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14. ABSTRACT

Increased incidence of treatment induced neuroendocrine prostate cancer (NEPC) is particularly alarming as this diagnosis is associated with poor prognosis and decades of cytotoxic chemotherapy as the only treatment option. We previously identified neuronal transcription factor BRN2 as a potent driver of neuroendocrine differentiation and an attractive target in NEPC. Our goal is to Evaluate the mechanism by which BRN2 alters chromatin architecture to support neuroendocrine lineage reprogramming. To facilitate any future clinical use of a BRN2 inhibitor in PCa, the project described herein aimed to identify a biomarker of BRN2positive (BRN2+) NEPC that can be readily detected in serum from patients. This would enable longitudinal monitoring of patients for development of BRN2+ NEPC, similarly to how prostate-specific antigen (PSA) levels are currently monitored to gauge PCa progression, and thus potentially provide the means for early detection of PCa patients who may benefit from a BRN2 inhibitor. This report documents progress made towards this aim. Using unbiased approach by combining ChIPseq, RNAseq and secreted protein analysis, we identified Neuronal pentraxin 1 (NPTX1) as a promising biomarker and validated that it can be detected in serum from PCa patients.

15. SUBJECT TERMS
None listed.

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Introduction

Prostate Cancer (PCa) is the most common male cancer and the 2nd leading cause of cancer deaths in North American men. While advanced PCa is initially controlled with hormonal therapies targeting the androgen receptor (AR) pathway, recurrence occurs due to emergence of lethal castration-resistant PCa (CRPC). Despite the potency of AR pathway inhibitors (ARPI) such us Enzalutamide (ENZ) that prolong survival, resistance ultimately emerges. Autopsy series suggest that up to 25% of CRPC patients resistant to ARPIs shed their dependence on the AR and exhibit a continuum of features associated with the neuroendocrine (NE) lineage (Bluemn et al., 2017). The diagnosis of Neuroendocrine Prostate Cancer (NEPC) is accompanied by a dismal overall survival measured in months, with decades old cytotoxic chemotherapy as standard treatment (Small et al., 2014, Aparicio et al., 2011, Robinson et al., 2015). Targeted treatments for this deadly disease are desperately needed. We were the first group to report that BRN2, a neuronal transcription factor, is sufficient and required to drive the evolution of an AR-driven adenocarcinoma into an aggressive and lethal AR-indifferent NEPC tumor (Bishop et al., 2017). We have since developed a highly specific and potent small molecule BRN2 inhibitor (BRN2i) and showed that it has activity in vitro and in vivo in cell lines and xenografts. We showed that BRN2 functions as a pioneer factor in NEPC: In t-NEPC 42DENZR model, BRN2 binds to both open and closed regions of chromatin. Moreover, the BRN2 cistrome is primarily linked to the transcriptionally active genes as 81% of H3K4me3 and H3K27Ac (Hyper-active) and 81% of H3K4me3 and H3K27me3 (Bivalent) genomic loci are co-occupied by BRN2. Importantly, these exact genes/pathways are simultaneously downregulated by BRN2 knockout and inhibition via small molecule. These data provide insight into how the epigenome is reprogrammed during the transition from an adenocarcinoma to NEPC in response to ARPIs by assessing fluxes in chromatin architecture and identifying the importance of BRN2 in mediating these processes.

To move forward with our BRN2 inhibitor to the clinic, we need to develop a serum marker that is crucial for a non-invasive approach for patient's selection and for BRN2 inhibitor pharmacodynamic in clinical trial. Here, we report the development of NPTX1 as serum marker that can be detected in serum from xenografts and from patients with NEPC and testing in additional NEPC PDX models to support the translational potential.

Key words

Prostate cancer, Neuroendocrine, Treatment, BRN2, small molecule inhibitor, ChIPseq, RNAseq, secretome, serum marker, ELISA

Key research accomplishments

- We have developed a computational analysis workflow, starting from BRN2 ChIP-seq data from two NEPC cell lines, to identify and rank candidates for a serum biomarker of BRN2+ NEPC.
- We have identified NPTX1 as a serum marker for BRN2+ NEPC.
- We have validated NPTX1 as a serum marker from mice bearing BRN2+ xenografts.
- We have performed a pilot study and measured NPTX1 in NEPC patients which was not detected in adenocarcinoma.
- NPTX1 will serve as a companion diagnostic serum marker for BRN2 expression/activity to guide patient selection in future clinical trials and a pharmacodynamic serum marker for BRN2 inhibitor.
- We are currently conducting the remaining in vivo studies in NEPC PDX models the test the activity of BRN2 inhibitor.

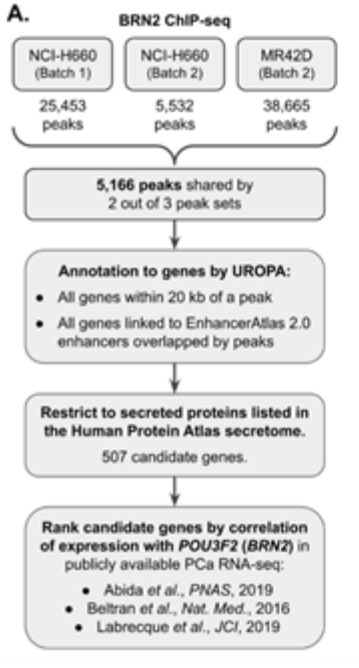
Aim 3: Development of a companion diagnostic marker for guided patient selection.

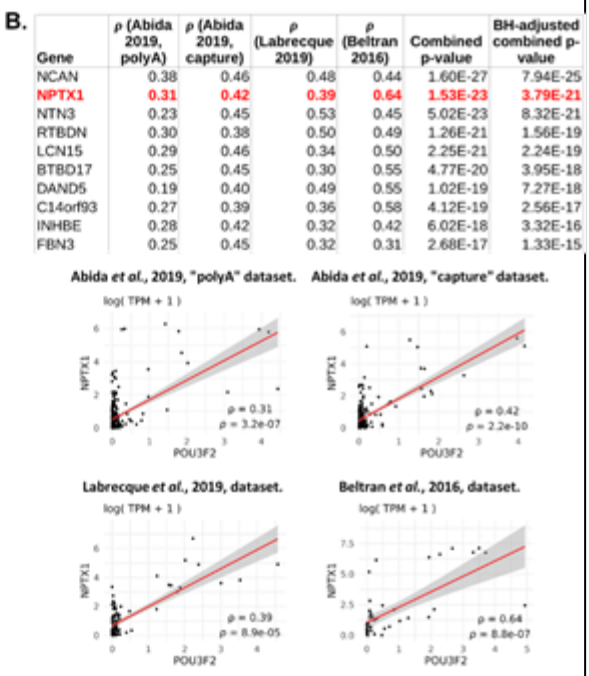
For this aim, our goal is to validate IGFBP5 as a pharmacodynamics marker for BRN2 activity as well as explore its potential as a marker for NE-trans-differentiation. We purchased over five different commercially available ELISA kits including some that were used in previous publications and some that were not. Unfortunately, all the kits failed to detect IGFBP5 efficiently; while they were able to detect positive control peptide, all the kits failed negative controls and demonstrated strong non-specific binding. We developed and optimized an assay in year 2 as indicated in our previous report. However, this IGFBP5 antibody lot is not available anymore and again we couldn't detect IGFBP5 in the serum.

Faced with these challenges we took a more comprehensive approach to identify another BRN2 serum marker. We analyzed new data generated in our labs and public data sets and used different approaches, and identified and validated NPTX1 as a serum marker for BRN2 expression/activity. Next, we evaluated patients serum samples and were able to detect NPTX1 in the serum of NEPC patients (details below).

Identification of NPTX1 as a candidate serum biomarker for BRN2^{+} NEPC

To identify candidate serum biomarkers of BRN2^{+} NEPC, we followed the workflow shown in **Fig 1A**. We performed chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) to identify sites of chromatin-bound BRN2 in the NEPC cell lines NCI-H660 and 42D^{ENZR}. MACS2(Zhang et al., 2008) was used to identify peaks (genomic regions where the ChIP target protein, in this case BRN2, is enriched compared to background) in ChIP-seq data. We analyzed BRN2 ChIP-seq peak sets from two independent NCI-H660 samples and one 42D^{ENZR} sample and identified a total of 5,166 peaks that were shared by at least two of the three peak sets. We then annotated these shared peaks with nearby genes using UROPA(Kondili et al., 2017). Specifically, we identified all genes within 20 kb of a BRN2 peak. We additionally, located all peaks that overlapped any enhancer in the EnhancerAtlas 2.0 database having known regulatory links to genes (in any human cell type). All genes linked to enhancers overlapped by BRN2 peaks were subsequently included in our peak annotation. We considered the complete list of genes annotated to peaks (12,924 genes) to be genes that are potentially regulated by BRN2 in NEPC. Because we were specifically interested in finding a serum biomarker for BRN2^{+} NEPC, we then filtered the list of potential BRN2 target genes to retain only genes that encode secreted proteins listed in the Human Protein Atlas secretome database. This resulted in 507 candidate genes for a serum





Gene	ρ (Abida 2019, polyA)	ρ (Abida 2019, capture)	ρ (Labrecque 2019)	ρ (Beltran 2016)	Combined p-value	BH-adjusted combined p-value
NCAN	0.38	0.46	0.48	0.44	1.60E-27	7.94E-25
NPTX1	0.31	0.42	0.39	0.64	1.53E-23	3.79E-21
NTN3	0.23	0.45	0.53	0.45	5.02E-23	8.32E-21
RTBDN	0.30	0.38	0.50	0.49	1.26E-21	1.56E-19
LCN15	0.29	0.46	0.34	0.50	2.25E-21	2.24E-19
BTBD17	0.25	0.45	0.30	0.55	4.77E-20	3.95E-18
DAND5	0.19	0.40	0.49	0.55	1.02E-19	7.27E-18
C14orf93	0.27	0.39	0.36	0.58	4.12E-19	2.56E-17
INHBE	0.28	0.42	0.32	0.42	6.02E-18	3.32E-16
FBN3	0.25	0.45	0.32	0.31	2.68E-17	1.33E-15

Figure 1: Identification of NPTX1 as a candidate serum biomarker for BRN2^{+} NEPC. A. Analysis workflow to identify and prioritize potential serum biomarkers of BRN2^{+} NEPC. Figure 1: **B.** Top: Spearman correlations, ρ, of mRNA expression of the top 10 serum biomarker candidates with *POU3F2* expression in four publicly available PCa RNA-seq datasets. The p-values were combined for each gene using Fisher's method, and adjusted for multiple hypothesis testing using the Benjamini-Hochberg procedure. Genes were ranked from most significant combined p-value to least significant. Bottom: scatter plots of *NPTX1* vs *POU3F2* mRNA expression in the four RNA-seq datasets. Linear trend lines are shown (red lines) with their 95% confidence intervals (shaded areas

biomarker. To prioritize candidate genes for investigation, we ranked the 507 candidate genes by their correlations with BRN2 mRNA (*POU3F2*) expression in PCa patient specimens in publicly available RNA-seq datasets (**Fig 1B**). For this, we used RNA-seq data from studies by Abida *et al.(Abida et al., 2019)*, Labrecque *et al.(Labrecque et al., 2019)*, and Beltran *et al.(Beltran et al., 2016)*. The study by Abida *et al.* used two different RNA-seq methods – enrichment for polyadenylated RNA and exon capture – which we treated as two separate datasets, labeled "polyA" and "capture" respectively. In each of the four RNA-seq datasets, we computed the Spearman correlation, ρ, of each candidate gene with *POU3F2*.

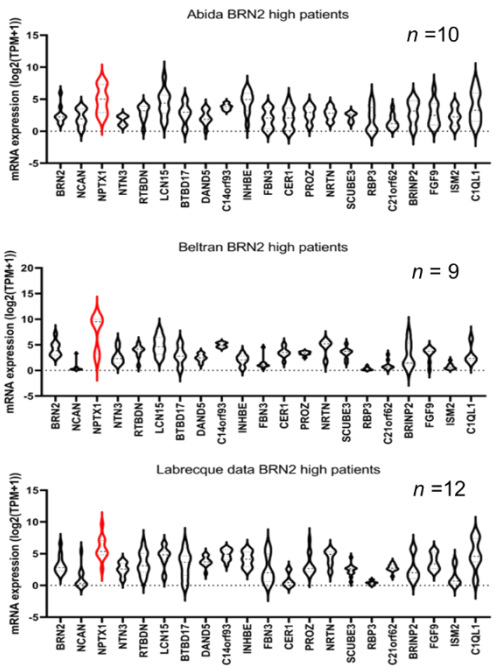


Figure 2: Expression of BRN2 potential serum markers in patients: Violin plots of mRNA expression of *POU3F2* (BRN2) and the top 20 serum biomarker candidate genes in PCa patient samples having *POU3F2* expression > mean + standard deviation in RNA-seq datasets (n = 10 for Abida *et al.*, n = 12 for Labrecque *et al.*, n = 9 for Beltran *et al.*).

We combined the p-values associated with the four correlation coefficients for each gene using Fisher's method and subsequently ranked the genes from most significant to least significant correlation. Neuronal pentraxin 1 (NPTX1) ranked second in this analysis, but its detected gene expression values (TPM values) were substantially higher than the top-ranked gene, NCAN, in *POU3F2*-high patients (**Fig 2)**, an important consideration for detection of the protein in serum.

Interrogation of our BRN2 ChIP-seq data confirmed the presence of a robust BRN2 peak downstream of the *NPTX1* gene in both, NCI-H660 and MR42D cell lines (**Fig 3 left**). This peak coincided with marks of an active promoter element (H3K27ac and H3K3me3; **Fig 3 left**) and in fact resided in the promoter of a long non-coding RNA (lncRNA) gene. H3K4me3 was also observed at the *NPTX1* promoter in both NCI-H660 and MR42D, as was H3K27ac in NCI-H660 (**Fig 3 left**). Intriguingly, the lncRNA promoter element was also highly active in V16D (**Fig 3 left**), a PCa cell line that represents an earlier stage of the disease and does not express BRN2. Nevertheless, across RNA-seq data from nine PCa cell lines, *NPTX1* expression was high only in cell lines that highly expressed *POU3F2* (**Fig 3 right**). Furthermore, we recently found by Western blot that overexpression of BRN2 in 22Rv1 cells, which already express both BRN2 and NPTX1, increases the protein levels of NPTX1. Considering all of these data, we hypothesize that recruitment of BRN2 to an already active cis-regulatory element, the promoter of a lncRNA gene, causes upregulation of the nearby *NPTX1* gene.

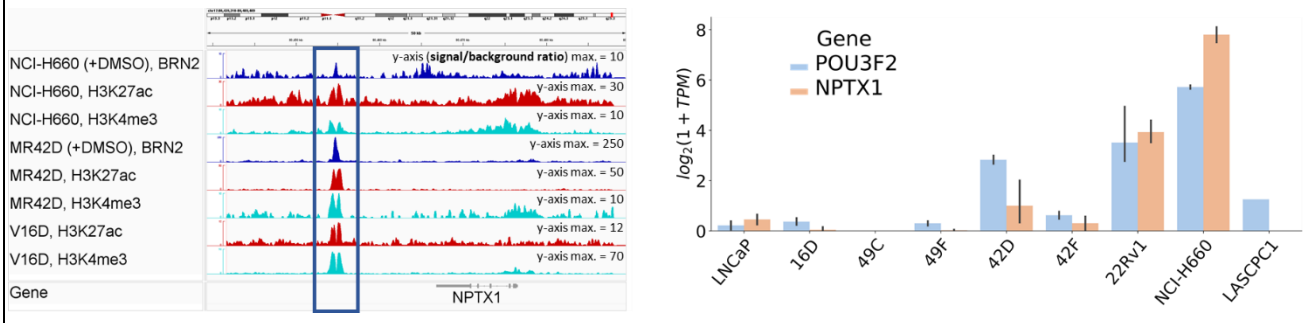


Figure 3: Left panel: ChIP-seq tracks for BRN2, H3K27ac, and H3K4me3 around the *NPTX1* locus in NCI-H660 and MR42D cells, and for H3K27ac and H3K4me3 in V16D cells. The *y*-axis shows the ratio of ChIP signal to background. **Right panel** *POU3F2* and *NPTX1* gene expression from RNA-seq data for nine PCa cell lines: LNCaP (n = 2), V16D (16D; n = 3), 49C (n = 1), 49F (n = 2), MR42D (42D; n = 9), 42F (n = 2), 22Rv1 (n = 3), NCI-H660 (n = 2), and LASPC1 (n = 1). Bar heights show mean expression values, error bars show 95% confidence intervals.

To further evaluate *NPTX1* regulation by BRN2, we downloaded publicly available BRN2 ChIP-seq data for LK2 and NCI-H520, two lung squamous cell carcinoma (LUSC) cell lines of a neural subtype defined by BRN2 and SOX2(Sato et al., 2019), and performed a refined version of our analysis workflow shown in Fig 1. Using the

peaks in the higher-quality of our two NCI-H660 BRN2 ChIP-seq samples, plus the BRN2 peak sets from 42D^{ENZR}, LK2, and NCI-H520, we identified 493 BRN2 peaks that were shared by at least three out of four peak sets. We then refined the first step of our peak annotation strategy to reduce the average number of genes per peak by annotating peaks in promoters of known protein-coding genes only to genes corresponding to those promoters, rather than all genes within 20 kb (annotation of all other peaks remained unchanged). Applying this refined analysis to the new set of 493 BRN2 peaks, and filtering the gene list to retain only secreted proteins resulted in identification of 51 genes. This list includes *NPTX1*, as the BRN2 peak downstream of *NPTX1* in NCIH660 and MR42D cells as well as in both the LK2 and NCI-H520 cell lines (**Fig 4**), even though only 302 non-blacklisted BRN2 peaks were detected in NCI-H520. After ranking the 51 BRN2 serum biomarker candidates, NPTX1 ranked as a top candidate (NCAN was lost from the list of candidates and no stronger candidates were gained), thus increasing our confidence in NPTX1 as the most promising candidate for a serum biomarker of BRN2^{+} NEPC.

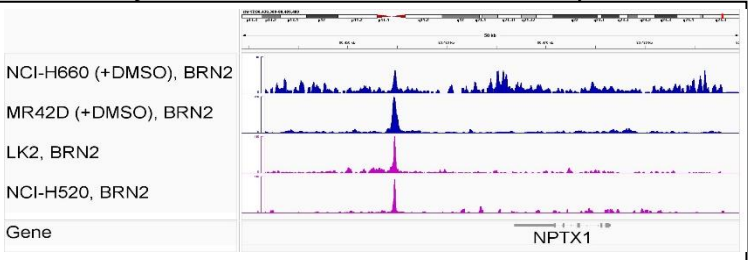


Figure 4: BRN2 ChIP-seq tracks around NPTX1 in the NEPC cell lines NCI-H660 and 42D^{ENZR} and the neural-type LUSC cell lines LK2 and NCI-H520.

Validation that NPTX1 in serum samples

To test whether NPTX1 protein is secreted by cells at a detectable level, we performed Western blots using either cell lysate or culture media from NCI-H660 cells. As anticipated, we detected NPTX1 from both cell lysate and media, whereas BRN2 was only detected only in cell lysate (**Fig 5A**). Using a commercially available ELISA Kit (R-PLEX Human NPTX1 Assay), we validated the presence of NPTX1 in culture media (**Fig 5B).**

Next, we performed an *in-vivo* experiment and collected the serum from mice bearing NCI-H660 xenografts over time. Our data showed that NPTX1 is present in the serum and that serum NPTX1 levels increased as NCI-H660 xenograft tumors increased in volume **(Fig 6).**

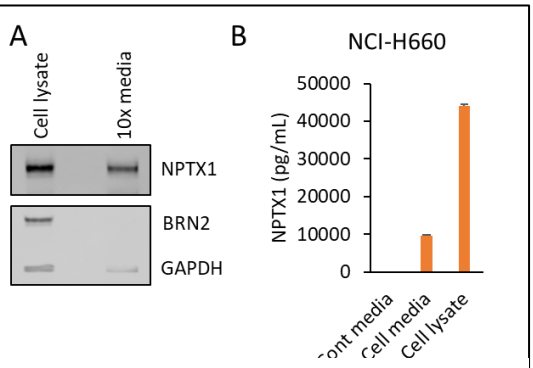


Figure 5: Expression of NPTX1 in the media of NCI-H660 cells. A. Western blots for NPTX1 and BRN2 from either cell lysate or culture media from cultured NCI-H660 cells. **B.** level of NPTX1 detected by R-PLEX Human NPTX1 Assay kit in control media, NCI-H660 cultured media, and NCI-H660 cell lysate

Having verified NPTX1 in culture media from NCI-H660 cells and serum from mice with NCI-H660 xenograft tumors, we next evaluated serum from LuCaP PDX models. We observed strong positive correlation between NPTX1 levels and mRNA NPTX1 expression multiplied tumor volume (r=0.96) (**Fig 7A**). Finally, we measured serum NPTX1 level in samples from patients with metastatic PCa from the University of Washington under the Prostate Cancer Donor Program. Overall, serum NPTX1 levels were correlated with tumor NPTX1 mRNA expression in same patients (r=0.56) (**Fig 7B**). Although we observed a positive correlation between BRN2 and NPTX1 mRNA expression in patients' tumor (r=0.69), we observed a weak positive correlation between BRN2 mRNA expression in tumors and serum NPTX1 (r=0.22). We hypothesize that this is due to the end-stage patients having multiple metastasis and tumor heterogeneity. Together our results are highly promising and support our hypothesis that NPTX1 is a suitable serum biomarker for BRN2+ NEPC, but more samples will need to be analyzed before conclusions can be drawn.

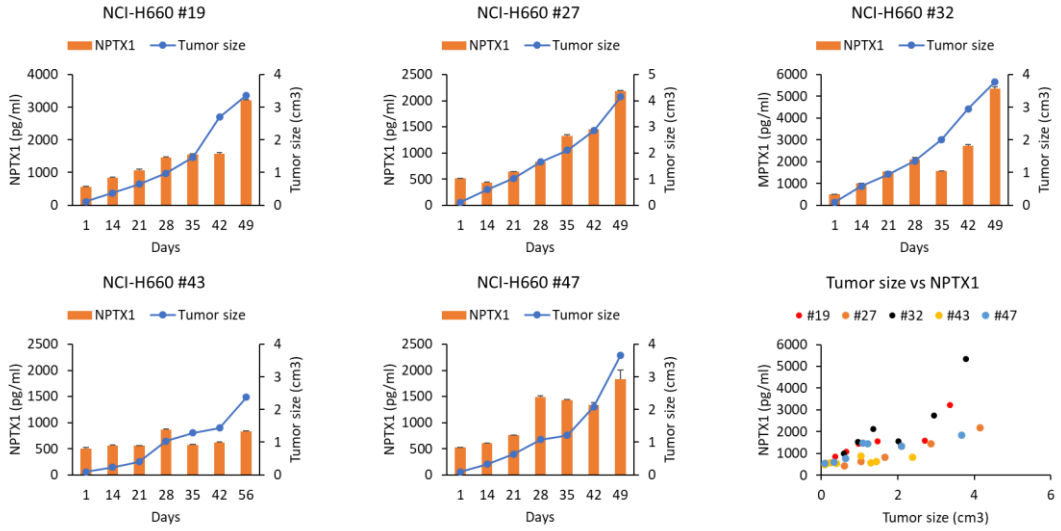


Figure 6: **Serum NPTX1 levels in vivo.** NCI-H660 cells were injected subcutaneously into mice and serum from mice was collected over time. NPTX1 was measured using ELISA Kit (R-PLEX Human NPTX1 Assay). NPTX1 correlates with tumor volume.

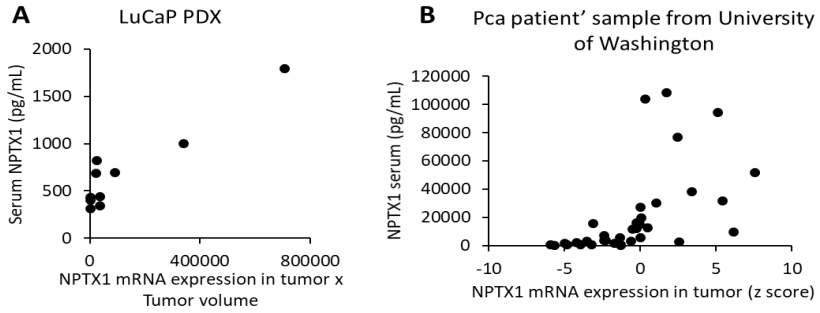


Figure 7: **A.** Correlation between serum NPTX1 level and NPTX1 mRNA expression in tumor multiplied tumor volume in LuCaP PDX models. **B.** Correlation between serum NPTX1 level and NPTX1 mRNA expression in human prostate cancer tissue. Tissue RNA-seq data (acquired after rapid autopsy) and serum from the corresponding patients were provided by the University of Washington under the Prostate Cancer Donor Program.

Conclusions

Through rigorous computational analysis of ChIP-seq and RNA-seq data sets, we identified NPTX1 as the strongest candidate as a serum biomarker of $BRN2^+$ NEPC. We have also validated that NPTX1 is present in serum from patients with NEPC, but not $BRN2^-$ adenocarcinoma CRPC patients, supporting our hypothesis that NPTX1 is a suitable serum biomarker of $BRN2^+$ NEPC.

Future Studies

We will continue to assess the potential for NPTX1 as a serum biomarker of $BRN2^+$ NEPC as we acquire serum from more PCa patients. We will also analyze RNA-seq data from lung cancer datasets to investigate whether our results could generalize to another cancer type and broaden the utility of NPTX1 as a biomarker beyond NEPC. Furthermore, NPTX1 has been reported to regulate neural lineage specification from human pluripotent stem cells(Boles et al., 2014). Therefore, we will investigate the biology of NPTX1 in PCa to determine whether NPTX1 is more than just a biomarker and in fact plays a functional role in the development of NEPC. We will also investigate whether the particular lncRNA whose gene promoter is located < 20 kb downstream of the *NPTX1* gene and bound robustly by BRN2 in NEPC and neural LUSC cell lines has any importance in PCa. We will perform the additional NEPC PDX studies of BRN2i to support the translational potential of BRN2 inhibition.

Changes and Problems

- In our last report, we encountered challenges with the PDX studies and we observed no activity of the BRN2i. When we went and analyzed the drug, we found that the drug lost activity.
- We synthesized new drug and the in vivo studies are currently ongoing under the supervision of Drs. Corey and Morrissey.

We have requested and were granted an additional one year no cost extension, which will allow us to complete both the remaining animal studies and perform the downstream analysis as described in the SOW, and further validate NPTX1 as a surrogate marker for BRN2i in patient samples.

Publications

Discovery and characterization of a first-in-field transcription factor BRN2 inhibitor for the treatment of neuroendocrine prostate cancer
Daksh Thaper, Ravi Munuganti, Adeleke Aguda, Soojin Kim, Shengyu Ku, Olena Sivak, Sahil Kumar, Sepideh Vahid, Dwaipayan Ganguli, Himisha Beltran, Colm Morrissey, Eva Corey, Amina Zoubeidi
bioRxiv 2022.05.04.490172; doi: https://doi.org/10.1101/2022.05.04.490172

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