Defining the Mechanism and Therapeutic Targets of lncRNAs in Castration Resistant Prostate Cancer

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Background

Currently, lowering hormone levels through androgen deprivation therapy (ADT) is the initial treatment for men with metastatic prostate cancer. However, despite initial response rates of 80 to 90 percent, nearly all men eventually develop progressive disease following ADT, referred to as castrate-resistant prostate cancer (CRPC). To date prostate cancer research has primarily focused on the deregulation of protein-coding genes to identify potential diagnostic and therapeutic targets. However, long non-coding RNAs (lncRNAs) represent an emerging class of transcripts that have a significant role in human cancers. Recently, we found 131 novel lncRNAs to be involved in prostate tumorigenesis. Due to their tissue-enriched expression many lncRNAs can serve as valuable biomarkers to stratify patients with aggressive prostate carcinomas. We are now faced with the critical challenge of therapeutically targeting each patient population. This represents a critical gap in our ability to translate these findings into the clinic. Although originally regarded as noise, several well- described examples indicate that lncRNAs may be master regulators in cancer biology. However, our understanding of how lncRNAs function in cancer is still in its infancy. Therefore, we hypothesize that a subset of lncRNAs altered in prostate cancer bind directly with a specific protein complex, alter its normal function, and cause aggressive disease. To address this, In Year 1 we have optimized a high-throughput assay to identify the subset of lncRNAs altered in prostate cancer that associate with the Polycomb Repressive Complex 2 (PRC2), which is known to promote aggressive disease. In Year 2, we focused on refining the list of PRC2-asociating lncRNAs through an integrative analysis that honed in on *PCAT-14* as a marker promoting aggressive prostate cancer. Overall, a better understanding of how lncRNAs enable primary tumors to invade and metastasize could lead to the development of more specific treatments to improve patient outcomes.

Progress in Year 1

Over Year 1 we focused on the global detection of Polycomb associated lncRNAs associated with prostate tumor progression. In order to accomplish this we optimized RNA immunoprecipitation coupled with RNA- Seq, or RIP-Seq, to capture PRC2-bound lncRNAs. We evaluated different aspects from previously published methods resulting in our optimized method identifying RNAs directly binding to a protein. Next, we performed deep sequencing of the RNA generated from our RIP method followed by integrative analysis using multiple published RIP-Seq analysis tools to maximize our confidence in identifying PRC2 associated lncRNAs Through this approach we discovered >20% of lncRNAs were interacting with the Polycomb Repressive Complex 2 (PRC2). Of these, we were able to validate the association of *prostate cancer associated transcript 1 (PCAT-1)* which we had previously reported to interact with the PRC2 complex and define a subgroup of patients with aggressive prostate cancer. Based on our hypothesis that a lncRNA modulates chromatin remodeling via PRC2 to promote metastasis, we expect that altering a lncRNA interacting with PRC2 will confer metastatic phenotypes similar to what has been observed when silencing PRC2 components.

Progress in Year 2

While our preliminary data demonstrated that 131 lncRNAs associate with prostate cancer progression to metastasis, we performed a multi-institutional analysis to focus on lncRNAs consistently altered across cohorts. Therefore, we first performed an integrative analysis of three patient cohorts: (i) transcriptome sequencing of 14 primary tumors and matched adjacent normal tissue (Ren cohort), (ii) transcriptome sequencing of 20 primary tumors and 10 matched adjacent normal tissues (Kannan cohort), and (iii) Affymetrix gene expression of 131 primary and 19 metastatic tumors (Taylor cohort). As shown in Figure 1, four lncRNAs were up-regulated and one lncRNA was down-regulated between the primary tumors and normal tissue across all three cohorts.

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Leveraging the clinical data associated with the Taylor cohort, we next assessed whether any of the five lncRNA candidates were associated with aggressive disease based on Gleason score. While multiple candidates were previously reported to have altered expression in prostate tumors (i.e., *DRAIC*, *PCAT-101*), *PCAT-14* is the only lncRNA that has a significant association between high (9) and low (6) Gleason scores (p=0.00013) (Figure 1B) and is anti-correlated with Gleason score (cor=-0.22). Furthermore, *PCAT-14* displayed altered expression throughout prostate tumor progression (Figure 1C) as exemplified by *PCAT-14* up-regulation in primary tumors compared to normal tissue (p=1.3e-15) and *PCAT-14* down-regulation in metastatic relative to primary tumors (p=9.7e-06).

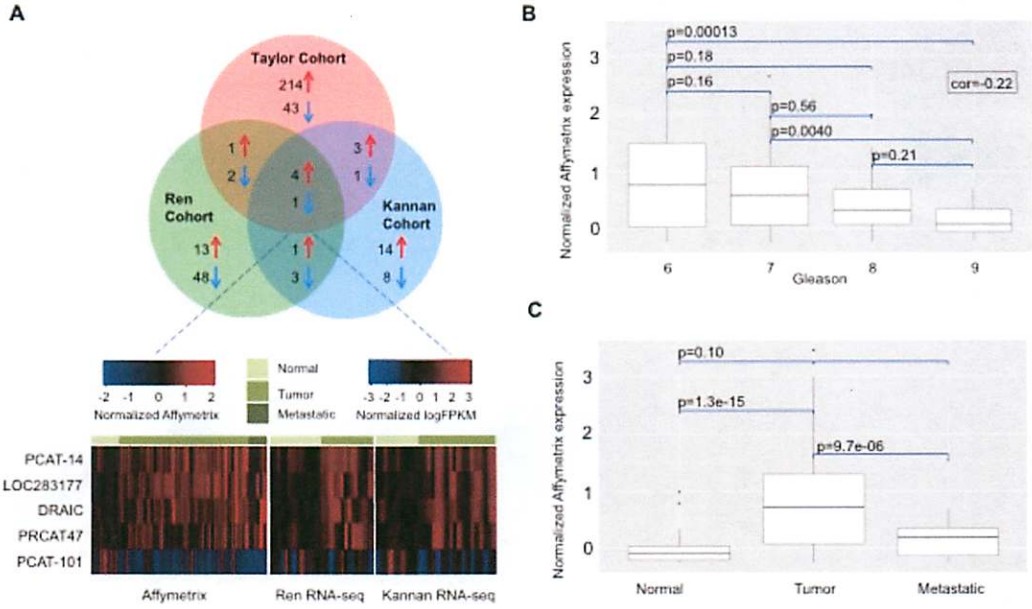

Figure 1 | Integrative analysis reveals *PCAT-14* association with aggressive prostate cancer. (A) Differentially expressed (DE) lncRNAs between normal and tumor samples of prostate cancer in one Affymetrix dataset and two RNA-seq datasets (Supplementary Table 2). The Venn diagram shows the number of up-regulated (red arrow) and down-regulated (blue arrow) DE lncRNAs identified in from one to three datasets. Heatmaps of the normalized Affymetrix expression and normalized RNA-seq log FPKM of the 5 DE lncRNAs from all three datasets are shown across normal, tumor, and metastatic samples in each datasets. 4 of the 5 DE lncRNAs are up-regulated in tumor and 1 is down-regulated in all three datasets. Among these 5 lncRNAs, *PCAT-14* is the only one shows association with tumor progression (Supplementary Table 2). **(B)** Boxplot of normalized Affymetrix expression of *PCAT-14* in Gleason 6 through 9. Expression of *PCAT-14* is anti-correlated with Gleason score (correlation=-0.22). Expression of *PCAT-14* is significantly getting down from Gleason 6 to 9 (p=0.00013). **(C)** Boxplot of normalized Affymetrix expression of *PCAT-14* in normal, tumor, and metastatic prostate samples. *PCAT-14* is up-regulated in tumor samples compared to normal samples (p=1.3e-15), and the its expression goes down again in metastatic patients from tumor (p=9.7e-06).

Notably, *PCAT-14* expression appears to be enriched in prostate cancer as shown in our pan-cancer expression analysis of 6,853 specimens across eighteen additional solid tumors, as part of The Cancer Genome Atlas consortium. This is further supported by a recent compendia study in which *PCAT-14* was reported, under the gene alias *PRCAT-104*, to have altered expression specifically in prostate cancer. Validation of *PCAT-14* expression in a prostate cancer cell line panel relative to the control cell line, RWPE, confirmed overexpression of *PCAT-14* in 22Rv1 and VCaP cell lines. Additionally, subcellular localization revealed that *PCAT-14* is enriched in the nucleus, which is common amongst lncRNAs associated with gene regulation.

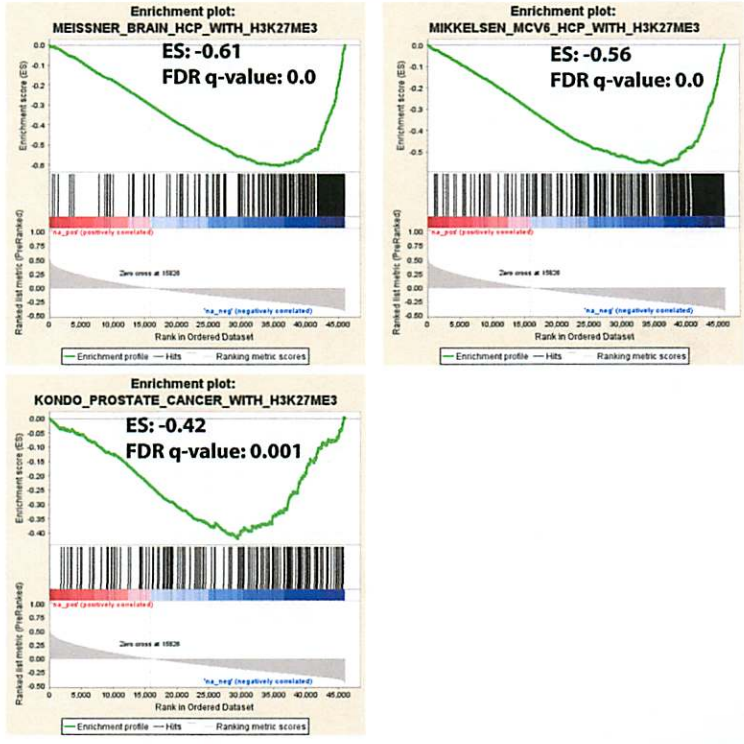

Figure 2 | PCAT-14 is enriched with genes involved in PRC2 regulation. Gene set enrichment analysis (GSEA) of protein-coding genes coexpressed with PCAT-14 revealed gene signatures associated with epigenetic regulation were inversely correlated with PCAT-14 expression. Enrichment Score (ES) and FDR q-value are shown for each concept.

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To elucidate the mechanisms of *PCAT-14* function, we utilized our large patient cohort to identify genes with correlated expression profiles to implicate *PCAT-14* in various biological processes (Figure 2). After ranking all genes according to their correlation value, we used Gene Set Enrichment Analysis (GSEA) to search for enrichment across the Molecular Signatures Database (MSigDB). ***Further supporting its role as an epigenetic regulator, we found an enrichment of H3K27 methylated target genes among the highest ranked concepts, which were inversely correlated with PCAT-14 expression*** (Figure 2). Therefore, *PCAT-14* is an ideal marker of aggressive disease and may be necessary for the Polycomb Repressive Complex 2 (PRC2) to methylate Lys^{27} of histone 3 (H3K27) to epigenetically silence target genes associated with metastasis and poor patient outcome. Furthermore, we observed numerous signatures associated with tumor microenvironment that could also be indicative of aggressive disease.

Given the altered expression of *PCAT-14* in prostate cancer, we hypothesized that there might be a relationship with clinical outcomes as well. Therefore, we assessed *PCAT-14* expression within a cohort of 910 radical prostatectomy specimens from three independent patient cohorts from the Decipher GRID: Mayo Clinic I (MCI, N=545), Mayo Clinic II (MCII, N=235), and Thomas Jefferson (TJU, N=130). We found that patients with high versus low expression of *PCAT-14* showed significantly different rates of distant metastasis free survival (DMFS) (MCI: P=0.0024, HR=0.66 [0.5-0.86], MCII: P=0.023, HR=0.59 [0.37-0.94], TJU (borderline): P=0.093, HR=0.33 [0.084-1.3]) in Figure 3A, overall survival (OS) (MCI: P=0.0044, HR=0.71 [0.56-0.9], MCII: P=0.14, HR=0.68 [0.41-1.1], TJU: P=0.0061, HR=0.35 [0.16-0.77]) in Figure 3B, and prostate cancer specific survival (PCSS) (MCI: P=0.00059, HR=0.54 [0.38-0.77], MCII: P=0.023, HR=0.44 [0.22-0.91], TJU unavailable) in Figure 3C. Consistent with these data, we also find an association with lower *PCAT-14* expression and Gleason score as well as lymph node invasion. The prognostic ability of *PCAT-14* is significant even after accounting for clinicopathologic variables on a pooled multivariable cox analysis of these cohorts (DMFS: P=0.002, HR= 0.68 [0.53-0.87], PCSS: P=0.015, HR= 0.68 [0.53-0.87], borderline significant for OS: P=0.056, HR= 0.81 [0.65-1.01]. In addition, we next investigated if *PCAT-14* was able to improve the performance of an existing prediction algorithm, CAPRA-S. We also found that when we added *PCAT-14* to CAPRA-S, it increased the ROC AUC for 10-year metastasis rates from 0.69 (CAPRA-S alone) to 0.71 (CAPRA-S+*PCAT-14*).

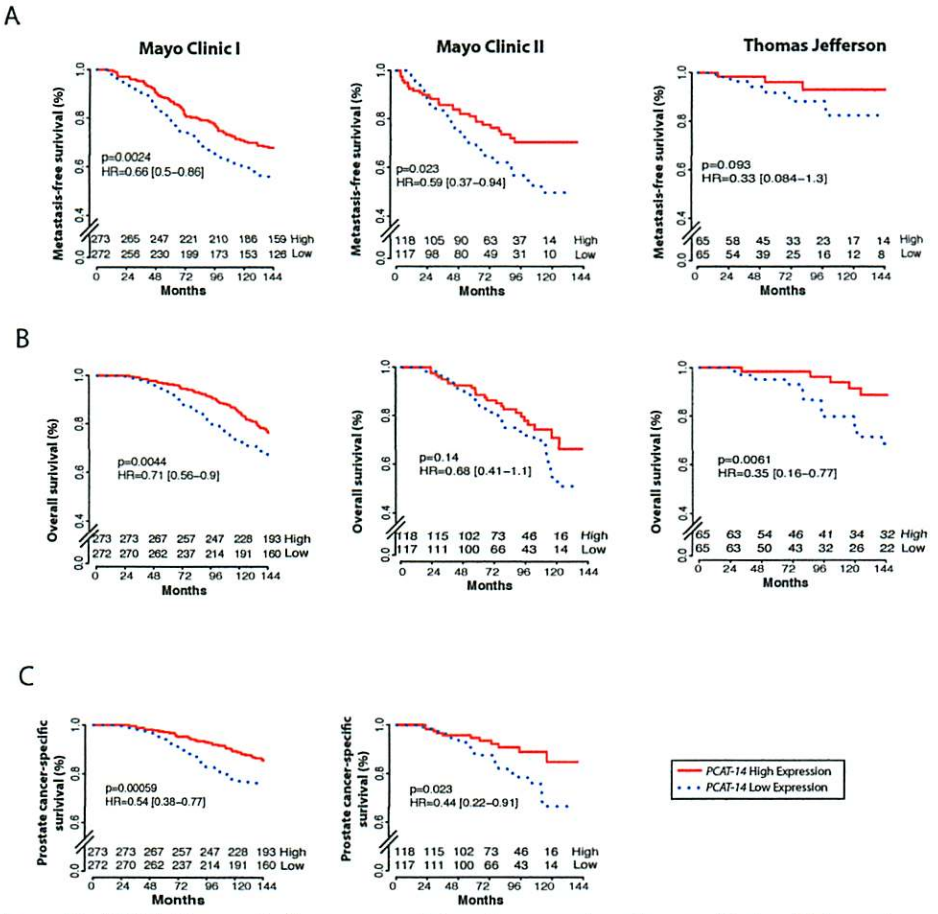

Figure 3 | *PCAT-14* as a single gene predictor of aggressive disease. Kaplan-Meier analyses of prostate cancer outcomes in the Mayo Clinic cohort. *PCAT-14* expression was measured using Affymetrix exon arrays, and subjects were stratified according to *PCAT-14* expression. Subject outcomes were analyzed for **(A)** distant metastasis-free survival, **(B)** overall survival, and **(C)** prostate cancer specific survival, across three patients cohorts (from left to right) Mayo I, Mayo II, and Thomas Jefferson. P values were calculated by a log-rank test. The number-at-risk is shown at the bottom of each plot.

Through GSEA, we also found that high *PCAT-14* expression had a positive correlation with genes involved in prostate cancer and androgen response. We also observed a moderate correlation (0.49) between *PCAT-14* expression and AR transcriptional activity. Therefore, we investigated if *PCAT-14* predicted response to therapies such as ADT in the MCI and MCII cohorts. Of 780 patients, 236 underwent post-operative ADT within 1-year of radical prostatectomy. We found that the distant metastasis-free survival prognostic differences between *PCAT-14* high and low expression are increased in patients treated with ADT (P=0.00082, HR=0.5 [0.34-0.76]), and these differences are attenuated in patients without ADT treatment (P=0.015, HR=0.69 [0.51-0.93]) as shown in Figure 4. As our cohorts are all retrospective, which confounds treatment by baseline risk, it is necessary to adjust for clinical and pathologic variables and other treatments such as RT. In our multivariate cox model, which accounts for these confounders, we find statistically significant interaction terms for ADT and *PCAT-14* (p=0.029) indicating that *PCAT-14* can potentially predict response to ADT after prostatectomy.

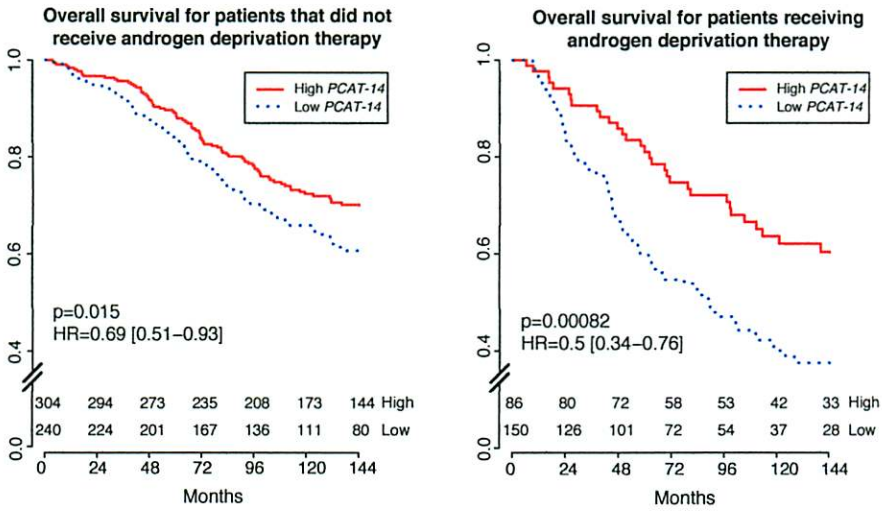

Figure 4 | *PCAT-14* is predictive of ADT response. Kaplan Meier curves show that high *PCAT-14* expression is differentially prognostic of distant metastasis-free survival in patients treated with (left) and without (right) androgen deprivation therapy.

To further support our clinical findings, we sought to evaluate whether *PCAT-14* promotes aggressive disease *in vitro.* To assess the functional significance of *PCAT-14*, we stably overexpressed it in the aggressive prostate cancer cell lines IGR-Cap1 and PC3 cells using two independent full-length clones. Mechanistic findings support the clinical observations of decreased *PCAT-14* expression associating with a more aggressive phenotype. *In vitro* experiments measuring the migration and invasion of cells overexpressing *PCAT-14* show a decreased migratory/invasive capacity of these cells relative to a cell line with low *PCAT-14* expression. *PCAT-14* overexpressing IGR-Cap1 cells and empty vector control IGR-Cap1 cells were plated in serum free media on a transwell membrane and allowed to migrate in a modified Boyden Chamber assay. After 24 hours there was a significant 45% decrease in clone 1 ($p \leq 0.00001$) and 26% decrease in clone 2 ($p \leq 0.001$) in migration of *PCAT-14* overexpressing cells compared to IGR-Cap1 control cells (Figure 5A). This decrease in migration was also similarly seen in PC3 *PCAT-14* overexpressing cells (Figure 5B). There was at least an 80% decrease in migrated cells in both PC3 *PCAT-14* overexpressing cell lines compared to the control cell line ($p \leq 0.00001$). Similar experiments were conducted with a Matrigel-coated transwell as further evidence for *PCAT-14* expression altering the aggressiveness of prostate cell lines. As seen with migration assays, *PCAT-14* overexpression in both IGR-Cap1 and PC3 cells significantly diminished the cellular invasion compared to the control cell lines. The changes in migratory and invasive cellular behavior highlight that the overexpression of *PCAT-14* in prostate cancer causes a less aggressive phenotype. Cellular proliferation was also monitored as an additional characterization of an aggressive phenotype. IGR-Cap1 cells overexpressing two different clones of *PCAT-14* showed a 30% decrease in cell growth relative to empty vector control infected cells at Day 2 ($p = 0.001$ and $p = 0.00001$). The diminished growth in *PCAT-14*-expressing cells continued to Day 6 with a 16% and 37% change in clone 1 and clone 2 cell growth respectively. Combined these data indicate the expression of *PCAT-14* leads to a less aggressive cancer phenotype in two well-studied aggressive cell lines. Moreover, these data support the strong clinical observations and associations that higher *PCAT-14* expression reduces aggressive metastatic disease.

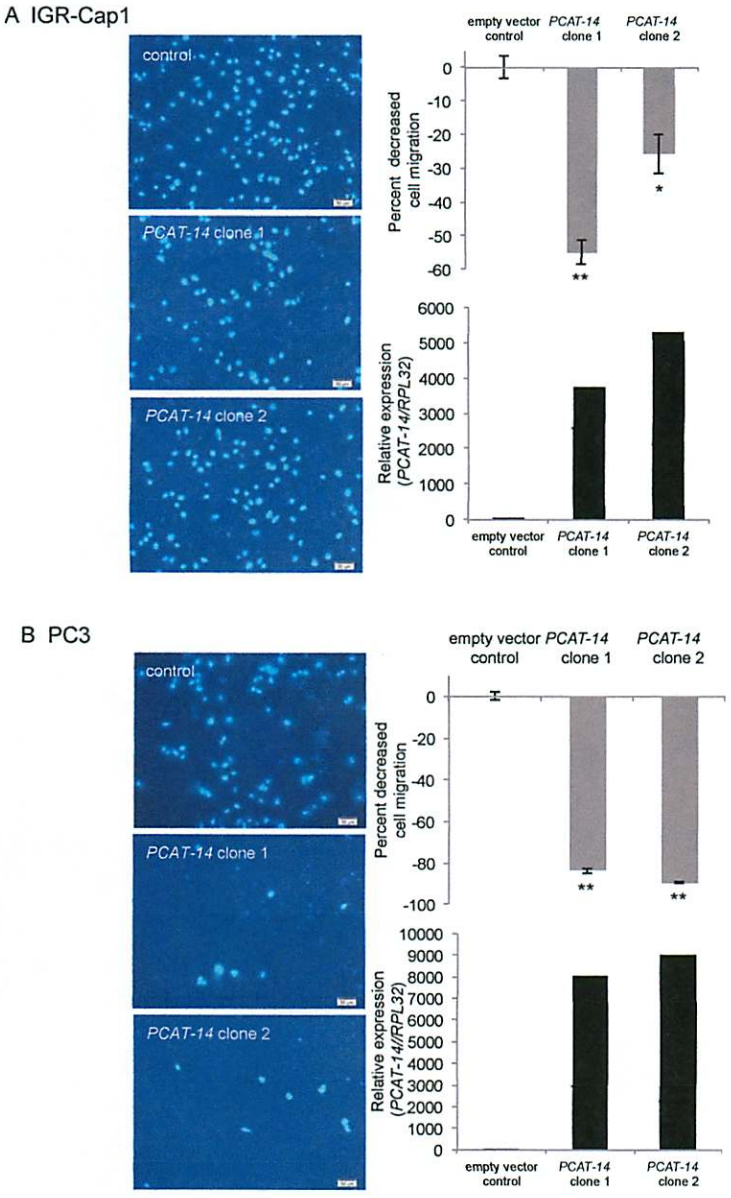

Figure 5 | *PCAT-14* promotes cellular migration *in vitro*. **(A)** IGR-Cap1 or **(B)** PC3 (B) cells were plated for 24 hours on a transwell membrane. Migrated cells (bottom of filter) were stained with DAPI and quantified. Quantitative RT-PCR confirmed overexpression of *PCAT-14* in both cell lines relative to empty vector control cell lines.

Overall, we discovered that *PCAT-14* is commonly up-regulated in primary tumors. Furthermore, low *PCAT-14* expression promotes aggressive oncogenic phenotypes through epigenetic regulation and is significantly prognostic for multiple clinical endpoints. In addition to predicting metastatic disease, we found that *PCAT-14* may be able to predict ADT response highlighting its potential use for improving patient management and prognosis.

This work was recently published in European Urology (which has an Impact Factor as 14.976 and is ranked it 1st in the fields of Nephrology and Urology):

White NM, Zhao SG, Zhang J, Rozycki EB, Dang HX, McFadden SD, Eteleeb AM, Alshalalfa M, Vergara IA, Erho N, Arbeit JM, Karnes RJ, Den RB, Davicioni E, Maher CA. Multi-institutional Analysis Shows that Low PCAT-14 Expression Associates with Poor Outcomes in Prostate Cancer. Eur Urol. 2016 Jul 22. PubMed PMID: 27460352.