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TITLE: A Changing Landscape of Advanced Prostate Cancer: Understanding Mechanisms of Resistance to Potent Hormonal Therapies

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14. ABSTRACT: Transition to a neuroendocrine prostate cancer (NEPC) phenotype has emerged as an important mechanism of treatment resistance to androgen receptor (AR) therapies for patients with metastatic prostate cancer. During the course of this Award, I have performed extensive, first–in-field molecular characterization of metastatic tumor biopsies from 81 patients with castration resistant adenocarcinoma and neuroendocrine prostate cancer (Beltran et al, *Nature Medicine, in press*). Whole exome, transcriptome, and CpG DNA methylation integrative analyses point to key drivers of NEPC including loss of RB1 and TP53 and primarily epigenetic changes. Clonality analysis of serial tumor biopsies in individual patients provides new insights into mechanisms of progression, favoring a model most consistent with divergent clonal evolution of NEPC from an adenocarcinoma precursor. These datasets represent the largest clinically annotated Biorepository of NEPC. As part of this Award, I have also evaluated circulating tumor cells (CTCs) from patients treated with abiraterone and enzalutamide for emergence of NEPC CTC characteristics and found that up to 10% harbor NEPC-like CTCs (characterized by low AR, smaller morphology, loss of CK), and the presence of NEPC CTCs was associated with poor prognostic features (Beltran et al, *Clinical Cancer Research, in press*). This has potential clinical implications for early detection, prognostication, and identification of patients less likely to respond to subsequent AR-targeted therapies.

15. SUBJECT TERMS: Prostate Cancer, AR independence, Neuroendocrine prostate cancer, Treatment resistance, Circulating tumor cells, Biomarkers

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1. **INTRODUCTION:** Although patients with castration resistant prostate cancer (CRPC) have, by definition, castrate levels of circulating testosterone, most advanced prostate tumors continue to remain dependent on androgens and on signaling from the androgen receptor (AR). Hence, it is generally accepted that most CRPC tumors are not truly hormone refractory. Based on this understanding, several new highly potent AR-targeted therapies have recently entered widespread clinical use for the treatment of patients with metastatic CRPC. While exciting, these drugs are not curative, and all patients ultimately develop resistance. Therefore, the clinical and molecular landscape of advanced prostate cancer is changing. Based on preliminary clinical and preclinical data, we posit two possible mechanisms of resistance to potent AR-targeting in CRPC: (1) continued activation of AR signaling through the development of additional AR alterations (AR gene amplification, alternative splicing, AR-activating gene mutations); and (2) the use of alternative genetically driven signaling pathways that lead to transformation to an AR-independent castration resistant neuroendocrine prostate cancer (CRPC-NE) (Beltran et al, CCR 2014). The purpose of this proposal is to systematically evaluate mechanisms of prostate cancer resistance to AR-targeted therapies using deep sequencing techniques of metastatic CRPC(Adeno) and CRPC-NE and non-invasively by evaluating circulating tumor cells (CTCs).

2. **KEYWORDS:** advanced prostate cancer, androgen receptor, resistance, abiraterone, neuroendocrine prostate cancer, circulating tumor cells, genomics, biomarkers

3. **OVERALL PROJECT SUMMARY:**

   **Aim 1.** To identify molecular determinants of acquired resistance to potent AR targeted therapies. The working hypothesis of this Aim is that advanced prostate tumors acquire genetic alterations in response to newer potent AR targeted therapies that enable them to continue to grow and proliferate. We will perform massively parallel whole exome sequencing of tumor tissue from abiraterone resistant prostate cancers to determine the spectrum of mutations associated with resistance to AR targeted therapies.

   **Aim 2.** To prospectively evaluate circulating tumor cells (CTCs) from patients receiving potent hormonal therapies for acquisition of gene alterations in response to therapy. The working hypothesis of this Aim is that evaluation of CTCs may provide a non-invasive method to detect genomic alterations of key genes that occur before or may be acquired on therapy that predict response or resistance. We will analyze CTCs from patients prior to starting abiraterone, during treatment, and at progression for gene amplification of AR, Aurora kinase A, and N-myc, and correlate with clinical response to therapy.

   **Aim 3.** To evaluate high-risk primary prostate tumors for mutations that may predispose to resistance to AR targeted therapy with comparison to matched metastatic tumors. The working hypothesis of this Aim is that specific genetic alterations occur early and predispose to the development of treatment resistance to AR targeted therapies, and these may be detected at the time of initial diagnosis in high-risk primary prostate tumors.

   **Aim 1 Progress:**
   I have been systematically evaluating different time points during treatment with potent AR targeted therapy and during progression from a hormone naive prostate adenocarcinoma to an AR-driven castration resistant adenocarcinoma (CRPC-Adeno) and/or AR-independent CRPC-NE. Metastatic biopsies have not been considered standard of care for patients with advanced disease; therefore this effort has required prospective enrollment of patients on a research protocol with informed consent. During Year 1, I developed an IRB protocol to perform metastatic biopsies and whole exome sequencing (WES) and other molecular analyses of tumor and germline DNA from patients with advanced disease and to follow patients prospectively to evaluate for response to subsequent therapies, optional re-biopsy at progression, long term follow up for PFS and OS endpoints, and optional participation in a rapid autopsy program. The design and initial results of this protocol were recently reported in Beltran et al, JAMA Oncology 2015. In addition, as study chair of a multi-center Phase 2 trial of MLN8237 for patients with neuroendocrine prostate cancer, I have obtained 60 pre-treatment metastatic tissue biopsies and blood samples from CRPC-NE patients. In Year 2, I focused on integrative clinical and molecular data analysis of these metastatic biopsies. I presented these data as an oral abstract.
presentation at the ASCO Annual Meeting (Beltran et al, ASCO 2015) and the manuscript is now in press in *Nature Medicine*, highlighting the interest of this study by the clinical and research communities.

We interrogated 114 metastatic tumor specimens from 81 patients including 51 patients with clinical and histologic features of castration resistant adenocarcinoma (CRPC-Adeno), 30 with CRPC-NE as confirmed by pathologic consensus criteria (Epstein et al, AJCP 2014), and 17 patients with multiple tumor biopsies. We hypothesized that CRPC-NE could be distinguished from CRPC-Adeno based on distinct molecular alterations and could thereby improve on the current often challenging diagnostic features reliant on morphology, and CRPC-NE that develops after therapy arises clonally from a CRPC-Adeno precursor (rather than selection of pre-existing neuroendocrine clones). We also hypothesized that CRPC-NE-specific molecular alterations are shared with select AR-independent prostate adenocarcinomas, which may represent tumors at high risk for progression or in transition. Biopsies were obtained from a wide range of metastatic sites, with a predominance of bone biopsies in CRPC-Adeno compared to CRPC-NE (Figure 1a). As expected, CRPC-NE demonstrated on average lower protein expression of the AR by immunohistochemistry. We also quantified AR signaling status by mRNA expression using a previously defined AR signature (Hieronymous et al, Nature Med 2005) and observed overall lower AR signaling in CRPC-NE compared to CRPC-Adeno (Figure 1b); however, there was significant overlap with a wide range of values observed within each subtype, suggesting that AR signaling alone is insufficient for subtyping metastatic tumors.

The mutational landscape of CRPC-NE was similar to CRPC-Adeno (Figure 1c), but also consistent with published studies of CRPC-NE including enrichment of *RB1* loss (deleted in 70% of CRPC-NE and 32% of CRPC-Adeno, p=0.003) and mutation or deletion of *TP53* (66.7% CRPC-NE versus 31.4% CRPC-Adeno, p=0.0043). Loss of *RB1* is common in primary small cell prostate and lung carcinomas, and promotes small cell carcinoma pathogenesis when concurrent with *TP53* mutation; in our series, concurrent *RB1* and *TP53* loss was present in 53.3% of CRPC-NE vs.13.7% of CRPC-Adeno (p<0.0004).
Another distinguishing feature of CRPC-NE compared to CRPC-Adeno was a paucity of somatic alterations involving the AR gene (p<0.0001). Genomic amplification, activating point mutations, and splice variants involving the AR are commonly observed in CRPC-Adeno and associated with treatment resistance to AR-directed therapies (Robinson et al, Cell 2014). This observation was confirmed in our cohort: 29 cases showed AR focal amplification or point mutation and 21 cases had alterations in known AR co-activators (FOXA1, NCOR1/2, ZBTB16). In contrast, AR point mutations were notably absent in CRPC-NE and gains when present were of low level and explained by tumor polyplody (Figure 1d). Although potentially affected by differences in prior therapies, we speculate that the absence of AR genomic alterations in CRPC-NE may be due to clonal selection of non-amplified CRPC-Adeno tumor subpopulations through selective pressure (in the context of AR-directed therapies).

The overall spectrum of genomic alterations was similar between the two castration resistant subtypes. For example, there were no significant differences in rate of non-silent point mutations, polyplody or copy number genomic burden between CRPC-Adeno and CRPC-NE (on average more than 30% of the genome was aberrant), and both subtypes showed a significantly higher number of clonal point mutations compared with clinically localized prostate cancer. The median number of non-silent SNVs in metastatic samples was 41 (range: 2-729). Five of the six samples with the highest number of SNVs (115-663) showed genetic and/or protein expression alterations involving DNA mismatch repair genes consistent with prior studies.

The significant overlap between CRPC-Adeno and CRPC-NE in terms of the overall somatic copy number landscape was noteworthy in light of the marked genomic differences between adenocarcinomas and small cell carcinomas in other tissue subtypes (e.g., lung, gastrointestinal tract). After correcting for admixture of non-tumor cells and for ploidy, we sought to identify regions of the genome differentially altered and noted deletions that are enriched in CRPC-NE and conversely preferential regions of gain in CRPC-Adeno (Fig 1e). The CYLD gene, deleted in 51% of CRPC-NE samples, encodes cylindromatosis, a deubiquinating enzyme reported as a tumor suppressor involved in negative regulation of multiple signaling pathways. We found that loss of CYLD is associated with decreased mRNA expression and a modest decrease in expression of AR signaling genes in this study as well as the CRPC SU2C/PCF cohort (Robinson et al, Cell 2014) and in cell lines, suggesting that CYLD loss alone may be insufficient but could cooperate with other alterations to promote AR-indifference. Extending the computational framework of CLONET (Prandi et al, Genome Biol 2014) to assess allele specific copy number clonality, we found both focal and broad copy-neutral or copy-aberrant loss of heterozygosity across our cohort.

To elucidate complex patterns of genetic evolution and infer clonal expansion dynamics, we studied serial tumor samples from individual patients during the course of their disease. Patient WCMC7520 underwent prostatectomy for clinically localized Gleason 9 prostate adenocarcinoma with local lymph node involvement treated initially with adjuvant androgen deprivation therapy (ADT) followed by chemotherapy at the time of metastatic disease and castration resistance. 29 months after starting ADT, he developed CRPC-NE diagnosed by pelvic soft tissue biopsy. Primary, lymph node, and CRPC-NE metastases from two time-points were evaluated. Homozygous deletion of BRCA2 and mutation of TP53 were present in all sites suggesting a common ancestor, while DNA allele specific analysis highlighted diverse genomic states of other key genes such as MYCN. MYCN, which encodes the N-myc oncogene, has been previously described as oncogenic in CRPC-NE. When sites were compared, the patient’s primary prostate harbored lesions that suggested divergent
but clonal paths towards lymph node and both distant metastases. However, from these data we cannot definitively rule out metastasis →to-metastasis seeding as recently suggested as a mechanism of tumor progression. Another patient, WCMC161, progressed after multiple lines of therapy for CRPC-Adeno including the development of new visceral metastases while on abiraterone and liver biopsy showing small cell carcinoma. Comparison of three different time points: CRPC-Adeno (adenocarcinoma, lymph node metastasis), CRPC-Adeno (adenocarcinoma, bone metastasis), and CRPC-NE (small cell carcinoma, liver metastasis at progression on abiraterone therapy) suggested divergent clonal evolution suggesting that the bone and liver metastases did not arise from the earlier lymph node.

Figure 2. Tracing CRPC-NE emergence through allele specific analysis. (a) Potential models of evolution that occur during prostate cancer progression towards the neuroendocrine phenotype: linear progression from primary untreated adenocarcinoma to CRPC-Adeno to CRPC-NE; independent progression of two distinct clonal populations within the primary or metastatic CRPC-Adeno towards either CRPC-Adeno or CRPC-NE; divergent clonal evolution of CRPC-NE from either primary adenocarcinoma or CRPC-Adeno. *indicates favored model. (b) Allele specific analysis of primary prostate adenocarcinoma and local lymph node metastasis removed at time of radical prostatectomy (RP) and two metastatic CRPC-NE (treated) tumors (3 years after RP) from subject WCMC7520. H&E pathology images and intervening therapies are shown in the timeline. Scale bars, 100 µm. (c) Allele specific analysis of tumors at three time points from patient subject WCMC161 during castration resistance: lymph node (CRPC-Adeno), bone biopsy (CRPC-Adeno), and liver biopsy (small cell CRPC-NE). H&E pathology images and intervening therapies are shown in the timeline. ADT= androgen deprivation therapy; EP= etoposide and cisplatin chemotherapy; Abi= abiraterone acetate with prednisone. Circos plots summarize genome-wide allele specific DNA quantity in tumor cells. Individual’s tumor phylogeny sketched upon allele-specific analysis including genome-wide amplification and ploidy assessment.

While informative, the observed DNA changes did not appear to fully explain the clinical aggressiveness of CRPC-NE. We therefore posited that this phenotype may also be mediated by epigenetic changes. Towards this end, we generated data to evaluate CpG-rich methylation genome wide by single cytosine resolution DNA methylation (eRRBS). In contrast to the largely similar genomic data, the CRPC-NE and CRPC-Adeno subtypes showed strong epigenetic segregation by unsupervised analysis using unselected methylation sites (Figure 3a). This raised the possibility that the transition to, or advent of, the CRPC-NE subtype is associated with epigenetic dysregulation. In fact, the epigenetic signal comprised an even stronger classifier than standard pathologic classification, as evidenced by the fact that it encompassed three cases that were initially binned as adenocarcinoma based on standard pathology. All three of these patients demonstrated radiographic progression in the setting of a stable or low serum level of the androgen-regulated protein prostate specific antigen (PSA). These data suggest that clustering prediction based on DNA methylation may provide additional information associated with AR independence and CRPC-NE that improves on tumor morphology.
By functional enrichment analysis evaluating regions of differential methylation, we identified epigenetically dysregulated pathways (Figure 3b) which included neuronal, cell-to-cell adhesion, developmental, epithelium-mesenchymal transition (EMT), and stem cell programs, which are pathways relevant to CRPC-NE progression. Amongst significant findings, we also observed hypermethylation and under-expression of the tumor suppressor gene SPDEF in CRPC-NE (Figure 3c). SPDEF (prostate-derived Ets factor) is a transcriptional activator and regulator of cellular differentiation involved in suppression of tumor metastasis through inhibition of EMT in prostate cancer. The DNA methyltransferase EZH2 was significantly overexpressed in CRPC-NE compared to CRPC-Adeno (p<10^-6, Wilcoxon test) (Figure 3d) and verified at the protein level, adding to its differential status we initially reported comparing CRPC-NE to localized prostate cancer which has since been confirmed by another study. Furthermore, EZH2 target genes are also downregulated in CRPC-NE. Treatment of cell lines with the EZH2 inhibitor GSK343 resulted in a preferential decrease in cellular viability in NCI-H660 compared to other prostate cancer cell lines with significant down-regulation of CRPC-NE associated genes after treatment including CD56, MYCN, and PEG10. Overall these data support a key role of the epigenome in promoting the emergence of CRPC-NE following AR-directed therapy.

Based on these findings and the current gap in the clinical and molecular assessment of CRPC-NE, we developed a 70 gene molecular classifier to potentially improve upon the often challenging clinical diagnosis of CRPC-NE that relies on pathologic features. This integrated neuroendocrine prostate cancer (NEPC) classifier was developed by exploiting expression data of genes prioritized by genomic, transcriptomic or epigenomic
status (Fig. 4a,b) and demonstrated both a precision and recall of >0.99 in identifying CRPC-NE in our discovery cohort (Fig. 4c,d). Interrogation of transcriptome data of 683 prostate samples using datasets from The Cancer Genome Atlas (TCGA 2015), Grasso et al 2012 (Michigan 2012), Robinson et al 2014 (SU2C/PCF, 2015), and internal published data (Beltran et al 2011) revealed an elevated CRPC-NE score in up to 8% of metastatic tumors (n=191) and none of treatment naïve prostate adenocarcinoma (n=460) or benign prostate (n=32) (Figure 4d). Of those with markedly elevated CRPC-NE score, we reviewed the pathology and found over 80% had pathologic features of CRPC-NE. Across datasets, the integrated neuroendocrine prostate cancer (NEPC) classifier demonstrated superior precision and recall compared to other variables such as AR mRNA expression, AR signaling status, or highly ranked differentially expressed genes (such as SPDEF) alone. Though recognizing the potential influences of prior therapies and the tumor microenvironment on gene expression changes, we posit that castration resistant tumors with moderate or rising CRPC-NE scores may potentially represent tumors with AR independent features either in transition or at high risk for CRPC-NE progression under treatment pressure with AR therapies. In fact, we found that a subpopulation of prostate adenocarcinoma cells (LNCaP) treated long term with enzalutamide demonstrated molecular features of CRPC-NE. These findings warrant larger prospective clinical evaluation to verify whether this classifier could be useful as a potential prognostic or predictive biomarker (associated with lack of response to AR therapies).

Incorporation of different layers helps apply the classifier to different datasets when only parts are available (DNA, RNA, or methylation) and paves the way for future studies that might apply the classifier to other methods (such as circulating tumor DNA) and formalin fixed tissues that are not highly suitable for extensive analyses. For instance, if CRPC-NE alterations could be detected earlier during CRPC-Adeno disease progression, such individuals could be selected for CRPC-NE-directed rather than AR-targeted systemic therapies or co-targeting therapeutic approaches. Further, these data set the stage for dynamic testing of the reversibility of the CRPC-NE state with early intervention or genetic/epigenetic modifiers possibly with EZH2 inhibitors.

In this comprehensive analysis of metastatic treatment resistant prostate cancer, we define an emerging subclass of advanced prostate cancer that undergoes neuroendocrine reprogramming during the course of AR targeted therapy with primarily epigenetic aberrations driving distinct signaling and differentiation characteristics raising the possibility of a class switch from CRPC-Adeno to CRPC-NE. Our data supports divergent evolution of CRPC-NE from one or more CRPC-Adeno cells (adaptation) rather than linear or independent clonal evolution, with selective pressure of AR-wild-type subclonal populations and acquisition of new genomic and epigenomic drivers associated with decreased AR signaling and epithelial plasticity. However, there are also other possibilities that cannot be fully excluded, such as de-differentiation of adenocarcinoma to a more progenitor-like cell state (with some cells subsequently adopting neuroendocrine features due to local effects). We identify a molecular classifier that improves on the current poorly defined diagnostic criteria based on heterogeneous morphologies and clinical features seen in CRPC-NE. Clinical evaluation of the CRPC-NE classifier as a potential biomarker to aid in the earlier detection of AR independence and patient selection for co-targeting approaches has broader implications for precision medicine and understanding the adaptive response mechanisms to targeted therapies that can occur in the advanced cancer setting. Overall, I feel that I have made tremendous progress in recruiting patients and in analysis of metastatic tumors from a large number of advanced prostate cancer patients during Year 2. I think that this information from valuable tissue specimens will become an important data resource to help fuel additional research in the field once made publically available. These data were recently accepted for publication the data should be publically available soon (Beltran et al, Nature Medicine, in press).
Figure 4: Integrative DNA, RNA and Methylation analysis. (a) Weighted Venn diagram with the number of protein-coding genes significantly differentially observed in the three data layers. The superimposed pie chart reports the estimation of the impact of each layer upon the following priority rule: methylation overall and DNA over RNA. (b) Integrated NEPC score analysis across 604 samples from four different RNA-Seq prostate cancer datasets (This Study, SU2C/PCF 2015, WCMC 2011/2014 and TCGA). Samples are ordered by decreasing values of Integrated NEPC score (only). Top, annotation tracks report original dataset and pathology classification. Middle, plot reports Integrated NEPC score (black line) and AR signaling (grey line) across samples. Bottom, heat map of normalized FPKMs for a selection of the 70 genes (in rows) across samples (in columns). (c) Prediction accuracy of CRPC-NE samples by precision and recall statistics for Integrated NEPC Score (circles), AR signaling (squares), mRNA level of SPDEF (diamonds), AR (triangles) in RNA-seq datasets: This Study (green), SU2C/PCF 2015 (orange), WCMC 2011/14 (violet) and all datasets (black). Grey curves represent F-measure levels, defined as the harmonic mean of precision and recall. Due to the absence of CRPC-NE samples (positive events), TCGA data were not reported here. (d) AR signaling versus Integrated NEPC score across 730 samples from five independent prostate datasets using transcriptome data as proxy. The old-rose shaded area refers to significant values of Integrated NEPC Score. Predicted CRPC-NE percentages calculated by excluding benign samples.

Aim 2 Progress:
I have been working to assess patients for circulating tumor cell (CTC) characteristics to help distinguish neuroendocrine prostate cancer (NEPC) patients. The diagnosis of NEPC remains challenging and currently relies on a combination of pathologic and clinical features suggestive of AR signaling independence. Before NEPC develops, metastatic tumor biopsies often show mixed features with both adenocarcinoma and neuroendocrine carcinoma cells present. There are no reliable serum markers to consistently diagnose patients...
transforming to the NEPC phenotype and the incidence of circulating tumor cells (CTCs) in these patients is unknown. Detection of NEPC has clinical implications, as NEPC patients would not be expected to respond well to currently approved AR-targeted therapies for CRPC and may be better served by therapies specifically directed to NEPC. CTCs provide the potential for non-invasive, real-time molecular characterization of cancer in patients with metastatic disease. To date, the only FDA-cleared test for CTC detection and enumeration is the CellSearch® technology, based on immunomagnetic enrichment of CTCs expressing the epithelial cell adhesion molecule (EpCAM). Several other platforms have recently been developed to improve sensitivity of CTC detection, most of which include enrichment and/or other physical selection methods (Ozkumar et al, 2013; Yap et al, 2014). There is mounting evidence that non-traditional populations of CTCs also exist, including EpCAM/cytokeratin (CK)-negative CTCs (Yu et al, 2013) and/or cells smaller in size than traditional CTCs, some even smaller than neighboring white blood cells (Philips et al, 2014). The Epic Sciences platform is a non-selection based platform that characterizes all nucleated cells and identifies CTCs based on a multi-parametric digital pathology process identifying abnormal cells among the normal white blood cells utilizing protein expression and cell morpholog. This technique has demonstrated the ability to identify distinct CTC populations including traditional (CK+, CD45-), apoptotic, CK-negative, and CTC clusters (Marrinucci et al, 2012; Werner et al, 2015). We aimed to characterize CTCs from patients with CRPC and NEPC utilizing the Epic platform and correlate results with patient-matched tumor biopsy and clinical features.

CTC collection
Under IRB approved protocols at Weill Cornell Medical College and Memorial Sloan Kettering Cancer Center, patients with metastatic CRPC including those with pure or mixed NEPC were prospectively enrolled. NEPC was defined by the presence of either a pure or mixed small cell high-grade neuroendocrine carcinoma histology in a metastatic tumor biopsy and confirmed by at least 20% positive immunohistochemical staining for a neuroendocrine marker (synaptophysin, chromogranin). CRPC was defined clinically, with or without a metastatic biopsy confirming prostate adenocarcinoma. CRPC patients were sub-classified as atypical CRPC if the biopsy showed adenocarcinoma and the patient had clinical features suggestive of an AR independent transition which included radiographic progression in the setting of a low PSA <1 ng/ml, visceral progression in the absence of PSA progression (defined by Prostate Cancer Working Group 2 criteria and/or elevated serum chromogranin A >3X upper limit of normal.

Clinical demographics including prior therapies, sites of metastases, PSA, serum neuroendocrine marker levels, and CTC number (CellSearch®, Raritan, NJ) were collected. Blood (10 mL) from each subject was shipped to Epic Sciences within 48 hours and processed immediately on arrival. Red blood cells were lysed, approximately 3 million nucleated blood cells dispensed onto 10-16 glass slides as previously described and placed at -80°C for long term storage.

CTC identification
Two slides from each patient were evaluated by immunofluorescence (IF) (Figure 5A) using antibodies targeting cytokeratins (CK), CD45, AR, and 4',6-diamidino-2-phenylindole (DAPI) counterstain. Slides were imaged using a platform that captures all 3 million cells per slide in less than 15 minutes, and analyzed by a proprietary software that characterizes each cell by parameters including cell size, shape, nuclear area, presence of macronucleoli, CK and AR expression, uniformity and cellular localization. CTC candidates were identified in an interactive report, reviewed by trained technicians. CK+/CD45- cells with intact, DAPI+ nuclei exhibiting tumor-associated morphologies were classified as traditional CTCs. CTCs with non-traditional characteristics were recorded, such as CK-/CD45- cells with morphological distinction and/or AR positivity, CK+/CD45-small cells, CTC clusters, CTCs with multiple macronucleoli and apoptotic CTCs (with nuclear or cytoplasmic fragmentation).

Pathologic evaluation
Patient-matched metastatic tumor biopsies were reviewed by two anatomic genitourinary pathologists and classified as adenocarcinoma or NEPC based on presence of either pure or mixed small cell high grade neuroendocrine carcinoma histology in a metastatic tumor biopsy and confirmed by at least 20% positive immunohistochemical (IHC) staining for the neuroendocrine marker chromogranin and/or synaptophysin. IHC was quantified on scale 0-3 and positive IHC was defined as any staining intensity seen of target cells above.
background. To assess AURKA amplification, we used a locus specific probe plus reference probe FISH assay as previously described (Beltran et al, 2011).

**Statistical Analysis**
CTC morphological/molecular data and clinical information were compiled into patient datasets (NEPC, CRPC, atypical CRPC) using KNIME, where cytokeratin expression, AR expression, presence of clusters and various nuclear and cytoplasmic morphological features were analyzed with single cell resolution. Kernel density estimates (KDE) of each CTC characteristic were performed to provide univariate distributions across each aggregate subtype. Patient samples were analyzed for frequency of cell types at calculated cell counts per mL of blood, and univariate distributions of CTC biomarkers were compared at the patient level for each diagnostic category. Supervised learning was performed using the Random Forest classifier algorithm (R package ‘randomForest’) built with 1,001 decision trees and configured to provide a probability output.

**Leave-One-Out Cross-Validation**
To evaluate the robustness of the Random Forest classifier, leave-one-out cross-validation was performed; CTCs from patients with atypical CRPC were removed from analysis, while CTCs from NEPC were labeled NEPC+ and CRPC were labeled NEPC-. For each blood tube, CTCs from every other sample were used to train a classifier, and CTCs from the blood tube being evaluated were held-out as a test set. CTCs from the test set were analyzed by the trained classifier, where the output is an estimated probability of class membership to NEPC+ and NEPC- for each CTC belonging to the held-out sample. This cycle was repeated iteratively for each sample, and the classifier output was collected at the end of each iteration. The criteria for patient-level class membership was established as at least 3 CTCs with a p(NEPC) score greater than 0.95.

**Atypical CRPC and Contemporary Cohort Analysis**
A classifier was first trained on NEPC and CRPC samples, without atypical CRPC samples. This classifier was then used to classify the atypical CRPC sample CTCs, as well as CTCs from a 159 patient validation cohort. In the validation cohort, the same criteria for patient positivity (at least 3 CTCs with p(NEPC) greater than 0.95) was applied to generate patient-level predictions from the classifier’s single-cell output. KDE curves were used to plot the distribution of NEPC+ class membership values for individual CTCs for each patient.

**Results:**
CTCs from 27 patients with metastatic prostate cancer were evaluated. The patients identified either pathologically as NEPC (n=12) or clinically as atypical CRPC (n=5) as defined above demonstrated a higher frequency of liver metastases and lower PSA compared to other CRPC patients. Overall, bone metastases were present in 24/27 (88.9%) of patients, and liver metastases were present in 8/12 (66.7%) of NEPC and 5/15 (33.3%) of CRPC of whom 4 had atypical clinical features. Median serum PSA level was 1.9 ng/ml in NEPC, 2.8 ng/ml in atypical CRPC, and 53.4 ng/ml in other CRPC patients. Serum neuroendocrine marker levels varied considerably within the NEPC subgroup and were also elevated in cases of CRPC.

**CTCs in NEPC vs. CRPC**
Enumeration of CTCs using both the CellSearch and Epic platforms was performed. Of note, 6/13 evaluated NEPC and atypical CRPC patients had CellSearch® CTC count of <5 CTC/7.5 mL (range 0-384, with 5 of these 13 patients having a CellSearch® CTC count of 0). In contrast, all 17 NEPC and atypical CRPC patients had CTCs ≥5 CTC/7.5mL using the Epic platform. Further characterization of the detected CTCs revealed heterogeneity of cytokeratin (CK) and AR expression in both NEPC and CRPC, with a significantly greater proportion of CK-negative and AR-negative CTCs in NEPC compared to CRPC (Figures 5-6). CTCs in NEPC patients overall had lower AR expression, higher cytoplasmic circularity, and higher nuclear to cytoplasmic ratio. The prevalence of CK-negative CTC subpopulations in NEPC patients is potentially consistent with a proposed epithelial-mesenchymal-transition (EMT).
Figure 5: (A) Epic platform workflow starting from a single blood tube to the identification and characterization of all nucleated cells. Steps include 1) Blood lysed, nucleated cells from blood sample placed onto slides; 2) Slides stored in -80C biorepository; 3) Slides stained with CK, CD45, DAPI and AR; 4) Slides scanned; 5) Multi-parametric digital pathology algorithms run; 6) Software and human reader confirmation of CTCs & quantitation of biomarker expression; (B) observed CK Expression and (C) AR Expression of each CTC subtype; traditional CTC (blue), clustered CTCs (green), apoptotic CTCs (red ) and CK- CTCs (purple) from each patient sample organized by their clinical diagnosis from biopsy.

Within the NEPC subgroup, there was a greater proportion of small cell CTCs in patients with metastatic biopsy confirming small cell carcinoma highlighting phenotypic similarities between tumor and CTCs. CTCs were tested by IF for the presence of the neuroendocrine marker CD56 (Figure 7A). Of those samples matched with metastatic biopsy showing neuroendocrine features, detectable CTCs, 7/12 (58%) had ≥1 CD56+ CTC and 0/8 (0%) non-neuroendocrine samples with detectable CTCs had ≥1 CD56+ CTC. Of patient samples with small cell carcinoma pathology by tumor biopsy, 5/7 (71%) had ≥1 CD56+ CTC. A confusion matrix demonstrates high specificity for small-cell NEPC patients, demonstrating concordance to tumor tissue. Additional molecular characterization of these CTCs using fluorescence in situ hybridization (FISH) for AURKA, a gene commonly amplified in NEPC (Beltran et al, 2011), showed concordance with matched metastatic biopsies in selected cases but was not present in all cases or all cells in positive cases.

Based on the observed differences in CTCs between groups, we sought to identify CTC characteristics specific to NEPC, as described in the Methods. KDE analysis of the patient groups’ CTCs in aggregate revealed significant differences in CK, AR and morphological characteristics when compared to CRPC (Figures 6-7B).

Figure 6: (Top panel) The kernel density estimate (KDE) curves for Cytokeratin Expression (left) and Androgen Receptor Expression (right) for CTCs aggregated from all NEPC (red), CRPC (blue), and atypical CRPC (green) patient samples. (Top Right Panel) (Lower Panel) Representative CTC images from patients with NEPC and CRPC, shown with the classifier output which is the estimated probability of the CTC’s class membership as NEPC+.

Identification of NEPC CTCs
To demonstrate the diagnostic potential of CTC characteristics in distinguishing NEPC, the observed differences between NEPC and CRPC were used to train a Random Forest classifier. From the density curve, the samples from patients with NEPC demonstrated a spike in the curves near the high end of the p(NEPC+) spectrum, with many curves peaking near a p(NEPC+) score of 95%. The number of CTCs/mL with p(NEPC+) scores greater than or equal to 95% are presented in a bar chart for each patient sample, where each column is colored by the actual clinical diagnosis that the classifier is trying to predict. Obtaining positive signals at the CTC level from samples that the classifier does not encounter during training demonstrates the classifier’s ability to detect NEPC from CRPC in a robust manner that mitigates the risk of over-fitting. These conditions simulate the environment that the classifier would face in practice, in the sense that any future blood sample sent in for NEPC analysis is presented to the algorithm as a series of CTCs that it has not encountered during training, which the classifier will then estimate the probability of class membership for each CTC from the new sample.
Figure 7: (A) Representative images of CTCs from CRPC and NEPC patients evaluated by IF for CD56 expression. All CTCs evaluated from CRPC patients were CD56 negative. Heterogeneous expression of CD56 was observed within and among NEPC patient samples. CTCs including CK+/CD56- (A-D, F), CK-/CD56+ (E), CK+/CD56+ (G,H), small CK+/CD56+ (G), and CK+/CD56+ clusters (I) were identified in patient samples. CRPC: Patient 21, CD56 negative B Patient 23, small CD56 negative, C Patient 23, CD56 negative, D Patient 28, CD56 negative NEPC: E Patient 24, small CK- CD56 positive, F Patient 12, CD56 negative, G Patient 12, CD56 positive, H Patient 10, CD56 positive, I Patient 26, cluster CD56 positive (B) The kernel density estimate (KDE) curves are shown for CTC morphological features ranging from nuclear/cytoplasmic ratio (left to p), nuclear area (center top), maximum cell area (right top), nuclear circularity (bottom left), cytoplasmic circularity (bottom center) and nuclear entropy (bottom right) for CTCs aggregated from all NEPC (red), CRPC (blue), and atypical CRPC (green) patient samples.

Atypical CRPC
The clinical significance of patients with castration resistant adenocarcinoma that develop progressive disease in the setting of low serum PSA <1ng/ml, visceral metastases in the absence of PSA progression, or elevated serum chromogranin is not well established. One hypothesis is that these tumors are less androgen responsive and may be in transition towards an AR negative/low or NEPC phenotype and/or demonstrate intratumoral heterogeneity with both adenocarcinoma and NEPC present within or between metastases. We applied the NEPC classification model trained to distinguish NEPC vs. CRPC CTCs to the 5 atypical CRPC patients and found that atypical CRPC is associated with an increase in heterogeneity of CRPC cells and a higher burden of NEPC-like cells compared to CRPC patients.

Patient Case Studies
Atypical CRPC patient 6, for example, harbored CTCs of various morphologies with a predominance of NEPC+ CTCs (Figure 8). Patient 6 is a 64 year old man who presented with metastatic hormone naïve prostate cancer, developed clinical progression within six months on primary hormonal therapy, was not responsive to subsequent abiraterone, radium-223, or docetaxel, and developed progressive bone metastases and new liver metastases in the setting of a stable PSA. Despite his bone biopsy at progression showing adenocarcinoma without neuroendocrine features (Figure 8A), his clinical history and CTC characteristics obtained at the time of bone biopsy supported AR independence.

Another example of how CTCs can be used to understand disease heterogeneity is illustrated in the case of Patient 12, a 68 year old gentleman with CRPC who had a bone biopsy at the time of castration resistance for research which showed prostate adenocarcinoma. He was treated with abiraterone and prednisone. Despite PSA stability, follow-up imaging at 3 months on abiraterone revealed new liver and lung metastases and his serum chromogranin was markedly elevated at 17,340 ng/ml (ULN 95 ng/ml). Liver biopsy was consistent with NEPC (small cell carcinoma) (Figure 8B). Similar to Patient 6, CTCs at time of liver biopsy showed heterogeneous CTC populations including both NEPC and CRPC cell characteristics, suggesting intra-patient heterogeneity. These cases support CTCs as potentially useful in capturing tumor heterogeneity that might not be assessed on metastatic biopsy.
Figure 8: Metastatic biopsy showing morphologic characteristics and IHC for synaptophysin (SYP) and androgen receptor (AR) of metastatic biopsies from patients 6 (A) and 12 (B). Atypical CRPC Patient 6 tumor was characterized as poorly differentiated adenocarcinoma, synaptophysin negative, AR positive; NEPC Patient 12 tumor was characterized as small cell carcinoma, synaptophysin positive, AR negative (C) Patient 6 distribution based on NEPC probability class memberships (D) Patient 12 distribution based on NEPC probability class memberships

Validation Cohort

We evaluated baseline CTCs from 159 CRPC patients prospectively enrolled in an independent patient cohort at MSKCC for the presence of NEPC+ CTCs (Figure 9A). NEPC+ CTC subpopulations were identified in 17 of 159 (10.7%) cases. A significantly higher proportion of CRPC patients with visceral metastases harbored NEPC+ CTCs compared to those that were NEPC- (35% versus 15%, respectively; p=0.04). Patients with NEPC+ CTCs also had an overall higher CTC burden (median CTC count 64.6 versus 4.2; p<0.01). To address whether the CTC classifier was a reflection of an overall higher CTC count, linearity was assessed with a Pearson’s coefficient showing a weak relationship between frequency of NEPC CTCs and total cell count. Representative images of NEPC+ CTC characteristics observed in the validation cohort are shown in Figure 9B.

Histologic and molecular subtyping of cancer often influences clinical decision making, and tissue confirmation is typically required at cancer diagnosis before treatment recommendations are offered. Prostate cancer is the most common cancer in men in the United States and Europe, and in nearly all cases diagnostic biopsies reveal adenocarcinoma upon initial diagnosis. Prostate adenocarcinomas are characterized by AR expression and activation, and therefore hormonal therapies targeting the AR are the mainstay of systemic therapy (23). Small cell neuroendocrine carcinoma of the prostate is a rare histologic subtype at diagnosis, representing less than
1% of all new prostate cancer diagnoses (Wang et al, 2008). However, in a subset of patients with metastatic prostate adenocarcinoma treated with AR targeted therapies, prostate adenocarcinomas can develop histologic transformation towards a predominantly neuroendocrine carcinoma likely as a mechanism of acquired resistance. The NEPC phenotype is associated with aggressive disease, frequent visceral metastases, and low or absent AR expression on metastatic tumor biopsy. In this setting, patients are often offered platinum based chemotherapy with regimens similar to small cell neuroendocrine carcinoma of the lung. Therefore, identification of advanced prostate cancer patients that have acquired NEPC has potential clinical implications.

However, the diagnosis of NEPC can be complex as there is a spectrum of morphologies seen in advanced prostate cancer with AR-positive adenocarcinoma and AR-negative small cell carcinoma representing the extreme phenotypes. Metastatic biopsies often reveal mixed features of both adenocarcinoma and neuroendocrine carcinoma, with variable AR or neuroendocrine marker protein expression. The clinical significance of mixed tumors is less clear and treatment decisions are often individualized based on a combination of pathologic and clinical features. Furthermore, for patients with an atypical clinical presentation such as rapid radiographic progression in setting of a low or modestly elevated PSA, platinum-based therapies are sometimes considered even in the absence of neuroendocrine morphology on biopsy. Another challenge in the diagnosis of NEPC is that metastatic biopsies are not always feasible for patients suffering from advanced prostate cancer, may carry additional risks for the patient including complications from biopsy procedure or delay of initiation of appropriate systemic therapy for NEPC, and does not always capture disease heterogeneity. Therefore, a noninvasive marker to detect NEPC progression and simultaneously capture intrapatient heterogeneity is an unmet need.

We found that CTCs from metastatic prostate cancer patients are often phenotypically heterogeneous. CTCs from patients with pathologically confirmed NEPC were predominantly of smaller size compared to other CRPC patients and demonstrated lower AR expression and abnormal nuclear and cytoplasmic features. There was also a higher prevalence of low cytokeratin expressing CTCs in NEPC, possibly related to EMT changes that can occur during metastatic transit and treatment resistance. When applied to an independent cohort, we found that up to 10% of CRPC patients also harbored similar NEPC+CTC subpopulations and their presence was associated with aggressive clinical features (i.e., visceral metastases, high CTC burden). These data support the possible detection of circulating neuroendocrine cancer cells in patients with metastatic CRPC with aggressive clinical features; however, the mixed cellular subpopulations observed reinforce the biologic and clinical complexity underlying disease progression and NEPC transformation. Future studies including single cell sequencing of CTCs will be important to molecularly characterize these heterogeneous populations and may improve our understanding of this complex resistance phenotype that can emerge during the course of AR-targeted therapies.

In this proof of principle study, we demonstrate that CTCs from patients with NEPC have distinct characteristics and thus their detection may potentially help identify patients that are developing NEPC-associated resistance. The results presented here indicate the feasibility of analyzing CTCs using the Epic platform and support the development of further studies to validate the clinical utility of CTCs for the early detection of patients transforming towards NEPC and the prognostic and potential predictive impact of CTC characteristics in predicting response to AR-directed therapies.

For Aim 3, I am currently working on systematic characterization of primary prostate tumors from our 81 patient cohort described above for early genomic alterations that may predispose to resistance to AR targeted therapy with comparison to matched metastatic tumors. The working hypothesis of this Aim is that specific genetic alterations occur early and predispose to the development of treatment resistance to AR targeted therapies, and these may be detected at the time of initial diagnosis in high-risk primary prostate tumors. In parallel to these efforts, I am functionally characterizing several putative drug targets and genes capable of driving neuroendocrine de-differentiation in the laboratory and developing co-targeting approaches. I am also actively developing new NEPC preclinical models. I have also recently generated AR resistance cell lines after short term and long term >60 days of enzalutamide treatment. I am isolating androgen dependent and neuroendocrine resistant subclones using labeled cell sorting as well as a novel computational based algorithm to assess clonality and distinguish adenocarcinoma and NEPC resistant cells. I am functionally characterizing
AR positive and NEPC resistant cells for growth patterns and molecular characteristics. In particular, I am focused on the development of co-targeting strategies including with hypomethylating agents and the EZH2 inhibitor GSK126 in preclinical models of NEPC transdifferentiation for future clinical trial development.

4. KEY ACCOMPLISHMENTS:

- Establishment of a large tissue Biorepository of castration resistant adenocarcinoma and neuroendocrine prostate cancer
- Extensive molecular analysis including whole exome, methylome, and transcriptome sequencing of CRPC-Adeno and CRPC-NE metastatic tumors (and matched primaries) with clinical correlation (Beltran, Prandi et al, Nature Medicine, in press)
- Collection and molecular characterization of CTCs from CRPC-Adeno and CRPC-NE patients (Beltran et al, CCR, in press)
- Establishment of a Precision Medicine Clinic and Rapid Autopsy program at Weill-Cornell-NYP for enrolling advanced cancer patients under an IRB approved protocol to understand mechanism of resistance during disease progression and at the time of death (five prostate cancer rapid autopsies performed to date).

5. CONCLUSION: This Award has allowed me to evaluate mechanisms of prostate cancer resistance to AR targeted therapies by performing integrative genomic and epigenomic analyses of metastatic tumors from patients with castration resistant prostate cancer. I have focused on the development of AR independence and the neuroendocrine phenotype, as this has recently emerged as an aggressive phenotype that is challenging to diagnose and treat. I will use this knowledge to develop biomarkers to improve diagnosis and early detection of patients developing NEPC. I have evaluated CTCs and more recently cell-free DNA in plasma of treated patients at different time points for the emergence of subsets of cells with resistance-associated alterations, as this may serve as a noninvasive method to detect altered genes in an individual patient. With continued work, this project has high potential for further validation and clinical development of biomarkers and could directly influence patient care by identifying patients less likely to respond to subsequent AR –directed therapy and who could be selected for alternative NEPC directed therapeutic approaches. This data has also identified novel drivers of treatment resistance and has nominated therapeutic targets for further preclinical development.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Lay Press:
1. Medscape Article, March 2014. NEPC: Emergence of a Deadlier Prostate Cancer?

(2) Peer-Reviewed Scientific Journals during Year 2:


5. Pauli C, Puca L, Mosquera JM, Beltran H, Rubin MA, Rao RA. An Emerging Role For Cytopathology In Precision Oncology, Cancer Cytopathology, in press.


(3) Invited Articles:

1. Alexander Wyatt and **Himisha Beltran**, Neuroendocrine prostate cancer, European Association of Urology-International Consultation on Urologic Disease (EAU-ICUD), Prostate Cancer Biology of Androgen Dependence and Castration Resistance, 2014. *Corresponding author*


(4) Abstracts: List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
**Defining a Molecular Subclass of Treatment Resistant Prostate Cancer**, Oral Abstract Presentation, ASCO Annual Meeting, 2015. Chicago, IL.


**Precision medicine program for whole-exome sequencing provides new insight on platinum sensitivity in advanced prostate cancer**. Poster presentation, AACR 2015, Philadelphia, PA

**Targeting EZH2 in the AR-independent advanced prostate cancer with neuroendocrine features**, Poster presentation, AACR 2015, Philadelphia, PA

**Development of 2D-cell strain and 3D-tumor spheroid models for Precision Medicine**, Minisymposium oral presentation, AACR 2015, Philadelphia, PA

**Myxofibrosarcoma: A move toward Precision Medicine**, Poster presentation, AACR 2015, Philadelphia, PA

**Phenotypic Characterization of Circulating Tumor Cells from Neuroendocrine Prostate Cancer and metastatic Castration Resistant Prostate Cancer Patients Identify a Novel Diagnostic Algorithm for the Presence of NEPC**. Poster presentation, GU ASCO 2015, Orlando, FL.

**Clonal heterogeneity in platinum-resistant metastatic urothelial cancer.** Oral abstract presentation, GU ASCO 2015, Orlando, FL.

**Rapid Autopsy of a Patient with Neuroendocrine Prostate Cancer,** Precision medicine androgens, September, 2014, London UK.

7. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report

8. **REPORTABLE OUTCOMES:** Nothing to report

9. **OTHER ACHIEVEMENTS:**
   Related Awards obtained during Year 2
   - The Alliance for Clinical Trials in Oncology Foundation Clinical Scholar Award In Honor of Dr. Emil “Tom” Frei III
   - Prostate Cancer Foundation Challenge Award
   - Best Poster Award, Precision Medicines Androgens Meeting, London UK
   - New York State Department of Health (NYSDOH) Grant

10. **REFERENCES:**


Werner SG, RP; Landers, M; Valenta DT; Schroeder, M; Greene, SB; Bales, N; Dittamore R; Marrinucci D Analytical Validation and Capabilities of the Epic CTC Platform: Enrichment-Free Circulating Tumour Cell Detection and Characterization. Journal of Circulating Biomarkers. 2015;4.


11. APPENDICES: N/A