCa regions.

Methods: Ten cases were processed using whole mount sectioning after radical prostatectomy. Regions of tumor were identified and demarcated by an uropathologist. The whole mount H&E stained prostate sections were scanned at high resolution (75μm/pixel). The scanned images were reconstructed into a "digital prostate map" interface and overlaid with a grid of tiles corresponding to voxel dimensions. Each grid tile was graded using the GG system. An experienced radiologist selected the slice from the presurgical T2 MR series that most closely corresponded to the plane of the histopathology section. Deformation of the histology section was corrected for by transforming the T2 and corresponding RSI-MRI slice to the size and shape of the histopathology section. The RSI-MRI cellularity index was calculated from the RSI-MRI data and presented as normalized z-score maps. In total, 2,795 tiles were analyzed and compared with RSI-MRI cellularity.

Results: Using a linear mixed-effect model with a random effect of subject, RSI-MRI cellularity index was found to distinguish between PCa and benign tumor (t=25.48,p<0.00001). Significant differences were also found between benign tissue and PCa classified as low-grade (GG=3; t=11.56,p<0.001) or high-grade (GG≥4 t=24.03,p<0.001). Furthermore, RSI-MRI differentiated between low and high-grade PCa (t=3.23,p=0.003).

Conclusions: Building on our previous findings of correlation between GG and the RSI-MRI among whole tumors, our current study reveals a similar correlation at voxel resolution within tumors. The relationship between GG and RSI-MRI means that RSI-MRI can be used as a component of active surveillance to non-invasively detect high-grade cancer and affect staging and treatment. Furthermore, because it can detect variations in tumor grade with voxel-level precision, RSI-MRI may have particular relevance for planning of focal procedures, such as MRI guided targeted biopsies and targeted radiotherapy, where identifying the area with the most aggressive disease is particularly important.

Impact: Use of an RSI-MRI index to differentiate between clinically relevant low- and high-grade categories of tumor prostate cancer aggressiveness may help improve and refine diagnosis and staging of prostate cancer. In addition, because it can detect intratumor variation, RSI-MRI may have particular relevance for planning targeted therapies such as radiation seed therapy placement, MR-guided focused ultrasound surgery, and MR-guided targeted biopsy.

Targeting GPR30 in Abiraterone- and MDV3100-resistant Prostate Cancer

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Background: Abiraterone (Abi) and MDV3100 (MDV) have been shown to extend the survival of patients with castration-resistant prostate cancer (CRPC). However, resistance to these treatments invariably emerges and little information is available on the novel treatment for this resistant disease. G protein-coupled receptor 30 (GPR30) is a seven-transmembrane estrogen receptor and activation by its specific agonist, G-1, inhibited growth in multiple CRPC xenograft models that were resistant to the first-generation androgen deprivation therapy (ADT). More importantly, GPR30 is an androgen-repressed target and its expression increased in clinical CRPC when compared to primary prostate cancer. Herein, we will conduct preclinical studies to test the efficacy of G-1 in inhibiting the growth of prostate cancer that are resistant to the new second-generation ADT including Abi and MDV. In addition, we will identify the tumor characteristics underlying responsiveness and potential resistance to GPR30-targeted therapy and determine the GPR30 expression pattern and its relationship to intracrine androgen levels in Abi- and MDV3100-resistant patients.

Methods: We evaluated the prevalence of GPR30 expression using immunohistochemistry in both CRPC and Abi- and MDV-resistant prostate cancer metastases from our rapid autopsy program and will measure the intracrine androgen levels of these metastases using mass spectrometry. We also investigated the G-1 efficacy on the growth inhibition using two newly characterized patient-derived xenograft (PDX) models that are resistant to Abi and MDV.

Results: GPR30 expression was detected in >90% of CRPC metastases, whereas 80% showed a moderate- to high-expression level. In our first few rapid autopsy patients who are Abi- and/or MDV3100-resistant, GPR30 was moderately to highly expressed in both lung and bone metastases. The analysis of intracrine androgens and the correlation with GPR30 expression is ongoing. In preclinical studies, LuCaP 35CR and LuCaP 82 were prostate cancer PDXes that were minimally responsive to both Abi and MDV treatments. G-1 significantly inhibited the growth and extended the progression-free survival of LuCaP 35CR ($p=0.0053$; LuCaP 86.2 study is underway). Interestingly, no survival benefit was observed with G-1 when LuCaP 35CR had been pre-treated with Abi or MDV, suggesting a defined window for G-1 therapy. Together with our previous findings, G-1 invariably inhibited four models of CRPC, independent of their sensitivity to Abi or MDV. No adverse side effect of G-1 was detected in these preclinical studies.

Conclusions: This study demonstrated that GPR30 was moderately to highly expressed in clinical CRPC metastases including Abi- and MDV-resistant disease. Targeting GPR30 with its agonist G-1 inhibited the growth of CRPC that is minimally responsive to Abi and MDV, preferably prior to Abi or MDV treatment.

Impact Statement: Treatments for the newly emerging Abi- and MDV-resistant prostate cancer have not been explored. Despite altered androgen and AR signaling, GPR30 remains highly expressed in these cancers. This study will define the optimal window for the G-1 therapy in these currently lethal Abi- and MDV-resistant prostate cancers.

Inducible Expression of Antigen Processing and Antigen Presentation Molecules in Human Prostate Cancer

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Background: Defects in immune surveillance have been correlated with tumorigenesis and poor clinical outcomes in cancer patients. Given recent advances in vaccine therapies and checkpoint inhibition, therapeutic targeting of defective antigen processing may play a critical role in improving the broad utility of immunotherapies. Epigenetic alterations have been found to alter expression of genes involved in antigen processing, presentation as well as neoantigen expression. We evaluated the ability of epigenetic modifying agents to increase expression of antigen presentation machinery (APM) molecules using *in vitro* and *ex vivo* drug culture using primary human prostate tumor tissue biopsies.

Methods: Prostate tissue biopsies were collected from patients with advanced, localized prostate cancer undergoing radical prostatectomy. Tumor biopsies were sectioned to $1mm^3$ slices and cultured with the hypomethylating agent decitabine (5AZA) and/or histone deacetylase inhibitor panobinostat (LBH589) for 72 hours followed by mRNA extraction and gene expression analysis. We further developed new microfluidic technology to evaluate expression of these molecules in circulating tumor cells (CTCs) from patients with advanced prostate cancer.

Results: 5AZA alone or in combination with LBH589 increased expression of MHC Class I molecules (HLA-ABC, B2M) and APM elements including TAP2, Tapasin and LMP7. Increased expression of neoantigens and MHC class I molecules was identified in human prostate cancer treated with 5AZA and the combination of 5AZA and LBH589. Significant inter-patient heterogeneity was also observed, suggesting other mechanisms by which prostate cancer downregulates MHC class I expression. We further identify increased expression of Cancer-Testis Antigens in these samples, including NY-ESO and SSX2, indicating multiple mechanisms by which epigenetic modifying agents may improve immune recognition of human prostate cancer. Finally, CTCs were identified that express these neoantigens indicating new blood based biomarkers of epigenetic therapies.

Conclusions: Immune evasion by prostate cancer occurs through many mechanisms, including down regulation of antigen presentation machinery. Epigenetic targeted agents can induce expression of these molecules as well as neoantigens in human prostate cancer samples. CTC biomarkers may serve as pharmacodynamic biomarkers of epigenetic therapies for prospective clinical trials.

Impact Statement: These results identify novel therapeutic and biomarker strategies that can be employed with other immune based therapies for men with localized and advanced prostate cancer.

The Impact of Self-Identified Race-Ethnicity and Genetic Ancestry on a Commonly Used Clinicopathologic Predictor of Biochemically Recurrent Prostate Cancer

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Background: Currently used clinicopathologic predictors of biochemically recurrent prostate cancer (BCR) were developed in populations of primarily European ancestry, and, to date, there has been limited evaluation of the effectiveness of such predictors in minority populations, such as African Americans. Further, within African Americans, an admixed population with genetic ancestry from both Africa and Europe, there has been little study of the importance of genetic ancestry in the prediction of BCR. The objectives of this study were to (1) evaluate the additional contribution of self-reported race-ethnicity to an accepted predictor of BCR and whether the effect of the predictor differed by self-reported race-ethnicity; and (2) among African Americans, evaluate the additional contribution of the proportion of genome-wide African ancestry to the predictor and whether the effectiveness differed based on the degree of African ancestry.

Methods: Our study of 176 African American and 231 European American prostate cancer cases treated with radical prostatectomy at Henry Ford Health System in Detroit, Michigan. Patients were recruited between July 1, 2001, and December 31, 2004, and were subsequently followed for BCR. The Cancer of the Prostate Risk Assessment Post-Surgical (CAPRA-S) score was used as the clinicopathologic predictor of BCR. Cox Proportional Hazards models were used to calculate hazard ratios (HR) to assess the additional contribution of self-identified race-ethnicity and genetic ancestry to CAPRA-S in the prediction of the BCR.

Results: Overall, CAPRA-S score was significantly associated with BCR ($p<0.001$), with each unit increase in score associated with a 1.47-fold (95% confidence interval [CI] 1.38-1.56) increased risk of recurrence. Inclusion of self-reported race-ethnicity did not significantly alter this estimate, and self-reported race-ethnicity was not associated with BCR ($p=0.839$). Also, the association between CAPRA-S and BCR did not depend on race-ethnicity (interaction $p=0.439$), with similar significant CAPRA-S effects in both African Americans (HR=1.44, 95%CI 1.30-1.60) and European Americans (HR=1.48, 95% CI 1.36-1.60). When restricted to African Americans, genetic African ancestry proportion did not significantly alter the association between CAPRA-S and BCR and was not associated with BCR risk ($p=0.519$). Further, the CAPRA-S and BCR association did not depend on African ancestry proportion (interaction $p=0.461$).

Conclusion: The results from this single institution, multi-ethnic, radical prostatectomy cohort suggest that the CAPRA-S BCR risk predictor is as effective in African Americans as it is in European Americans, and among African Americans, genetic African ancestry does not alter the association between CAPRA-S and BCR.

Impact Statement: Clinically, our results suggest that CAPRA-S applies equally to both African American and European Americans. For our newly funded Department of Defense study "Integrated Genomic Biomarkers to Identify Aggressive Disease in African Americans with Prostate Cancer" (PC141414), our results show that it is valid to use CAPRA-S in our analytic strategy to identify genomic alterations that add to this clinicopathologic predictor of BCR, leading to further improvements in clinical recurrence risk prediction in African Americans and more refined identification of those likely to benefit from earlier aggressive therapy.

Incorporation of Novel MRI and Biomarkers into Prostate Cancer Active Surveillance Risk Assessment

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Background: Active surveillance is a strategy used to monitor low-risk prostate cancer in order to delay or avoid aggressive therapies, with the option to intervene yearly in the disease process of progression if detected with curative intent. Unfortunately, the initial and secondary prostate biopsies suffer from a 30% sampling error. Progression is usually detected by repeating the prostate biopsy, in some cases yearly. Prostate biopsies can cause significant pain, bleeding, infection, and anxiety. The primary objective of this project is to investigate if a novel screening MRI can predict prostate biopsy outcomes and eventually replace the prostate biopsy as the primary means to follow patients. The secondary purpose is to use biomarkers from blood, urine, or prostate tissue to identify those men who are likely to progress on active surveillance.

Methods: Our primary population is men who are diagnosed with prostate cancer and choose active surveillance. We plan to enroll 160 subjects to undergo a prostate MRI prior to their TRUS prostate biopsy. Both conventional MRI with IV gadolinium contrast and Restriction Spectrum Imaging (RSI) techniques will be employed. Images will be evaluated using a five-point scale (PI-RADS) to determine suspicion of clinical significant prostate cancer. PI-RADS will also be used to grade the RSI images with secondary radiology review. After the MRI, the patient will undergo targeted and template prostate biopsy and pathology compared to PI-RADS. Other study endpoints will include Gleason 6 tumor (low-grade) or a negative biopsy. After pathologic review, the paraffin-embedded tissue will be sent to the Center for Prostate Disease Research in Rockville, Maryland.

Results: To date, we have completed the IRB protocol and attained approval for imaging in men undergoing active surveillance for prostate cancer and who have an upcoming prostate biopsy. We have enrolled 19 subjects to obtain an MRI and undergo cognitive or MRI-Fusion targeted prostate biopsy from 2/1/2016 to present. Of these men, nine have completed their biopsy, four have had an MRI and are scheduled for biopsy, and two are pending their MRI. Two men have dropped out of the study as screening fails at the MRI scanner (elevated creatinine and claustrophobia). Three scans showed “No suspicious lesions;” 66% (2/3) were completely negative; and one had one core of low-grade, low-volume prostate cancer (Gleason 3+3, 5% of the core). Three targeted biopsies were negative in the targeted lesions with all having PI-RADS scores of 3. In these samples, the standard 12-core biopsy only revealed a “missed lesion” in one patient with one core of Gleason 3+3 (<5% of the core). One PI-RADS 4 lesion had a positive target with Gleason 3+3 (80% of the core) and a positive 12 core (same Gleason) and a PI-RADS 5 that showed Gleason 4+4.

Conclusions: We have successfully implemented our Prostate MRI study at the University of Texas HSC San Antonio. From our preliminary results, PI-RADS 4 and 5 lesions are more significant findings on prostate biopsy. PI-RADS 3 lesions on MRI are commonly negative on biopsy.

Impact Statement: Our research is directed towards improving the quality of life of prostate cancer patients in the form of reduction of prostate biopsies and more accurate selection of active surveillance candidates.

Co-targeting EGFR and Survivin with a Bivalent Aptamer-dual siRNA Chimera Effectively Suppresses Prostate Cancer

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Background: Current single-agent therapies for prostate cancer (PCa) do not have significant benefits due to heterogeneity of PCa and capability of cancer cells to switch to alternative survival signals. That highlights the importance of a combination therapy to co-target multiple oncogenic pathways.

Current targeted therapies using tyrosine kinase inhibitors (TKIs) and antibodies have inherent disadvantages. TKIs have high toxicity and off-target effect, while antibodies have high immunogenicity and cannot access intracellular signaling molecules.

SiRNA has provided unprecedented opportunities for cancers since it enables sequence-specific silence of any oncogenes. Aptamer-siRNA chimera (AsiC) provides a new platform for bringing siRNA to target cells via receptor-mediated internalization. Aptamer is ssDNA or RNA that can bind to target with high affinity and specificity. AsiC features high potent, nontoxic, low immunogenic ease of synthesis and tumor specificity.

EGFR overexpression is associated with castration-resistant and bone metastasis of PCa. Survivin plays a pivotal role in the progression of PCa. It is reported that resistant to EGFR inhibitors may switch to the survivin network for survival. Therefore, co-targeting EGFR and survivin may provide a novel strategy to effectively inhibit multiple oncogenic signals.

We design to simultaneously silence EGFR and survivin with AsiC. PSMA aptamer, which specifically binds to prostate-specific membrane antigen (PSMA), has been well characterized for siRNA and drug delivery and is selected to guide siRNA to enter the PCa tumor.

Methods: We have engineered an aptamer-siRNA chimera, in which EGFR siRNA and survivin siRNA are fused between two PSMA aptamers. Bivalent aptamer binds PSMA via an antibody-like structure to enhance siRNA internalization. The PSMA aptamer-survivin siRNA-EGFR siRNA-PSMA aptamer chimera (PSEP) are synthesized by *in vitro* transcription, and 2'fluoro-pyrimidines are incorporated into PSEP during transcription for preventing nuclease degradation.

Results: PSEP is able to inhibit EGFR and survivin simultaneously and induce apoptosis effectively *in vitro* and *in vivo*. In the C4-2 PCa xenograft model, the treatment with PSEP significantly suppresses tumor growth and angiogenesis. We also demonstrate that the inhibition of angiogenesis is mediated by an EGFR-HIF1α-VEGF-dependent mechanism.

Conclusions: Co-targeting EGFR and survivin with AsiC is an effective approach for PCa xenograft treatment. With further development, PSEP promises to translate to the clinic for treating PCa patients. The bivalent aptamer-driven delivery of two siRNAs could be a new combination strategy to effectively inhibit multiple targets.

Impact Statement: This research will directly contribute to suppression of growth and metastasis of prostate cancer, prolong the lifespan, and improve life quality of prostate cancer patients.

Molecular Markers to Distinguish Intraductal Carcinoma of the Prostate from High-Grade Prostatic Intraepithelial Neoplasia

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Background: Although non-invasive, intraductal carcinoma of the prostate (IDC-P) has long been recognized by pathologists as an extremely high-risk feature. Defined by the presence of malignant cells spreading within intact prostatic ducts and acini, IDC-P occurs almost exclusively in high Gleason grade and stage tumors and is a consistent independent risk factor for tumor progression and death in cohorts treated with surgery or radiotherapy. Importantly, however, IDC-P is currently systematically under-diagnosed in needle biopsies because it has significant morphologic overlap with another intraepithelial lesion, high-grade prostatic intraepithelial neoplasia (PIN). Since PIN is a morphologically similar lesion with virtually no prognostic significance, *we propose that the systematic under-diagnosis of IDC-P in needle biopsies results in the under-recognition of potentially aggressive prostate tumors.* Here, we tested whether molecular aberrations in PTEN and ERG, which are common in invasive prostate cancers but relatively rare in PIN, might be useful to distinguish IDC-P from PIN.

Methods: Genetically validated PTEN and ERG immunostains were performed on biopsies containing morphologically identified IDC-P, isolated PIN, or borderline intraductal proliferations more concerning than PIN, but falling short of morphologic criteria for IDC-P. Immunostains were also performed on isolated PIN occurring in cystoprostatectomy specimens that did not contain invasive prostatic adenocarcinoma.

Results: In biopsies, IDC-P occurring with concurrent invasive tumor showed the highest rate of PTEN loss, with 76% (38/50) lacking PTEN and 58% (29/50) expressing ERG. Of biopsies containing isolated IDC-P, 61% (20/33) showed PTEN loss and 30% (10/33) expressed ERG. Of the borderline intraductal proliferations, 52% (11/21) showed PTEN loss and 27% (4/15) expressed ERG. Of the borderline cases with PTEN loss, 64% (7/11) had carcinoma in a subsequent needle biopsy specimen, compared to 50% (5/10) of PTEN-intact cases. In contrast, none of the PIN cases on biopsy showed PTEN loss or ERG expression (0/19). In foci of isolated PIN occurring in cystoprostatectomies without invasive cancer, PTEN loss was not seen in any PIN lesion (0/88) and only 7% (8/107) were positive for ERG.

Conclusions: PTEN loss and ERG expression are common in morphologically identified IDC-P, yet are very rare in isolated high-grade PIN. Borderline intraductal proliferations, especially those with PTEN loss, have a high rate of carcinoma on resampling.

Impact Statement: If confirmed in larger prospective studies, these results suggest that PTEN and ERG status may provide a useful molecular assay to distinguish IDC-P from high-grade PIN in this setting. Ongoing experiments will profile the gene expression signature of IDC-P and systematically compare it to PIN, identifying additional candidate markers for distinguishing the two lesions, and integrate IDC-P into the molecular landscape of invasive carcinoma, both at the gene expression and genomic levels, using a combination of bioinformatics, targeted next-generation sequencing, and copy number variation analysis.

Combination Targeted and Immune Checkpoint Blockade Therapy Inhibits Metastatic Castration-Resistant Prostate Cancer

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Background: Prostate cancer (PCa) is the most common noncutaneous malignancy in men in the United States. A significant fraction of advanced PCa treated with androgen deprivation therapy (ADT) experience relentless progression to lethal metastatic castration-resistant prostate cancer (mCRPC). The PCa tumor microenvironment is comprised of a complex mixture of epithelial and stroma cell types engaged in multifaceted heterotypic interactions functioning to maintain tumor growth and immune evasion. We recently uncovered the important role played by myeloid-derived suppressor cells (MDSCs) to mediate tumor immune evasion in aggressive PCa (Wang, Lu, et al. *Cancer Discovery*, 2016). Immune checkpoint blockade (ICB) has elicited durable therapeutic responses across a number of cancer types. However, the impact of ICB on mCRPC has been disappointing, which may signal the need to combine mechanistically distinct ICB agents and/or override immunosuppression in the tumor microenvironment. Our objective is to determine if robust immunotherapy responses in mCRPC may be elicited by the combined actions of ICB agents together with targeted agents that neutralize MDSCs yet preserve T cell function.

Methods: We created a novel embryonic stem cell (ESC)-based chimeric mouse model of mCRPC engineered with signature mutations to study the response to single and combination immunotherapy.

Results: Consonant with early stage clinical trials experience, anti-CTLA4 or anti-PD1 monotherapy failed to impact disease progression. Similarly, modest antitumor activity was observed with combination ICB as well as monotherapy with targeted agents including Cabozantinib (tyrosine kinase inhibitor), BEZ235 (PI3K/mTOR inhibitor), and Dasatinib (tyrosine kinase inhibitor). In contrast, mCRPC primary and metastatic disease showed robust responses to dual ICB treatment together with either Cabozantinib or BEZ235, but not with Dasatinib, which impaired T cell infiltration in the tumor. Detailed intratumoral immune profiling with mass cytometry (CyTOF) showed that combined ICB and Cabozantinib or BEZ235 was associated with significant depletion of MDSCs. Cabozantinib and BEZ235 blocked the PI3K signaling activity in MDSCs and reduced their immunosupppresive activity. Moreover, Cabozantinib or BEZ235 abolished secretion of multiple key cytokines produced by mCRPC cancer cells, which are known to promote MDSC activation in a paracrine manner.

Conclusions: By employing a new chimeric model of mCRPC, we demonstrated that an antibody cocktail targeting immune checkpoint proteins CTLA4 and PD1 was insufficient to generate effective antitumor response, but combination of ICB with targeted therapy Cabozantinib or BEZ235 displayed superior synergistic efficacy through impairing MDSCs in the tumor microenvironment.

Impact Statement: These observations provide a clinical path hypothesis for combined targeted and ICB therapy in mCRPC and illuminate the importance of incorporating agents with specific anti-MDSC activity yet minimal impact on T cells.

Targeting Prostate Cancer with Multi-Functional Nanoparticles

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Background: Prostate cancer is one of the most common malignancies in men, with distant metastases being a poor prognostic predictor of limited survival. Hence, there is a need for effective diagnostic and therapeutic tools that can track and treat metastatic prostate cancer. Our poly(lactide-co-glycolide) (PLGA) NPs that are being designed to target claudin-3 (Cldn3) and claudin-4 (Cldn4) tight junction proteins on prostate cancer are encapsulated with superparamagnetic iron oxide (SPIO) contrast for magnetic resonance (MR) imaging. The truncated *clostridium perfringens enterotoxin* (C-CPE) has been shown to have high affinity for Cldn3 and Cldn4 receptors that are overexpressed in prostate cancer cells, and therefore, may serve as a novel tumor-homing peptide for human prostate cancer. The goal of this project is to develop a NP system that has the specificity to target metastatic prostate cancer.

Methods: Immunohistochemistry was performed on human prostate cancer specimens by deparaffinization, rehydration, and antigen retrieval of paraffin-embedded sections. The sections were blocked in 1% animal serum, incubated overnight with primary antibodies and developed using Vector NovaRed.

Human prostate cancer PC-3 cells were transfected with Cldn3/4 siRNA for 48 and 72 hrs at concentrations of 25, 50, and 100 nM, and with control siRNA duplexes, using Lipofectamine RNAiMAX and cell viability, was measured using WST-1.

NPs were prepared by dissolving PLGA and hydrophobic SPIO in chloroform before being added dropwise to a 5% polyvinylalcohol solution while vortexing. The resulting mixture was sonicated three times for 10 s. NPs were hardened during solvent evaporation in 0.2% PVA for 3 hr.

A prostate cancer xenograft flank tumor model was developed in a murine system as a proxy for a metastatic model. Five million PC-3 cells were subcutaneously injected into the flank of Foxn1 nu/nu mice, and NPs were systematically injected for MRI and biodistribution studies. The C-CPE peptide also was injected for localization studies.

Results: Cldn3/4 were expressed in human prostate cancer specimens as well as in human prostate cancer PC-3 cells. Transfection of PC-3 cells with Cldn3/4 siRNA resulted in a 60% decrease in cell viability.

Phantom gels containing a mixture of PC-3 cells and NPs were visualized in an *in vivo* imaging system for NP uptake. In addition, a Mini 4T magnet was used to acquire MR images of NP localization to xenograft prostate tumors. Biodistribution data suggested that the NPs were localized mainly to the tumor, but also were observed in the liver, testes, seminal vesicles, and bladder. In contrast, biodistribution of the C-CPE peptide alone demonstrated a strong fluorescence accumulation in the tumor, with very little accumulation in the liver.

Conclusions: Targeting Cldn3/4 appears to select for a specific population of prostate cancer cells. In addition, knocking down Cldn3/4 *in vitro* decreased cell viability. Non-functionalized NP formulations were able to detect distant prostate tumors. However, targeting with FITC-labeled C-CPE peptide alone improved detection. Therefore, it is expected that C-CPE functionalized NPs will increase detection of advanced prostate cancers.

Impact Statement: By combining both diagnostic and therapeutic properties in a single platform, we will have the ability to generate a valuable tool for noninvasive imaging and drug delivery that can enhance the well-being of men with prostate cancer.

An Innovative Approach to Extinguish Androgen Receptor (AR) Axis Signaling in Prostate Cancer

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Background: The androgen receptor (AR) is a ligand-activated transcription factor (TF) that plays critical oncogenic roles and is a validated therapeutic target in prostate adenocarcinoma (PrCa). Medical castration therapy is the mainstay treatment for advanced PrCa, but the AR signaling axis frequently remains active in castration-resistant PrCa (CRPC). Despite advances in AR targeting with abiraterone and enzalutamide, disease progression re-occurs in the form of CRPCs that still express AR-dependent genes. This highlights the need for novel approaches to block AR signaling and CRPC growth. These ligand-independent mechanisms of AR activation cannot be inhibited by ligand-depleting (abiraterone) or LBD-targeting (enzalutamide) approaches and represent an unmet need for novel treatment approaches.

The GATA family of TFs contains six members in mammals, all of which bind a consensus DNA sequence (A/T)GATA(A/G) to regulate gene expression. GATA2 is the predominant family member in prostate luminal epithelial cells. In a search for TFs that control AR expression, we found that GATA2 induces AR gene expression as well as promotes the recruitment of coactivators to the AR transcriptional complex.

Methods: We performed immunohistochemical (IHC) analysis for GATA2 in 383 clinically localized PrCa specimens. We also examined the role of GATA2 in PrCa cell lines using siRNA and chromatin immunoprecipitation-sequencing (ChIP-Seq).

Results: GATA2 immunostaining was nuclear and stronger in malignant than in benign luminal epithelial cells. GATA2 protein expression was strongly correlated with AR protein expression (Spearman's correlation coefficient: 0.323, $P<0.0001$) in our PrCa specimens. The intensity of GATA2 immunostaining in our BCM patient cohort positively correlated with Gleason score ($R=0.37$, $p=0.008$) and was a significant predictor of biochemical recurrence ($p=0.0004$). We also demonstrated that GATA2 directly promotes expression of both full-length and splice-variant AR, resulting in a strong positive correlation between GATA2 and AR expression in both PrCa cell lines and patient specimens. Conversely, GATA2 expression is repressed by androgen and AR, suggesting a negative feedback regulatory loop that, upon androgen deprivation, derepresses GATA2 to contribute to AR overexpression in CRPC. Simultaneously, GATA2 is necessary for optimal transcriptional activity of both full-length and splice-variant AR. GATA2 colocalizes with AR and Forkhead box protein A1 (FOXA1) on chromatin to enhance recruitment of steroid receptor coactivators and formation of the transcriptional holocomplex. A GATA2 small molecule inhibitor (SMI) suppressed the expression and transcriptional function of both full-length and splice-variant AR and exerted potent anticancer activity against PrCa cell lines.

Conclusions: We propose a "first-in-field" approach to target AR expression and function, including ligand-independent AR, for the treatment of CRPC by inhibiting GATA2.

Impact Statement: Targeting the AR axis at the level of AR expression and transcriptional activity will overcome resistance caused by multiple upstream mechanisms, and, thus, will have wide therapeutic implications in CRPC. Future studies will dissect further the mechanism of anticancer activity of GATA2 SMIs in PrCa in vitro models and will evaluate their anticancer activity and safety profile in PrCa animal models.

RNA-Seq of Single Prostate CTCs Implicates Noncanonical Wnt Signaling in Antiandrogen Resistance

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Background: Prostate cancer is initially responsive to androgen deprivation therapy, but the effectiveness of androgen receptor (*AR*) targeted therapies in castration-resistant prostate cancer (CRPC) is variable. Isolation of circulating tumor cells (CTCs) in the blood may enable noninvasive tumor sampling and molecular analyses to reveal drug resistance mechanisms.

Methods: Patients with a diagnosis of prostate cancer provided informed consent to an IRB-approved protocol to donate blood for CTC analysis. Using a microfluidic device (CTC-iChip), we captured CTCs from blood and selected a total of 221 single candidate CTCs from 22 patients for micromanipulation and single cell RNA-sequencing, based on cell surface staining for epithelial (EpCAM) and mesenchymal (CDH11) markers combined with absence of staining for the leukocyte marker CD45. Of these, 133 single cells had RNA of sufficient quality for amplification and RNA-sequencing, and 77 single cells (from 13 patients) were defined as lineage-confirmed CTCs based on high expression of prostate lineage-specific genes and low expression of leukocyte transcripts. Digital gene expression profiles of CTCs were compared with each other, with primary prostate tumors, and with annotated markers of cellular signaling pathways.

Results: RNA sequencing of single prostate CTCs revealed considerable heterogeneity in transcriptional profiles, but clustered according to patient of origin, indicating higher diversity in CTCs across different individuals (mean correlation, 0.10 for CTCs within patients vs. 0.0014 for CTCs across patients, P=2.0x10E-11). Compared to primary tumors, CTCs were significantly enriched in 21 molecular pathways (FDR <0.1), with the majority implicated in growth factor, cell adhesion, and hormone signaling. Alternative splice variants of the *AR* gene, including AR-V7, were commonly expressed in CTCs from patients with CRPC, although their expression was variable among individual cells from each patient. Retrospective analysis of CTCs from patients with progressive disease despite treatment with the AR inhibitor enzalutamide exhibited activation of noncanonical Wnt signaling, compared with enzalutamide-naïve patients (P = 0.0064). Ectopic expression of WNT5A in cultured prostate cancer cells attenuates the antiproliferative effect of AR inhibition, whereas its suppression in drug-resistant cells restores partial sensitivity, a correlation also evident in an established mouse model.

Conclusions: Single cell molecular analysis of CTCs from the blood of patients with prostate cancer reveals considerable heterogeneity in the transcriptional program of each CTC, and identifies noncanonical Wnt signaling as contributing to disease progression and enzalutamide resistance. Multiple heterogeneous signaling pathways may cooperate in mediating disease progression and treatment failure in advanced prostate cancer.

Impact Statement: This study demonstrates that single-cell RNA-seq of CTCs is feasible to identify clinically relevant AR alterations and key alternative pathways associated with treatment resistance in patients with CRPC. These findings set the stage for future prospective clinical trials to evaluate novel CTC biomarkers as predictors of response to therapy and lay the groundwork for the use of non-invasive liquid biopsies to monitor the molecular status of tumors as they evolve during treatment, allowing for individualized precision therapeutic decisions for patients over time.

Semenogelin I as an Androgen Receptor Coactivator Is a Novel Molecular Target for the Treatment of Prostate Cancer

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Background: Seminal plasma proteins, semenogelins, contribute to sperm clotting, upon binding to Zn^{2+}, and can be proteolyzed by prostate-specific antigen (PSA), resulting in release of the trapped spermatozoa after ejaculation. In contrast, the role of semenogelins in the development and progression of any type of malignancy remains largely unknown. In the current project, we aim to determine biological functions of semenogelins, especially semenogelin I (SgI), in conjunction with zinc and androgen/androgen receptor (AR) signaling, in prostate cancer cells.

Methods: We assessed the expression of semenogelins in radical prostatectomy specimens by immunohistochemistry as well as in prostate cancer cell lines by quantitative PCR analysis (qPCR) and western blot (WB). We then assessed the effects of SgI overexpression in prostate cancer lines cultured in the presence or absence of zinc and dihydrotestosterone (DHT) on cell viability (by MTT assay)/invasion (by transwell assay), PSA expression (by qPCR and WB), and AR transcriptional activity (by luciferase assay). We also performed co-immunoprecipitation assay to determine AR-SgI interactions.

Results: Immunohistochemistry in tissue specimens revealed overexpression of SgI in prostatic carcinoma, which was significantly correlated with biochemical recurrence after the surgery. In prostate cancer LNCaP cells, zinc treatment was found to enhance SgI expression. Meanwhile, SgI overexpression protected AR-positive cells, but not AR-negative cells, from zinc cytotoxicity and resulted in significant increases in AR-positive cell proliferation/invasion and PSA expression. Luciferase reporter gene assay showed even slight inhibitory effects of SgI in the absence of zinc versus its significant stimulatory effects in the presence of a high level of zinc on DHT-mediated AR transactivation. Co-immunoprecipitation then demonstrated DHT-induced physical interactions between AR and SgI. Additionally, as seen in some of other steroid hormone receptor co-regulators, the LxxLL motif (L=leucine; x=any amino acids) present in SgI appeared to be critical for mediating its interaction with AR.

Conclusions: SgI was found to serve as an AR co-activator only in the presence of zinc and could thereby promote androgen-mediated prostate cancer progression.

Impact Statement: We demonstrate molecular evidence to answer why prostate cancer tissue contains high levels of zinc, which, by itself, was known to have a strong inhibitory effect on tumor growth. Furthermore, our findings may provide a new therapeutic target for androgen-sensitive prostate cancer as well as castration-resistant tumor.

Treatment Decision-making and Adherence to Active Surveillance in Prostate Cancer Patients

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Background: Active surveillance (AS) is recommended for patients with low-risk prostate cancer (LrPC) to prevent overtreatment and maintain sexual and urinary functioning. However, approximately 90% of AS-eligible patients opt for curative treatment and 25%-50% discontinue AS within 2-5 years without clinical evidence of cancer progression. Research is necessary to examine patients' unmet informational/supportive care needs and barriers and facilitators of informed decision-making and adherence to AS.

Methods: LrPC patients (N=28; mean age, 63.7 years, range: 54-74; 100% Caucasian) were on an AS protocol at the Icahn School of Medicine at Mount Sinai. Data was collected through focus groups, individual interviews, and chart review between January and May 2016. All focus groups and interviews were audio-recorded, transcribed, and analyzed using Atlas.ti software. Qualitative analyses used an immersion/crystallization approach to examine factors contributing to participants' treatment decision-making and continuation/discontinuation of AS.

Results: The majority of patients followed physicians' recommendations (90%) and few searched the Internet for additional information on AS (30%). Factors influencing patients' decisions to opt for AS include trust in their physician's expertise, good intentions, and skills in detecting cancer progression in a timely manner, as well as avoidance of sexual and urinary deterioration associated with LrPC treatment options. The partner's approval of AS played a significant role in the decision to opt for AS. Although no desire to discontinue AS or decisional regret was reported, participants reported increased anxiety at the time of clinical testing for cancer progression. Lack of information about follow-up care, AS management plan, and delays in follow-up surveillance appointments also contribute to anxiety levels. Financial barriers to AS emerged as a significant concern affecting patients' ability to stay on AS protocol in the future, despite being willing to continue AS.

Conclusions: This study provides insight into patients' treatment decision-making and adherence to AS. We recommend further examination of these issues in ethnic minorities with LrPC and their partners. Psycho-educational interventions and specialist referrals are needed to provide information, regulate emotions, and enhance disease self-management.

Impact Statement: This study has significant implications for LrPC care. Our findings suggest areas for healthcare improvements including enhancing possible system-level determinants of trust, physician-patient communication and closeness, and shared decision-making. Healthcare facilities may be able to adopt organizational changes promoting physician communication skills to increase trust and understanding of patients' and partners' values and preferences.

Gene Expression Profiling of Circulating Tumor Cells to Understand Mechanisms of Therapeutic Resistance in Advanced Prostate Cancer

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Background: The trend towards precision-based therapeutic approaches dictated by genomic alterations in a given tumor continues to offer substantial promise for men with metastatic castration resistant prostate cancer (mCRPC). However, current approaches for genomic characterization are primarily tissue-based, necessitating serial biopsies to understand changes over time and potentially limited by the challenges inherent to extracting genomic material from bone metastases. We sought to develop a circulating tumor cell (CTC)-based assay to determine gene expression across a panel of clinically relevant and potentially actionable prostate cancer-related genes.

Methods: CTCs were isolated from 5 mL whole blood using anti-EpCAM-conjugated microbeads. Following cell lysis, mRNA from CTCs was captured using Oligo(dT)25 mRNA Dynabeads followed by reverse transcription. Multiplex qPCR was then performed to evaluate a panel of 96 genes including WNT5A, TMPRSS2:ERG, SChLAP1, NKX3.1, EGFR, AR, PSA, and cytokeratins (KRT 8, 18, 19). To account for WBC contamination, blood processed from 27 healthy controls was used as a baseline referent. Gene expression data from prostate cancer cells (VCaP, PC3, and LNCaP) spiked into normal control blood was used for assay development, and CTCs from 41 patients with mCRPC were subsequently evaluated. The Halabi nomogram (JCO 2014) was utilized to account for baseline clinicopathologic variables (opioid analgesic use, disease site, ECOG PS, albumin, hemoglobin, alkaline phosphatase, and PSA). Cox regression was used to identify genes independently associated with overall survival (OS), and receiver operator curves were constructed to assess model performance.

Results: For platform validation, 300 GFP-labeled VCaP and LNCaP cells were each sorted into 5 mL of venous blood from a healthy donor and recovery was visually confirmed by microscopy. Spiking experiments were then performed using <200 cells added into 5 mL of blood. Gene expression was assessed by qPCR and was in line with the known expression profile of each cell line, confirming cell recovery and mRNA capture. For patient studies, out of 41 patients with mCRPC, we identified 27 (63%) with detectable CTCs, defined by expression of EpCAM, EGFR, KRT8, KRT18 and/or KRT19. There have been 21 deaths to date. The Halabi nomogram was strongly associated with OS (HR 1.74, 95%CI 1.20-2.54), and AURKA (HR 3.40, 95%CI 1.47-7.85), BMP7 (HR 2.10, 95%CI 1.25-3.52), and WNT5A (HR 2.71, 95%CI 1.43-5.13) were independently associated with OS after adjusting for the Halabi nomogram score ($p<0.01$, FDR<15%). A model including the Halabi nomogram, AURKA, BMP7, and WNT5A expression had an AUC of 0.92 for predicting OS at 6 months in comparison with an AUC of 0.74 for the nomogram alone.

Conclusions: The present platform for CTC analysis in men with mCRPC allows multiplex expression profiling across a panel of prostate cancer-related genes. A score based on an established nomogram plus AURKA, BMP7, and WNT5A expression in CTCs was highly prognostic and needs to be validated in additional patient cohorts. We anticipate that, with serial patient assessments over time, we will also be able to gain a better understanding of key mechanisms of resistance to therapy in men with advanced prostate cancer.

Impact Statement: Both prognostic and predictive biomarkers are needed for men with mCPRC, and this work demonstrates the potential for a liquid biopsy approach to predict OS in these patients.

Protein Synthesis-Dependent Activation of the Unfolded Protein Response Enables Prostate Cancer Development and a Druggable Target for Cancer Therapy

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Background: The acquisition of oncogenic lesions stimulates biosynthetically and bioenergetically, demanding cellular processes such as protein synthesis to drive cancer cell growth and proliferation. The hijacking of these key processes by oncogenic pathways triggers cellular stress that requires an adaptive or evasive response in order for cancer cells to survive and continue proliferating. We have previously demonstrated that deregulated protein synthesis, which is induced by oncogenic signaling pathways such as Myc and PI3K that are commonly activated in prostate cancer, activates one of the key cytoprotective stress response pathways, known as the unfolded protein response (UPR). The UPR is a cellular homeostatic program engaged when an excess of unfolded/misfolded proteins accumulate within the lumen of the endoplasmic reticulum. It is carried out by three major signaling arms: PERK, IRE1, and ATF6. However, whether and how each of these distinct signaling arms of the UPR is specifically activated by deregulated protein synthesis upon oncogenic insult is poorly understood.

Methods: Here, we show that prostate cancer initiation and maintenance, following combined loss of the PTEN tumor suppressor and overexpression of the Myc oncogene, rely on protein synthesis-dependent activation of the UPR to facilitate tumor cell survival. Specifically, using the genetic mouse model coupling PTEN loss with MYC overexpression in the prostate, we observe that overexpression of Myc synergizes with PTEN loss to dramatically stimulate the PERK and IRE1 signaling arms of the UPR pathway, which correlates with enhanced PIN formation and invasive carcinoma. To dissect the mechanism by which these oncogenic lesions promote UPR signaling, we have developed a cell culture model employing human prostate epithelial cells overexpressing MYC and harboring an shRNA targeting PTEN. Using this cell culture model, we demonstrate the activation of UPR arms PERK and IRE1 upon oncogenic transformation by Myc overexpression and loss of PTEN. Interestingly, blocking the cytoprotective UPR using PERK or IRE1 inhibitors resulted in a significant increase in cell death and decreased clonogenic potential in cells harboring both oncogenic lesions (MYC/PTEN), but not in normal cells. Furthermore, down-regulation of protein synthesis by the ATP-site mTOR inhibitor MLN0128 further attenuates the UPR response in these cells and synergistically promotes cell death in the presence of a UPR inhibitor.

Conclusions: Experiments are currently underway to test UPR inhibition in a preclinical trial utilizing our *in vivo* PTEN loss/MYC overexpression model. Furthermore, we aim to test UPR inhibition in a patient-derived xenograft (PDX) model of high-risk prostate cancer. In addition, we are utilizing gene expression analysis to understand the mechanistic connection between protein synthesis and the specific arms of the UPR. Taken together, our results suggest a critical role of the UPR in ensuring prostate cancer cell progression and serve as a promising opportunity for therapeutically targeting this cancer-specific vulnerability to stress adaptation in order to elicit synthetic lethality.

Impact Statement: Delineate the mechanisms underlying stress response pathways, in particular, the UPR, in novel mouse models (MYC hyperactivation/PTEN knock down) and human prostate cancer, and investigate novel therapies to target stress response pathways.

Racial Differences in Prostatic Vitamin D Metabolism in a Cohort of African-American and European-American Prostate Cancer Patients

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Background: African American (AA) men are disproportionately affected by prostate cancer (PCa), with 60% higher incidence and twice the mortality rate compared to European American (EA) men. Vitamin D deficiency has not only been linked to increased PCa aggressiveness and mortality; it is also more prevalent in the AA population. Therefore, it has been hypothesized that Vitamin D deficiency may contribute to the disparity in incidence and mortality of PCa between AA and EA men. All of the studies to date have relied on serum levels of 25-hydroxyvitamin D_3 ($25D_3$) to evaluate vitamin D status. However, prostate cells are capable of local production of the active hormone,$1\alpha,25(OH)_2D_3$ ($1,25D_3$), as they express the 25-hydroxylase and 1α-hydroxylase enzymes.

We hypothesized that long-term vitamin D deficiency in AA men results in differences in the prostatic vitamin D axis and response. To assess this hypothesis, the relationship between serum and prostate tissue levels of vitamin D metabolites was investigated in a racially diverse cohort of PCa patients.

Methods: Serum, whole blood, and frozen prostate tissue were obtained from 50 PCa patients (AA n=29, EA n=28) via a tissue biorepository. DNA was isolated from the whole blood and the percentage of West African ancestry was determined by single-nucleotide polymorphism (SNP) analysis. The levels of vitamin D precursor ($25D_3$) and active hormone ($1,25D_3$) were measured in the serum and tissue by LC-MS-MS. Gene expression analysis was carried out by microarray on RNA isolated from laser-capture micro-dissected epithelium from 26 patients.

Results: The serum results emulated previous studies and showed that serum concentrations of $25D_3$ were significantly lower in AA men (95% CI, 16 - 23 ng/mL) compared to EA men (95% CI, 28-38 ng/mL). Unexpectedly, prostatic concentrations of $1,25D_3$ were significantly lower in EA men (95% CI, 18-28 pg/mL) compared to AA men (95% CI, 27-40 pg/mL) whereas prostatic $25D_3$ was lower in AA men. There were significant differences in the expression of vitamin D receptor and two vitamin D metabolism enzymes. Analyses to compare gene expression and SNPs in the vitamin D metabolism genes as potential contributors to the prostatic $1,25D_3$ levels are ongoing.

Conclusions: The major finding of our study is that prostate tissue levels of vitamin D metabolites did not correlate with serum levels. We found that the AA men had higher levels of prostatic $1,25D_3$ compared to EA men despite having lower serum $25D_3$. AA and EA men had significant differences in the expression of vitamin D metabolizing enzymes, suggesting differential metabolism.

Impact Statement: These findings highlight the challenges in assessing vitamin D status in patients and demonstrate that there are differences between AA and EA men. They suggest that systemic vitamin D deficiency may lead to a compensatory response within the prostate to increase local levels of $1,25D_3$ via alterations in the vitamin D metabolism enzymes. The significance of this compensation is yet to be determined.

Identifying DNA Methylation Features that Underlie Prostate Cancer Disparities

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Background: In the U.S., there are pronounced racial/ethnic disparities in prostate cancer incidence and mortality. African Americans (AA) are more likely to be diagnosed with prostate cancer and have lower survival rates after diagnosis as compared to European Americans (EA). Ethnicity-based differences in prostate cancer risk factors are likely to result in tumor characteristics that vary by ethnicity. Understanding these ethnicity-based differences in tumor biology will improve our ability to address prostate cancer disparities. One such feature, tumor DNA methylation, is known to play a critical role in prostate carcinogenesis, but it is unknown if cancer-related DNA methylation events vary by ethnicity.

Methods: We are conducting a genome-wide study of DNA methylation patterns in prostate tumor tissue and paired normal tissue derived from both AA and EA prostate cancer patients. We will generate genome-wide DNA methylation profiles (>450,000 CpG sites) for 144 prostate cancer patients (72 AA and 72 EA with Gleason ≥7), using DNA samples derived from tumor and matched normal prostate epithelium and Illumina's HumanMethylation450 BeadChip. We will determine if DNA methylation patterns in prostate tissue (both cancer and normal) differ between AA and EA individuals. In addition, we will identify methylation features that differ between tumor and normal tissue and those that correlate with clinical features (i.e., Gleason, stage). We will also attempt to determine if variation in these epigenetic features is associated with genetic and environmental factors that vary by ethnicity.

Results: We have currently recruited >150 prostate cancer patients to our study, including >40 AA patients (for whom recruitment continues). We are currently dissecting both tumor and normal tissue to obtain DNA samples representing tumor epithelium, tumor stroma, normal epithelium, and normal stroma for all participants. Our study will work with DNA extracted from both FFPE and frozen tissue, in order to compare how these storage conditions may impact DNA methylation measurements. DNA methylation data will be generated once all DNA is collected.

Conclusions: The proposed project will create a comprehensive understanding of racial/ethnic differences in methylation patterns in both cancerous and normal prostate tissue. In conjunction with the data we're collecting on clinical features and environmental/lifestyle factors, these data are likely to yield novel insights into prostate cancer biology and etiology.

Impact Statement: Developing an understanding of ethnicity-based differences in DNA methylation in prostate tissue is a critical and necessary step towards understanding and addressing prostate cancer disparities. The features identified here can be further investigated in future studies of disparities in prostate cancer outcomes (such as biochemical recurrence and survival). The utility of such markers will need to be evaluated in larger studies of diverse prostate cancer patients.

Hypermutation in Advanced Prostate Cancer: From Mechanism to Implementation of Genomic Testing for Precision Therapy

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Background: Recent sequencing efforts have revealed that a subset of metastatic prostate cancers are hypermutated, but mechanisms that lead to hypermutation in prostate cancer and clinical implications have not been well-studied. Hypermutation due to mismatch repair deficiency associated with microsatellite instability (MSI) has been shown to predict response to immune checkpoint inhibition in other cancer types. The goal of this research is to characterize the mechanisms leading to hypermutated prostate cancer and to integrate tumor hypermutation status with clinical decision making and therapy to improve the care of men with advanced prostate cancer.

Methods: We identified hypermutated cases by exome sequencing of metastatic prostate cancer specimens obtained through a rapid autopsy program at the University of Washington. We then interrogated 30 DNA repair pathway genes in hypermutated and non-hypermutated cases using a targeted deep sequencing approach that included intronic capture. We developed a custom bioinformatics pipeline to accurately detect MSI (mSINGS) and hypermutation status from targeted sequencing data, as well as structural variation, copy number variation, and insertion/deletion mutations of all sizes. We incorporated diagnostic testing for hypermutation and MSI status in advanced prostate cancer into the UW-OncoPlex program for precision medicine.

Results: We identified 10/103 patients (9.7% of men) with hypermutated advanced prostate cancers in the UW rapid autopsy series. Targeted deep sequencing revealed mutations in mismatch DNA repair (MMR) genes in all hypermutated tumors. Unlike hypermutated colorectal and endometrial cancer, mutations were primarily in *MSH2* and/or *MSH6* (9/10) rather than *MLH1* epigenetic silencing (0/10). MMR mutations were frequently large structural rearrangements (8/10) rather than point mutations or small insertion/deletions. All hypermutated cases had associated microsatellite instability and corresponding loss of MMR protein by IHC. We next developed a novel bioinformatics approach to detect MSI and hypermutation directly from targeted next-generation assays (mSINGS) and incorporated this method into the clinically validated UW-OncoPlex platform. In a pilot study using the UW-OncoPlex assay for advanced prostate cancer patients we prospectively identified 4/53 (7.5%) men with hypermutated tumors, all of which had evidence of MSI by mSINGS, and an underlying MMR gene mutation. This work facilitated a PD-L1 inhibitor trial for men with hypermutated prostate cancer, which will open soon.

Conclusions: Hypermutation is present in up to 10% of advanced prostate cancers. Complex mutations in *MSH2* and *MSH6* are a key mechanism underlying hypermutation in advanced prostate cancer and result in microsatellite instability. Targeted genomic testing can accurately identify prostate cancer with hypermutation, MSI, and mismatch repair gene defects. Future research should focus on determining if patients with mismatch repair gene defects exhibit a distinct clinical course and are differentially responsive to immune checkpoint inhibition.

Impact Statement: The results of this research are likely to immediately and directly impact men with advanced prostate cancer by allowing accurate and cost-effective screening for hypermutation status using clinically validated genomic testing to select men for immunotherapy trials.

MERTK as a Marker of Disease Aggressiveness and Regulator of Prostate Cancer Metastasis

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Background: The molecular processes driving prostate cancer metastasis are only beginning to be understood. MERTK belongs to the TAM family of receptor tyrosine kinases and has been shown to be up-regulated in cancer and mediate metastasis in breast cancer. Here we aimed to define MERTK as a marker for prostate cancer aggressiveness and determine whether its activation can potentiate metastasis.

Methods: MERTK expression was explored in prostatectomy tissue by immunohistochemistry as well as DASL and Affy microarray, and in the serum from men with and without prostate cancer by ELISA. Expression of MERTK in prostate cancer cell lines (DU145, PC-3, LNCaP) as well as PrECs and the murine prostate was investigated by western blotting and RT-PCR in the presence and absence of Gas6, androgen deprivation, and treatment with radiation or docetaxel. A transgenic mouse was generated by cloning MERTK into the pS2S inducible vector.

Results: MERTK was found to be expressed at increased RNA and protein levels in the prostatectomy tissue of men treated locally for prostate cancer that eventually developed clinical metastasis. Serum levels of MERTK did not positively correlate with presence of cancer or clinical outcome. MERTK was expressed in prostate cancer cell lines at far greater levels than PrECs. Presence of the GAS6 ligand appeared to decrease expression of MERTK in LNCaP cell lines without significant modification in androgen-insensitive cell lines (DU145 and PC-3). MERTK levels decreased in the murine prostate upon castration could be partially restored by addition of androgen. Exposure of prostate cancer cell lines to docetaxel did not modify MERTK expression while radiation exposure appeared to decrease protein levels while increasing MERTK RNA expression; however, this requires validation. We were able to successfully generate a transgenic mouse with inducible expression of MERTK.

Conclusions: MERTK is overexpressed in prostate cancer and levels correlate positively with metastatic potential. MERTK may be regulated by androgens, but this requires further exploration. The biologic influence of MERTK on prostate cancer metastasis can be best explored in a mouse model.

Impact Statement: MERTK could potentially be used as a tissue-based marker for individuals at risk for clinical progression of localized disease. An understanding of the direct influence of MERTK on metastasis could encourage the therapeutic use of small molecule inhibition of MERTK in aggressive prostate cancer.

A Relationship between Mast Cells and the Racial Disparity of Prostate Cancer

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Background: African American (AA) men are more likely to be diagnosed with advanced prostate cancer and are nearly 2.5 times more likely to die from the disease than White (WH) men. This disparity cannot be fully accounted for by disparities in access to care, financial barriers, and socio-economic status – indicating that the increased mortality may also be due to a more aggressive molecular phenotype of the tumors of AA men. The etiological factors driving this more aggressive prostate cancer phenotype in AA men may be multifactorial, and may include underlying genetics, diet, and lifestyle factors, and environmental exposures. A very consistent finding in previous studies conducted on prostate cancer tissues from AA versus WH men is a stark discrepancy in the expression of genes involved in inflammatory pathways that are more prevalent in tumors from AA men than those of WH men. Studies have also shown that AA men are more likely to present with inflammation in benign prostate biopsies. One immune cell type of particular interest in the context of prostate cancer is mast cells, which have the potential to play a number of roles in cancer and have also been shown to increase in number in prostate cancer versus benign regions of the prostate. In the present study, we aimed to determine if there are differential numbers of mast cells in the tumor microenvironment of AA versus WH men.

Methods: We used dual-immunostaining for mast cell tryptase and epithelial cytokeratin-8 (CK8) to directly visualize and localize mast cells in the prostate microenvironment of AA and WH men in a tissue microarray (TMA) containing tumor and benign tissues from 75 AA and 75 WH men matched on tumor grade and stage. Automated counting of mast cell numbers as well as the calculation of mast cell-to-epithelial ratio (MC/Ep) and mast cell numbers per total tissue area (density) was aided by novel digital image analysis using a software framework integrating whole slide imaging, virtual microscopy, and ImageJ-based analysis algorithms.

Results: Key results to date include the finding that mast cell density in prostate tumor tissues of AA men in the TMA set analyzed was significantly lower than that of WH men ($p < 0.0001$). This same trend was observed for total mast cell numbers and MC/Ep.

Conclusions and Impact Statement: Our findings follow a similar trend with a separate study that we have been conducting to determine if mast cell numbers are predictive of PSA progression, e.g., that lower intratumoral mast cell numbers seem to predict worse outcomes (manuscript in process). These results suggest the intriguing possibility that a decrease in tumor-infiltrating mast cells may contribute to the racial disparity observed in association with prostate cancer and additional studies are underway to determine the biological function of mast cells in the prostate tumor microenvironment.

The Effects of Neuropeptides on Prostate Cancer Progression

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Background: Metastatic progression of disseminated tumor cells (**DTCs**) in the bone may be directed by influences from the bone marrow microenvironment. Full understanding of the complex interactions that result in active bone metastases, however, remains elusive. Interestingly, we have discovered that early in the metastatic process, prostate cancer (**PCa**) cells target and commandeer the specific microenvironment for hematopoietic stem cells (**HSCs**), using mechanisms similar to those involved in HSC homing. It has also been shown that the involvement of sympathetic and parasympathetic nerve fibers is crucial for both HSC maintenance and dissemination of PCa to bone, suggesting that the interactions between DTCs and nerves are at least, in part, responsible for controlling metastatic progression. Interestingly, sensory neurons which express neuropeptides, such as substance P (**SP**) and calcitonin gene-related peptide (**CGRP**), are known to innervate bone. However, the influence of neuropeptides on PCa progression, including tumor dormancy and the reactivation of the dormant cells, has not previously been examined.

Methods: The effects of neuropeptides on proliferation, the sphere-forming ability, and the migration of prostate cancer cells were assessed *in vitro*. Antibody-based cell signaling pathway arrays were performed to determine the further downstream pathways.

Results: Contrary to our expectations, SP failed to stimulate *in vitro* proliferation and sphere forming ability of the prostate cancer cell lines examined (DU145, PC3, LNCaP). On the other hand, CGRP significantly enhanced the proliferation of DU145, while little or no proliferation was observed in PC3 or LNCaP. In addition, a small molecule antagonist for CGRP, CGRP 8-37, inhibited the proliferation of DU145 mediated by CGRP. Moreover, *in vitro* migration assays revealed that DU145 migrated towards CGRP. Finally, antibody-based cell signaling pathway arrays revealed possible molecular mechanisms whereby CGRP activates the proliferation and migration of DU145.

Conclusions: Our studies suggest that CGRP influences the proliferation and migration of prostate cancer. Our next attempts will be to determine whether CGRP controls the process of bone metastasis *in vivo*, and define the molecular mechanisms involved in the crosstalk between DTCs and the bone marrow microenvironment.

Impact Statement: Results from this work will lead to a greater understanding of the mechanisms whereby the bone marrow microenvironment regulates bone metastatic progression, and could result in valuable new treatment approaches.

Circulating Tumor DNA as a Biomarker Predictive of Aggressive Disease and Early Recurrence

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Background: Liquid biopsies for the assessment of circulating tumor DNA (ctDNA) have recently emerged as a popular, minimally invasive strategy for detecting mutations that appear at high frequency in various cancer types for either the prediction of disease subtypes or selection of a targeted therapy. However, in primary prostate cancer, where recurrent point mutations occur in less than 10% of patients, this strategy is not feasible. However, we hypothesize that assessing for ctDNA may identify patients with high-grade disease who might benefit from surgical or radiological intervention and, in the post-radical prostatectomy (RP) setting, indicate which patients are at high risk of recurrence. In preliminary experiments, we established the feasibility of using *a priori* sequencing of the cancer to enable allele-specific identification of ctDNA molecules, on a patient-specific basis, to test these hypotheses. Critical to this assay is identifying mutations that are truncal and clonal, which emerged early during tumor initiation, and thus would most likely be present in micrometastases and ctDNA. In this preliminary group, we also detected ctDNA when PSA levels were below the limit of detection. In the subset of men with high grade (Gleason score $\geq$7) who recur, as well in all patients who have undergone RP, our objective is to assess how often the detection of ctDNA correlates with the appearance of adverse pathologic features in the RP specimen, and determine the precision interval at which detection of ctDNA precedes PSA levels rising.

Methods: This prospective study aims to enroll a total of 100 men who will provide plasma specimens prior to RP and every 3 months for 3 years following RP. We are collecting blood in Streck BCT tubes. A pathologist maps the extent of tumor in the entire RP specimen and selects blocks containing tumor tissue that corresponds to the index lesion. We perform laser capture microdissection (LCM) of multiple foci of tissue from multiple blocks, and then perform whole genome sequencing of these multiple regions. Using computational and empiric approaches, we identify the mutations that are truncal. We then use these mutations to design patient-specific assays for detecting ctDNA. In these assays, we achieve precise enumeration of ctDNA molecules by performing a single cycle of PCR with primers containing unique molecular identifiers and a short region of the Illumina adaptor sequence, followed by library amplification and paired-end Illumina sequencing. Bioinformatic approaches count the number of distinct ctDNA molecules present in each sample.

Results: We have received multi-site IRB approval for this project and have begun to enroll subjects. LCM and sequencing of tumor specimens is ongoing. No subjects from this study have yet recurred. At this meeting, we hope to report on the status of sequencing serial ctDNA from at least 3 patients with high-risk disease.

Conclusions: None.

Impact Statement: If our first hypothesis is correct, higher levels of circulating tumor DNA may identify those men who are more likely to have a more aggressive prostate cancer and would benefit from immediate surgery rather than enrolling in active surveillance. In addition, if circulating tumor DNA is a more sensitive and accurate biomarker than PSA for detecting recurrent disease in men who have undergone surgery, earlier salvage radiation therapy or hormonal therapy may improve overall survival.

Targeting TMPRSS2-ERG Using High-Throughput Gene Expression Profiling

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Background: The TMPRSS2-ERG translocation is present in approximately half of all prostate cancers and results in aberrant ERG overexpression and subsequent tumorigenesis. Despite being an attractive therapeutic target, transcription factors such as ERG have been difficult to target with small molecules as they lack active sites and function primarily through protein-protein interaction. To address these challenges, we used a novel high throughput gene expression method to measure ERG activity in prostate cancer cells. We used this method to measure the effect of inhibiting 800 kinases by RNAi to identify kinases that modulate ERG activity. To identify small molecules that bind to and inhibit ERG activity, we tested the activity of compounds that scored from a small molecule microarray. Lastly, we measured the effect of a set of novel small molecules from a diversity oriented synthesis (DOS) collection.

Methods: We used a novel high throughput gene expression method referred to as L1000 (unpublished). The L1000 assay measures the transcript levels of a set of 1,000 genes using barcoded nucleotide probes. The probes were chosen based on gene expression correlations from publicly available gene expression microarray data. By knowing the expression of these representative 1,000 genes, the expression of the remaining 14,000 genes can be inferred computationally. We used this assay to profile prostate cancer cells that were transduced with viruses expressing shRNA or treated with small molecules. We then compared the expression profiles with a reference signature of ERG activity to identify shRNAs or small molecules that modulate ERG activity.

Results: Using a kinome lentiviral shRNA library, we identified multiple kinases involved in ERG signaling including the p38 and protein kinase C pathways. Small molecule microarrays were used to identify small molecule interactors with ERG that are currently being tested in secondary assays. We confirmed one small molecule that inhibits ERG transcription by inhibiting AR-dependent transcription of ERG.

Conclusions: High throughput gene expression profiling can be used to identify genetic and chemical modulators of ERG activity in order to provide new insight into ERG biology as well as target its function with small molecules.

Impact Statement: Identification of kinases or small molecules that modulate ERG activity could serve as potential leads for novel drugs for prostate cancer. Although multiple lines of evidence have demonstrated an essential role of ERG in prostate cancer, currently there are no drugs that target its activity. If successful, our approach may also be generalizable to any potential cancer target.

Targeting WAS Protein Family Member 3 to Suppress Prostate Cancer Metastasis

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Background: Death due to prostate cancer (PCa) results largely from metastatic spread of the disease. Defining novel proteins and pathways that lead to metastatic progression will expand opportunity to provide systemic adjuvant therapies during the early stages to limit the spread of cancer cells. One such pathway involves hyperactivation of WAS protein family member 3 (WASF3), which promotes PCa metastasis, and inactivating it can suppress invasion and metastasis of PCa cells. Although there are no drugs that target WASF3 directly, WASF3 regulates many pathways and targeting critical nodes in these pathways using genetic approaches can suppress metastasis. Drugs are available to target the same nodes in these pathways but these drugs have not been considered in the context of metastasis. In this study, we aim to investigate whether drugs that target WASF3 regulatory network can be purposed in an attempt to suppress PCa metastasis.

Methods: Transwell invasion assays were used to determine the drug effects on cell invasion. Immunoprecipitation and Western blot analysis were used to determine the WASF3 levels after drug treatment. Zebrafish-metastasis models were used to screen and identify the synergistic treatment that inhibits metastasis. The efficacy of the best performing drugs was validated using mouse models of PCa.

Results: The SRC inhibitor Dasatinib and the tubulin inhibitor CYT997 suppress PCa cell invasion more potently than other examined drugs that are proposed to target WASF3-dependent molecular pathways. Mechanistic studies found that CYT997 significantly increases phosphor-activation of WASF3, while Dasatinib produces an opposite effect on WASF3. Intriguingly, Dasatinib effectively antagonizes WASF3 activation by CYT997 in PCa cells. Using Zebrafish models, we demonstrate that treatment of either Dasatinib or CYT997 inhibits PCa cells to metastatic spread throughout fish body. Most importantly, the combination of these two drugs prevents cells from metastasizing more efficiently than either drug alone. We then transferred this synergistic treatment from zebrafish to mouse and evaluated the anti-metastasis activity in the NSG-xenograft model. Consistent with the observations from zebrafish, Dasatinib significantly improves CYT997 efficacy against metastasis in mouse.

Conclusions: These findings suggest that the synergistic strategy by co-treatment of Dasatinib and CYT997 is a potential therapeutic for metastatic PCa.

Impact: WASF3 is a very compelling target to limit metastasis. Suppressing WASF3-dependent metastasis or significantly delaying it will lead to improvement in overall survival of cancer patients. Our study has significant impact on the design and execution of effective therapy of patients with high risk or metastatic PCa.

Clonal Evaluation of Prostate Cancer Foci in Biopsies with Discontinuous Tumor Involvement by Dual ERG/SPINK1 Immunohistochemistry

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Background: The presence of two or more prostate cancer foci separated by intervening benign tissue in a single core is a well-recognized finding on prostate biopsy. Cancer involvement can be measured by including intervening benign tissue or only including the actual cancer involved area. Importantly, this parameter is a common enrollment criterion for active surveillance protocols. We hypothesized that spatially distinct prostate cancer foci in biopsies may arise from separate clones, impacting cancer involvement assessment.

Methods: We used dual ERG/SPINK1 immunohistochemistry to determine the frequency of separate clones—when separate tumor foci showed discordant ERG and/or SPINK1 status—in discontinuously involved prostate biopsy cores from two academic institutions.

Results: In our cohort of 97 prostate biopsy cores with spatially discrete tumor foci (from 80 patients), discontinuous cancer involvement including intervening tissue ranged from 20 to 100% and Gleason scores ranged from 6 to 9. Twenty four (25%) of 97 discontinuously involved cores harbored clonally distinct cancer foci by discordant ERG and/or SPINK1 expression status: 58% (14/24) had one ERG^{+} focus, and one ERG^{-}/$SPINK1^{-}$ focus; 29% (7/24) had one $SPINK1^{+}$ focus and one ERG^{-}/$SPINK1^{-}$ focus; and 13% (3/24) had one ERG^{+} focus and one $SPINK1^{+}$ focus. ERG and SPINK1 overexpression were mutually exclusive in all tumor foci.

Conclusions: Approximately 25% of discontinuously involved prostate biopsy cores showed tumor foci with discordant ERG/SPINK1 status, consistent with multiclonal disease.

Impact Statement: The relatively frequent presence of multiclonality in discontinuously involved prostate biopsy cores impacts assessment of tumor volume and active surveillance eligibility. The remainder of our project will focus on assessing the clinical impact of multiclonality in other scenarios from additional multi-institutional cohorts.

Function and Clinical Utility of the Hox Protein Co-Factors Meis1 and Meis2

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Background: The exciting identification of germline HoxB13(G84E) mutations within a subset of familial prostate cancers highlights a novel pathway to understand prostate tumor etiology and develop new treatment modalities to combat prostate tumor initiation and progression. The G84E mutation is located within the Meis-interacting domain of HoxB13 and thus emphasizes the importance of Meis-Hox protein interactions in prostate tumor biology. Independent bioinformatic, clinical, and molecular analyses conducted by our lab demonstrated that Meis proteins also have a significant role in prostate cancer. Hence, there is a clear need to elucidate the function of Meis proteins in normal prostate epithelial cells and the role of Meis proteins in prostate cancer. Our objective is to elucidate the function of Meis1 and Meis2 in prostate cancer initiation and progression and to identify compounds that increase their expression.

Methods: Meis1/2 expression was used to stratify outcomes of men with Gleason 7 tumors in a retrospective bioinformatics analysis of microarray data obtained from the Swedish Watchful Waiting cohort. RNA expression in normal and cancerous cells and tissues was determined by qRT-PCR. The impact of re-expression of Flag-tagged Meis proteins in prostate cancer cells was measured. The expression of Meis in response to de-methylating agents, inhibitors of histone acetylation, and Androgen Receptor pathway modulation was measured. Publicly available RNAseq data from primary prostate tumors and metastases were analyzed for changes in Meis and Hox expression.

Results: First, Meis1 and Meis2 expression is decreased in prostate tumors and cancer cell lines compared to normal prostate epithelial cells. Second, re-expression of either Meis1 or Meis2 in prostate cancer cells suppresses growth. Third, RNA sequencing demonstrated multiple Meis2 splice variants in normal and malignant prostate cells, including the presence of the dominant-negative type E isoform. This E isoform lacks a DNA-binding domain and can thus bind and prevent DNA-binding of partnering Hox proteins. When expressed in LNCaP and CWR-22Rv1 cells, Meis2E did not suppress cell growth. Fourth, Meis1 expression appears to be suppressed by promoter methylation, while Meis2 expression is suppressed by histone de-acetylation and AR pathway activation. Fifth, decreased Meis expression in Gleason 7 tumors resulted in a 40 month decreased in overall survival compared to Gleason 7 tumor with high Meis expression. Furthermore, decreased Meis2 was associated with metastatic progression.

Conclusions: These data implicate Meis proteins, their partnering Hox co-factors, and the genes regulated by Meis/Hox proteins as novel prostate cancer tumor suppressor pathways. Increased understanding of Meis and HoxB13 functional interaction, mechanisms leading to decreased Meis expression, and the functional role of loss of Meis expression in poor-prognosis tumors will be a crucial steps toward elucidating the predictive and therapeutic role of Meis expression in prostate cancer initiation and progression.

Impact: This work has the potential for significant impact as it will elucidate Meis-mediated mechanisms contributing to prostate cancer formation and progression, thus implicating Meis proteins, and genes regulated by Meis proteins, as targets for therapy and biomarkers to predict metastatic progression.

Multiregion Analysis Demonstrates Local Enrichment of Multiple Clinically Significant Genetic Alterations within Index Foci of Localized Prostate Cancer

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Background: Prostate cancer is a multifocal disease, with early divergence or complete independence between spatially distinct foci. Yet, metastatic, lethal prostate cancer is typically derived from a single index focus. Given its clinical importance, we sought to evaluate the genomic heterogeneity of the index focus by studying the clonal relationships of spatially separate regions of contiguous index foci.

Methods: We performed exome sequencing and low-coverage whole genome sequencing to identify single nucleotide variants and copy number variation in the index focus of ten cases of potentially lethal, localized prostate cancer, with up to nine separate tumor regions sampled per index focus.

Results: Exome sequencing demonstrated branched evolution characterized by marked heterogeneity among regions sampled, with 91% of mutations being subclonal. Early mutations included those in known prostate cancer driver genes, *SPOP*, *FOXA1*, *TP53*, and *PTEN*. Copy number analysis also demonstrated branched evolution, and multiple examples of convergent evolution were identified, both in non-cancer and known cancer genes. Mutation and copy number analysis demonstrated subclones are comingled within multiple regions, and within an individual region branch and trunk mutations can be found in subtotal cell fractions. Importantly, multiple locally enriched subclonal alterations (average 11.5/ case) were identified in genes altered in advanced disease with statistical or clinical significance, including genes in androgen receptor, cell cycle, PI3K, Ras/Raf, WNT, DNA repair, and chromatin pathways.

Conclusions: There is marked genomic heterogeneity within an individual, contiguous index focus. Most known prostate cancer driver mutations are early events and identified in most regions of the focus. Mutations in other cancer genes, and alterations in pathways that are altered in advanced disease, are typically subclonal events found in one or a few regions. These locally enriched, subclonal events likely contribute to therapy resistance.

Impact Statement: Our research supports a model whereby advanced disease emerges from enrichment of pre-existing subclones, rather than the accumulation of additional variants, in response to therapeutic pressure. Moreover, it is difficult to identify clinically relevant subclones, and predictions regarding targets for future therapy should be made with caution.

The Development of Peptide-Based Positron Emission Tomography Agents for Prostate Cancer Imaging

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Background: A physician's ability to detect early stage prostate cancer (PCa) and predict which patients will develop aggressive disease is fundamental to reducing PCa mortality rates. However, the heterogeneity of PCa tumors has made developing successful agents for clinical imaging problematic.

Tumor associated fibroblasts (TAFs) represent a major component of a PCa tumor, and play a critical role in tumor development. However, unlike PCa cells, they are not transformed genetically and differ from normal fibroblasts found in benign adult tissues in morphology and gene and protein expression profiles. Fibroblast activation protein alpha (FAP) is one such protein that is expressed on TAFs, in over 90% of common epithelial cancers including PCa, but is undetectable in benign, adult tissues. Accordingly, positron emission tomography (PET) radiopharmaceuticals, which target FAP, would enable physicians to more accurately diagnose and stage PCa using currently approved, clinical imaging technologies.

Methods: Peptide conjugates designed to target the FAP active site were synthesized using standard solid phase peptide synthesis. Conjugate specificity for the FAP active site was evaluated using a plate-based Fluorescence Resonance Energy Transfer (FRET) assay. Peptide conjugates were radiolabeled with copper-64 according to known protocols and their stability in human serum was evaluated.

Results: All conjugates demonstrated specificity for the FAP active site and could be easily radiolabeled with copper-64. All agents were stable in human serum at physiological temperature and did not show evidence of degradation for more than three days.

Conclusions: All agents demonstrated specificity for the FAP active site and excellent stability *in vitro*. The most promising radiopharmaceuticals are now being tested to determine biodistribution in an appropriate mouse model bearing FAP^+ tumors.

Impact Statement: Successful completion of this research proposal will positively impact efforts to reduce PCa mortality rates in four ways:

(1) It creates new tools that will give researchers valuable insight in PCa tumor biology by allowing them to non-invasively image the interactions between tumor cells and the tumor stroma.

(2) It will provide the foundation for developing a new generation of clinical PET agents, which target the stable expression of FAP on TAFs in the PCa tumor stroma.

(3) It will demonstrate the ability of cryo-microscopy, a new multimodal imaging technique, to enhance PET radiopharmaceutical development by more effectively unifying preclinical and clinical data to dramatically accelerate new compounds into clinical trials.

(4) The distinct expression of FAP provides a new targeting paradigm for the non-invasive detection of cancer using current imaging technologies.

Design and Development of Prostate Cancer-Responsive Nanotherapeutics

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Background: Advanced prostate cancer frequently leads to skeletal complications that are very difficult to treat and result in pain, bone fractures, nerve compression, morbidity, and often mortality, and it is considered to be an incurable disease. Our research has addressed such a challenge by innovative design of bio-responsive and prostate-specific drug delivery systems. Our research strategy is based on recent advances in the development and optimization of prostate-specific antigen (PSA) that have provided more specific ligands to prostate cancer. The major objective from our research is to develop effective therapeutic approaches that are favourably responsive to prostate cancer-induced microenvironment, thus improving prostate cancer therapy.

Methods: Three approaches in order to improve specificity and responsiveness of nanotherapeutics to prostate cancer have been designed. The first one is to take advantage of a prostate-specific membrane antigen (PSMA) with high affinity (K_i = 8 nm), namely 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid (DUPA). The second approach is to utilize a PSA-cleavable peptide, specifically Arg-Ser-Ser-Tyr-Tyr-Ser-Leu-Lys, as a linker for selectively releasing an anticancer agent (docetaxel, DTX) from nanotherapeutics. The third approach is to explore a charge reversal strategy by using a substrate possessing both features of PSA-specificity and bone-targeting capacity. The chemical and biophysical properties of nanotherapeutics, including chemical structure and composition, size, surface charge, drug loading capacity, drug release profile, and binding affinity to hydroxylapatite, have been determined. The nanotherapeutics have further been investigated in terms of cellular uptake and cytotoxicity in cultured prostate cells. Evaluation of nanotherapeutics for biodistribution and antitumor efficacy in animals is ongoing.

Results: The designed biomaterials have been successfully synthesized. DTX has been loaded into biopolymers via a chemical conjugation strategy with approximately 10% drug loading content in polymers. Their molecular structures have been confirmed by nuclear magnetic resonance. The obtained biomaterials are able to spontaneously form nanosized constructs with the size of approximately 100 nm. DUPA-modified block polymer has shown moderate improvement over controls in terms of cellular uptake by a PSA-expressing prostate cancer cell line (LNCaP). Interestingly, Arg-Ser-Ser-Tyr-Tyr-Ser-Leu-Lys modified biopolymer has shown selective and effective drug release in LNCaP cells. More importantly, after functionalization with a substrate containing both polyaspartic acid and PSA-cleavable peptide, nanotherapeutics not only exhibit rapid hydroxyl apatite binding capacity, but also demonstrate surface charge reversal profile, indicating their potential application for prostate cancer-induced bone metastasis.

Conclusions: The combination of bone-targeting moiety and PSA-cleavable linker in nanotherapeutics is a feasible and promising strategy to improve their bioavailability, skeleton-selectivity, and PSA-specificity for prostate cancer therapy.

Impact Statement: The encouraging results from this study may prompt the development of bone-targeted, enzyme-triggered drug delivery systems to improve their affinity to skeletal tissue, enhance selectivity for prostate cancer, and improve efficacy of anti-cancer agents, thus facilitating development of therapeutic strategy for prostate cancer.

Resistance Mechanisms of Prostate Cancer: Identification of Master Regulators of Advanced and Castrate-Resistant Prostate Cancer under Treatment with Abiraterone

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Background: Curative treatment options for advanced prostate cancer (PCa) are limited; androgen-deprivation therapy has been a mainstay of palliative therapy. Abiraterone (A) is approved for the treatment of castrate-resistant PCa. Most patients will ultimately fail this treatment, while the resistance mechanisms are not understood. Our hypothesis is that genetic/epigenetic features may be different between patients that respond to A versus those that do not, and these features may change under the treatment with A; their identification would give insights into the cause for resistance to this drug. Interrogating interactomes and identifying master regulator genes before and after treatment, and comparing them between different responders would allow us to better understand the disease process.

Methods: Castrated nude mice with LuCaP35CR xenograft tumors were treated with A orally, and tumor growth was compared to the control group. After harvesting the tumors, immunohistochemistry and -fluorescence were performed to determine expression patterns of typical prostate cancer and epithelial markers, and tissue was submitted for RNA sequencing.

Results: Tumors were harvested at time of resistance. Histologically, the treatment arm showed slightly more stromal features, neoangiogenesis, and fibroblast invasion. Ki67 was only marginally detected in either group. CK8 and 18 were vastly positive, Cleaved Caspase 3 was only minimally detected. Both groups showed significant PSA tissue expression. On IHC, androgen receptor (AR) expression in the A group was mainly cytoplasmic; AR expression in the control group was more nuclear. CYP17A1 was vastly expressed in both groups; it was seen significantly overexpressed in the A group. Synaptophysin and Chromogranin A were seen expressed significantly greater in the A group. RNA sequencing was subsequently performed, which did not yield conclusive results on underlying master regulators responsible for resistance mechanisms.

Conclusions: Histopathological analyses, IF, and IHC confirmed inherent PCa features under treatment with A. Proliferative ability and lack of apoptotic markers in the xenograft tissue without significant difference between both A and control group signified the proliferative potential of tissue in the A arm in the setting of gained resistance to the drug. Positive staining for neuroendocrine markers in the A arm only indicated that neuroendocrine features might play a role in gaining resistance to A. Certainly, this will be taken into consideration during our currently ongoing RNA sequencing analyses of subsequent xenograft studies for genomic profiling and bioinformatic analyses.

Impact Statement: The major impact of our studies will be a comprehensive understanding of different response rates to the treatment with Abiraterone, and the interrogation of interactomes involved in resistance mechanisms to A will help us understand underlying resistance mechanisms. These would then allow us to distinguish better between more indolent versus more aggressive cancers. Our broader long-term goal will be the identification of master regulator genes that are responsible for these resistance mechanisms. Identifying these genes, and validating them using tissue microarrays to assess their relevance for human cancer, as well as investigating their functional significance, would then allow us to contribute to develop new drugs that target PCa differently and more effectively.

Monoamine Oxidase A Promotes Prostate Cancer Neuromimicry by Activation of Neurotrophic and Axon Guidance Genes

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Background: Perineural invasion (PNI), a complex process of neoplastic nerve invasion, has been recognized as a significant route for prostate cancer (PCa) metastasis and associated with poor clinical outcomes. Recent studies, including ours, have shown that PCa cells develop neuromimicry by expressing neuronal genes and exhibiting neuron-like phenotypes, which enable cancer cells to mimic and reciprocally interact with nerves to promote metastatic spread in and along nerves. This represents a new opportunity to develop novel therapeutics for PCa metastasis.

Methods: PCa tissue microarrays (N=74) were used for immunohistochemical and quantum dot labeling (QDL) analyses of monoamine oxidase A (MAOA) expression, a key enzyme metabolizing monoamine neurotransmitters, to explore its association with PNI. A separate clinical cohort of both early- and late-stage PCa cases (N=65), including paired normal/tumor tissues, underwent whole-genome and -transcriptome sequencing analysis to identify novel disease-related genetic alterations. MAOA overexpression in human PCa PC-3 cells allowed us to determine the expression of downstream genes mediating axonal growth and migration by qPCR, Western blot, and ELISA. The expression of select adrenergic and cholinergic receptors known to promote PNI and PCa metastasis was also assessed in prostate stromal cells from wild-type/MAOA-knockout and vehicle-/clorgyline (an MAOA inhibitor)-treated mice by qPCR. In addition, orthotopic xenograft models were established by using PC-3 (vector and MAOA-overexpression) and $ARCaP_M$ (control and MAOA-knockdown) PCa cells to determine MAOA-mediated prostate tumor growth, invasion, and metastases in mice, with nerve fiber densities within tumors further assessed by QDL analyses of nerve-specific markers.

Results: Increased tumor MAOA expression was associated with PNI in PCa patient samples, with intense expression observed in (1) nerve-invasive tumor areas in the entire cohort and (2) PNI areas relative to other non-PNI cancerous areas in the same patient sample. Sequencing analysis identified axon guidance pathway genes, such as semaphorin signaling-related genes, to have the highest genetic alteration rate in >70% samples. Overexpression of MAOA increased the secretion of nerve growth factor threefold to fourfold and also activated select class 3 semaphorin signaling molecules, including semaphorin 3C, Plexin A2, and neuropilin 1 (NRP1), which act as the ligand, receptor, and co-receptor, respectively, in PC-3 cells. We further confirmed a positive co-expression correlation between MAOA and NRP1 in clinical samples and demonstrated that NRP1 is a downstream effector mediating MAO's function in PCa cells. Additionally, genetic deletion or pharmacological inhibition of MAOA in mouse prostate stromal cells reduced the expression of select adrenergic (Adrβ2 and Adrβ3) and cholinergic (Chrm1) receptors. The role of MAOA in mediating prostate tumor growth/progression and tumor-nerve interactions in xenograft mouse models will be further discussed.

Conclusions: We demonstrated that MAOA and its associated neurotrophic and axon guidance genes induce PCa neuromimicry and may serve as a driver promoting perineural PCa metastasis.

Impact Statement: Our results will provide insights into understanding how PCa neuromimicry mediates PCa-nerve communications and also lay mechanistic foundations on developing rational strategies for improved treatment of PCa metastasis.

A Selective and Active Mechanism for Androgen Uptake by Prostate Cancer Cells

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Background: Androgen-stimulated androgen receptor (AR) activity is crucial to proliferation of prostate cancer (CaP) cells. However, how CaP cells take up androgens remains unclear. **C**onventional wisdom is that androgens enter the cells by passive diffusion, which is not an efficient mechanism for regulating cellular availability and homeostasis of important steroid hormones. Accumulation of androgens by CaP cells suggested the existence of an active transport system for the uptake of androgens by CaP cells. Verification and characterization of an androgen uptake mechanism is important for understanding androgen-AR signaling-associated CaP biology, with respect to development and growth of castration-recurrent CaP (CRPC). Androgen deprivation therapy (ADT) remains an effective modality of treatment for locally advanced and metastatic CaP. Despite the favorable initial response, most CaPs progress to CRPC, which is lethal. Intracrine steroidogenesis in CaP cells produces the testicular androgens testosterone (T) or dihydrotestosterone (DHT) to sustain AR activity. Following ADT, CaP cells may rely on active uptake mechanisms to maintain AR activity using residual circulating T or DHT, and obtain adrenal androgens as precursors for intracrine synthesis. In this study, CaP cell lines were used to characterize underlying mechanisms for androgen uptake.

Methods: Prostate cancer cell lines were treated with ^{3}H-labeled androgens to determine the kinetics of androgen uptake. Treated cells were rinsed and lysed in lysis buffer. Radioactivity of cell lysates were assessed using a scintillation counter. Non-radioactive steroids were used as competitors for ^{3}H-T uptake. Chemical inhibitors were used to obtain clues for potential uptake mediators.

Results: Uptake of T was mediated by an active and high affinity transport mechanism. Uptake of T, DHT, estradiol, and progesterone shared the same or similar transport mechanism. Uptake of adrenal androgens was mediated by different mechanisms. An uptake mechanism for T may be driven by ATP and mediated by membrane proteins that reside in the lipid rafts.

Conclusions: Androgen uptake is mediated by an active and selective transport mechanism on the cell membrane.

Impact Statement: An active androgen uptake mechanism may provide CaP cells the ability to increase AR activity by accumulating more intracellular androgens. Androgen uptake mechanisms may be crucial for CaP cells to sustain AR activity after ADT by scavenging residual testicular androgens or obtain adrenal androgens for intracrine production of T/DHT. Targeting uptake mechanisms offers a new approach for a more complete ADT. In addition, differences in the active uptake mechanism may contribute to racial disparity in CaP. Compared to European-American (EA) men, African-American (AA) men suffer higher incidence of, and greater mortality rate from CaP. AA men tend to have CaP diagnosed at more advanced stages and at younger ages. Racial differences in CaP aggressiveness are evidenced by findings of studies on disease characteristics at diagnosis and studies on cancer progression or recurrence. CaP cells in AA men may be equipped with the ability to acquire more androgen for AR stimulation, which leads to AR stabilization and hence over-expression, and more aggressive CaP.

TRAF4-mediated AR Ubiquitination and Castration-Resistant Prostate Cancer

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Background: It is now well-recognized that AR remains to be a critical player in castration-resistant prostate cancers. Reactivation of AR in CRPC is usually linked to AR amplification, overexpression, or hypersensitivity to low androgen levels. It was suggested that the function of AR in CRPC is not to turn on the same transcriptional targeted genes in the absence of androgen but to turn on a distinct set of genes independent of androgen. However, it was not clear what triggers the functional switch of AR, leading to androgen-independent growth. Here we report another pathway to bypass androgen dependency through AR ubiquitination.

Methods: AR ubiquitination was identified using mass spectrometry and site-directed mutagenesis. The expression levels of AR target genes were assessed through real time RT-PCR.

Results: We found that TRAF4, a RING domain E3 ubiquitin ligase, is overexpressed in CRPCs. Overexpression of TRAF4 but not its RING domain deletion mutant in LNCaP cells promoted androgen-independent cell growth. Through mass spectrometry we identified AR as a TRAF4-interacting protein and further found that TRAF4 promoted AR ubiquitination at the C-terminal tail. It enhanced AR targeted gene UBE2C and CDC20 expression, which are important for CRPC progression. The expression levels of canonical AR regulated genes such as PSA and TMPRSS2 are not altered by TRAF4.

Conclusions: Through an uncommon K27 ubiquitin linkage, TRAF4 overexpression alters AR-regulated transcription profile, leading to the activation of a distinct set of genes and resulting in androgen-independent cell growth.

Impact Statement: Our study revealed a novel pathway regulating AR activity that is important for CRPC progression. Results from our study will provide potential therapeutic targets for treating CRPCs.

Phosphatidylserine-Targeting Antibody Enhances the Antitumor Responses to Programmed Death -1 Blockade in a Mouse Model of Prostate Cancer

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Background: Phosphatidylserine is a phospholipid normally residing in the inner leaflet of the plasma membrane and becomes exposed on tumor vascular endothelial cells and tumor cells. Phosphatidylserine exposure becomes enhanced in response to chemotherapy, irradiation, and oxidative stresses in the tumor microenvironment. Phosphatidylserine exposure in tumors promotes an immunosuppressive microenvironment, which includes the recruitment of myeloid derived suppressor cells (MDSCs), immature dendritic cells, and M2-like macrophages as well as the production of anti-inflammatory cytokines. The objective of this research is to test if blockade of the distinct inhibitory signaling induced by phosphatidylserine and programmed death-1 (PD-1) results in therapeutic synergy against prostate cancer.

Methods: We evaluated phosphatidylserine-targeting antibody combined with anti-PD-1 antibody in an established murine transgenic adenocarcinoma of mouse prostate (TRAMP)-C2 prostate tumor model. Antitumor effect was determined by tumor growth inhibition. Changes in the presence of tumor-infiltrating lymphocytes were assessed by flow cytometry. The generation of a tumor-specific immune response was assessed by splenocyte-produced interferon gamma.

Results: Our data show that treatment with phosphatidylserine-targeting antibody significantly enhances the antitumor activity of anti-PD-1 antibody. Tumor growth inhibition correlates with infiltration of immune active cytolytic T-cells in tumors and reduction of tumor-infiltrating MDSC. Combination of phosphatidylserine blockade and anti-PD-1 significantly increases production of interferon gamma. Toxicity to the mice was not observed.

Conclusions: Antibody therapy targeting phosphatidylserine-associated immunosuppression can significantly enhance immunotherapy targeting the PD-1 pathway in murine castration-resistant prostate cancer.

Impact Statement: These results establish a novel paradigm in which targeting phosphatidylserine and PD-1 synergize to control antitumor immune responses, thus providing a rationale for targeting phosphatidylserine in combination with other immune checkpoint pathways for long-term control of advanced prostate cancer.

Immunotherapeutic Targeting of Prostate Cancer Using a Small Molecule Ligand

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Background: Despite the recent progress in the development of chemotherapies for the treatment of prostate cancer, hormone refractory prostate cancer (HRPC) still contributes to a large number of mortalities each year. Therefore, the development of novel targeted therapies that can eliminate refractory prostate cancer cells, independent of mechanisms that lead to chemotherapy resistance, is highly desirable. Towards this end, we have exploited the use of a small molecule ligand for prostate specific membrane antigen (PSMA) named "DUPA" – originally developed as an imaging agent for prostate cancer – to redirect T cell-based immunotherapies. In the first proof of concept we demonstrated that a bispecific antibody-like molecule can be developed by conjugating DUPA to an anti-CD3 antibody (DUPA-CD3) to redirect native T cells to lyse and eliminate PSMA-positive prostate cancer cells. The objective of this work is to demonstrate the DUPA ligand can be used to redirect highly potent, chimeric antigen receptor-engineered (CAR-T) cells using a novel switchable technology that allows tunable control over the engineered cells.

Methods: The switchable CAR-T cell (sCAR-T) platform is a two-component system in which an scFv is incorporated into the CAR, which recognizes a non-endogenous peptide or ligand. The peptide or ligand is then fused to the DUPA molecule to form a bridge (referred to as the "switch") between the sCAR-T cell and PSMA-positive tumor cell. In this way, the activity of the sCAR-T cell is strictly controlled by the dosage of the switch and can be adjusted for maximum efficacy while avoiding toxicities related to over- or off-target activation of the engineered cell.

Results: For our bispecific molecule, we have developed a humanized, cynomolgus monkey cross reactive anti-CD3 bispecific-DUPA conjugate and demonstrated its efficacy in traditional xenografts and primary, patient-derived xenografts (PDX). This candidate is currently entering into GMP manufacture and is scheduled to be tested in a clinical trial in the next 18-24 months. Preliminary data for translating DUPA-targeting to the sCAR-T system have demonstrated single-digit picomolar potency for lysis of PSMA-positive prostate cancer cells *in vitro* and good preliminary data in xenograft models. We are currently evaluating this platform in expanded xenograft models and PDX models.

Conclusions: The DUPA ligand can be effectively used as a seek-and-destroy small molecule for prostate cancer when coupled to agents that can redirect either native or engineered T cells. The application of this molecule to immunotherapy is expected to be effective in HRPC for its ability to circumvent chemotherapy-resistance mechanisms and eliminate hard-to-kill cancer stem cells.

Impact Statement: The switchable CAR-T cell technology proposed here provides a single, universal design that can be used in any patient. We expect that the universality of this platform will ultimately decrease treatment costs associated with cellular therapy by standardizing cell manufacture and treatment regimens. Thus, we expect the number of patients that can benefit from cell therapy will increase due to the ability to treat high-risk patient groups who would not otherwise be eligible for conventional CAR-T cell therapy due to inherent risks.

Arginyltransferase1 Regulates Prostate Cancer Progression

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Background: Post-translational arginylation, mediated by Arginyltransferase (Ate1), is an important protein modification involved in stress response yet remains poorly studied. Our lab recently reported that a loss of Ate1 is sufficient to induce tumorigenesis and loss of contact inhibition in fibroblasts. However, the role of Ate1 in cancer initiation and progression is not clear. This study examines the effects of Ate1 loss in prostate cancer models of tumorigenesis and progression.

Methods: Genetic knockout (KO) and shRNA-mediated knockdown (KD); cellular viability assay; matrigel invasion assay, soft-agar growth assay; orthotopic xenografts in immune-compromised mice.

Results: By KO and KD of Ate1 in human and mouse fibroblasts, as well as in yeasts, we found that Ate1 is required for growth arrest and cell death following oxidative stress, hypoxia, UV and gamma radiation, and apoptosis-inducing drugs. In prostate cancer cell line LnCap, a reduction of Ate1 leads to increased colony formation. In another prostate cancer cell line, PC3, a down-regulation of Ate1 leads to increased resistance to H_2O_2-induced cell death as well as increased invasiveness in matrigel, similar to those of PC3-ML, a highly metastatic cell line derived from PC3. More importantly, a down-regulation of Ate1 in PC3 is sufficient to induce local and remote metastasis from orthotopic xenografts in prostate of immune-compromised mouse. In addition, a survey of patient samples suggested that a significant down-regulation of Ate1 is specifically seen in metastatic sites from prostate cancer.

Conclusions: Our data suggests that the loss of Ate1 in prostate cancer serves to stimulate cancer progression including metastasis.

Impact Statement: Our research is expected to reveal a previously unknown mechanism driving prostate cancer progression. The results of our research has the potential to lead to novel biomarkers for predicting the metastatic potential of prostate cancer, which is one of the most important issues in prostate cancer patient care.