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14. ABSTRACT

Prostate cancer (PCA) is a clinically and genetically heterogeneous and the development of a molecular classification is critical to distinguish lethal from indolent tumors and minimize overtreatment. Genomic alterations of the PTEN and ERG genes are among the most common in PCA and there is an interest in exploiting these alterations for routine risk assessment. We found that PTEN loss is most strongly associated with PCA death in patients whose tumors do not carry an ERG gene rearrangement, suggesting that ERG absence strengthens PTEN loss association with lethal progression. Despite the widely accessible PTEN/ERG molecular classification, our understanding of their biological interaction along PCA progression remains very limited. Hence, in our study we will perform a comprehensive molecular profiling of well-annotated PCA samples in relation to PTEN and ERG status. Our goals are threefold: 1) to confirm that PTEN/ERG double negative tumors are the most aggressive; 2) to characterize the expression profiles associated with PTEN and ERG alterations; and 3) to determine whether such expression profiles can be used to improve PCA patient stratification into different risk groups.

15. SUBJECT TERMS

Prostate cancer, PTEN, ERG, ETS, MYC, cell cycle, gene expression, Cap Analysis of Gene Expression (CAGE)

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1. INTRODUCTION

Prostate cancer (PCA) is a clinically and genetically heterogeneous and the development of a molecular classification is critical to distinguish lethal from indolent tumors and minimize overtreatment. Recent technological advances have enabled extraordinary insights into molecular changes occurring in PCA and the *PTEN* and *ERG* genomic alterations have emerged as the most common in PCA. Furthermore, we have found that PTEN loss is associated with PCA death most strongly in patients carrying *ERG* rearrangements, hence there is an interest in exploiting such alterations for routine risk assessment. Furthermore, despite the fact that PTEN and ERG molecular classification is widely accessible, our understanding of their interaction during disease progression is very limited, and a molecular signature of PTEN/ERG loss in PCA is still lacking.

To address these issues, we have formed a collaborative, multi-disciplinary team – led by a urologic pathologist and computational biologist with expertise in PCA molecular pathology and cancer genomics – to perform a comprehensive molecular assessment of well-annotated prostate cancers in relation to PTEN and ERG status using existing and novel data. Our objectives are threefold: 1) to confirm that the tumors with loss of PTEN and lacking *ERG* rearrangement are among the most aggressive; 2) to characterize the *expression profiles* associated with PTEN and ERG alterations; and 3) to determine whether these *expression profiles* can improve the way we stratify prostate cancer patients into different risk groups.

Findings from our proposed research have the potential for both immediate and long-term clinical and translational research applicability. First, by analyzing several large clinical cohorts from multiple institutions, we will be able to confirm the performance of these biomarkers in patient risk stratification. Second, we will also be able to assess if and how PTEN/ERG *molecular signatures* correlate with lethal disease risk in comparison to currently available prognostic assays. Third, we expect to identify novel molecular alterations responsible for the distinct clinical and biological behavior of tumors based on PTEN and ERG status. Lastly, we will also generate a wealth of information about the biologic drivers of prostate cancer behavior, which shall then be utilized by the entire PCA research community.

2. KEYWORDS

Prostate cancer, PTEN, ERG, ETS, MYC, cell cycle, gene expression, RNA sequencing, Cap Analysis of Gene Expression (CAGE)

3. ACCOMPLISHMENTS

Below are listed tasks, subtasks, and accomplishments for research sites 1 (coordinated by the initiating PI, Dr. Marchionni), and site 2 (coordinated by the partnering PI Dr. Lotan).

SPECIFIC AIM 1 (Dr. Lotan)

Expected tasks and milestones are summarized below.

Specific Aim 1: Validate association of PTEN and ETS status with risk of lethal prostate cancer	**Timeline (Months)**
Major Task 1: Assessing prostatectomy cohorts on multiple tissue microarrays (TMA) for PTEN, ETS, and cell proliferation rate	1-36
Subtask 1: Perform immunostaining for PTEN, ERG and Ki-67 and in situ hybridization on tissue microarrays (TMAs) from JHU and MSKCC cohorts; immunostaining for Ki-67 on HPFS/PHS cohort	1-12
Subtask 2: Score immunostaining and in situ hybridization from Subtask 1 • Digitally scan all slides using Aperio CS slide scanning system in Johns Hopkins OTS Core facility • Segment TMAs and upload to web-based browser, TMAJ (http://tmaj.pathology.jhmi.edu/) • Dr. Lotan, Dr. Gopalan (MSKCC), and pathology fellow supervised by Dr. Lotan perform scoring. Image analysis software (FRiDA on TMAJ) to be used for Ki-67 scoring	13-24

Subtask 3: Analysis of immunostaining and in situ hybridization data from Subtask 2 • Multivariate models to assess association of PTEN/ETS status with metastasis and survival in JHU and MSKCC cohorts • Correlation of PTEN/ETS status with proliferation in JHU, MSKCC and HPFS/PHS cohorts	18-30
Milestone #1: Co-author manuscript on association of PTEN/ETS status with cell cycle gene expression, proliferation rate and risk of metastasis and death in multiple validation cohorts	31-36

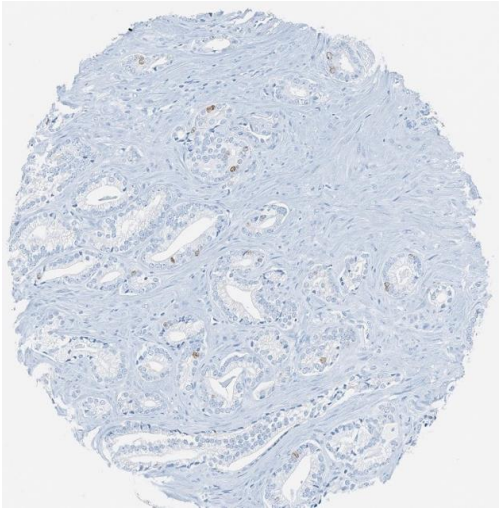

Figure 1: Ki-67 labelling in JHU cohort (200x magnification)

Progress on Major Task 1 – Subtask 1: Major activities for this activities included performing immunostaining and in situ hybridization on the JHU and MSKCC tissue microarray (TMA) cohorts. We have therefore performed and scored PTEN and ERG immunostaining on the JHU and MSKCC TMA cohorts, in addition to ETV1, ETV4 and ETV5 in situ hybridization on the JHU cohorts. Ki-67 immunostaining has been performed as proposed on both cohorts and automated scoring is pending. We also analyzed the PTEN/ERG/ETS data for association with metastasis and death from prostate cancer in the JHU and MSKCC cohorts. We have also correlated PTEN/ERG/ETS status of tumors in these cohorts with gene expression data from the same cohorts.

Progress on Major Task 1 – Subtask 2: The scoring for ETS gene rearrangements (ETV1/4/5) (as well as PTEN and ERG) on the JHU tissue microarrays has been completed and analyzed. Ki-67 immunostaining on those arrays is completed. PTEN and ERG staining as well as Ki-67 staining is completed for these arrays as well. All arrays have been digitally scanned and are viewed on our TMAJ viewer (**Figure 1**). However digital automated scoring of Ki-67 has been challenging since it is difficult to normalize the number of positive detected nuclei (brown) to the negative tumor nuclei (blue). This is because it is difficult to detect all negative tumor nuclei. After examining the possibility of simply normalizing the number of positive tumor cells to the total area of the spot (*i.e.*, density of positive cells per mm^2 of tumor), which is the easiest method, we have decided that this is suboptimal since it can be confounded by the amount of tumor nuclei sampled in the TMA spot (**Figure 1**). In this analysis, we find that lower grade tumors, with fewer tumor nuclei will have inappropriately low Ki-67 density scores. Thus, it is necessary to manually annotate all tumor-containing spots. To pilot this manual annotation algorithm, we used a smaller JHU cohort of ~200 prostate cancer cases where tumor spots could be manually annotated more easily. In this analysis (**Figure 2**, below), we found that median Ki-67 levels (% tumor nuclei staining) were significantly elevated in tumors with PTEN loss compared to those with intact PTEN (p=0.006), regardless of ERG status (p=0.006). Interestingly, cases with PTEN loss that were ERG negative showed increased variability in Ki-67 levels, potentially consistent with our finding of their heterogeneous molecular status (see below). However, we found this to be extraordinarily time-consuming and impractical to perform on 400 spots x 12 TMAs in the MSKCC cohort and 9 TMAs in the full JHU cohort.

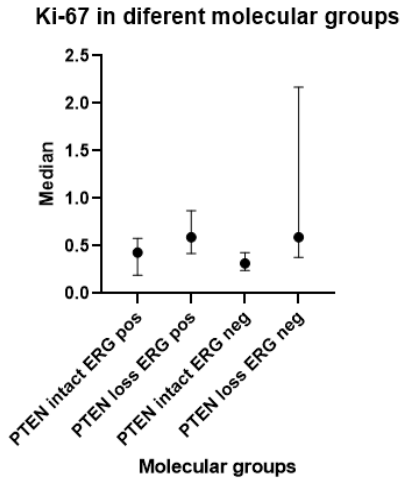

Figure 2: Ki-67 labelling in JHU cohort by PTEN-ERG status. Median Ki-67 is median % tumor nuclei staining.

To automate the tumor annotation process, we piloted a new protocol for dual staining for Ki-67 (brown) and AE1/AE3 keratin (red) with p63 (brown) in the JHU TMAs. This enabled us to train HALO image analysis software to identify the epithelial (glandular) components on each slide and automatically annotate them. Then, using the p63 immunohistochemistry, we can manually exclude benign glands from the analysis. Though still requiring a manual step, this is much more efficient than full manual annotation. We have annotated all JHU TMAs and are now in the process of quantifying the Ki-67 using this algorithm.

Table 1. Ki-67 labeling stratified by PTEN/ERG status in HPFS/PHS cohort

	Ki-67 (% positive tumor nuclei)	P-value	P-value
Any PTEN intact/ERG negative	0.8%	Reference	0.02
Any PTEN intact/ERG positive	0.3%	0.13	0.26
Complete PTEN loss / ERG negative	1.3%	0.02	Reference
Complete PTEN loss / ERG positive	1.1%	0.03	0.68

Progress on Major Task 1 – Subtask 3: For the HPFS/PHS tissue microarrays, we have completed and analyzed Ki-67 immunostaining. Data comparing the percent of tumor cells labeling for Ki-67, stratified by PTEN and ERG status are presented in **Table 1**. Exactly as reported in the JHU cohort described above, there was a statistically significant increase in Ki-67 labelling when comparing tumors that have PTEN loss and those that are PTEN intact, however there was not a significant difference in Ki-67 labeling between tumors with PTEN loss and ERG expression and those with PTEN loss that do not express ERG (p=0.68). Thus, we anticipate we will further validate this finding in the full JHU and MSKCC cohorts as well.

Table 2: Multivariable models of association of PTEN-ERG status with lethal prostate cancer in the MSKCC cohort.

			Univariable		Multivariable*	
	No. Cases	No. Controls	HR (95% CI)	p Value	HR (95% CI)	p Value
PTEN:						
Intact	46	542	Referent	–	Referent	–
Loss	47	156	3.25 (2.16–4.88)	<0.001	1.87 (1.15–3.04)	0.012
ERG:						
Neg	62	384	Referent	–	Referent	–
Pos	30	300	0.64 (0.41–0.99)	0.043	0.64 (0.36–1.11)	0.113
PTEN/ERG:						
PTEN intact/*ERG* neg	35	328	Referent	–	Referent	–
PTEN intact/*ERG* pos	10	200	0.47 (0.23–0.96)	0.037	0.48 (0.18–1.26)	0.136
PTEN loss/*ERG* neg	27	56	3.76 (2.27–6.21)	<0.001	2.31 (1.29–4.14)	0.005
PTEN loss/*ERG* pos	20	100	1.84 (1.06–3.18)	0.030	1.09 (0.56–2.12)	0.809

We have finalized the analysis of PTEN and ERG on the MSKCC cohort and examined the correlation of these molecular alterations with clinical outcomes (lethal prostate cancer) in multivariable models. These results are presented in **Table 2** and **Figure 3** below, and were recently published in *Journal of Urology* (Haney NM, Faisal FA, Lu J, Guedes LB, Reuter VE, Scher HI, Eastham JA, Marchionni L, Joshu C, Gopalan A*, Lotan TL*. PTEN loss with ERG-negative status is associated with lethal disease after radical prostatectomy. *J Urol*. 2020, 203(2):344-350. *Equal Contribution. PMID: 31502941).

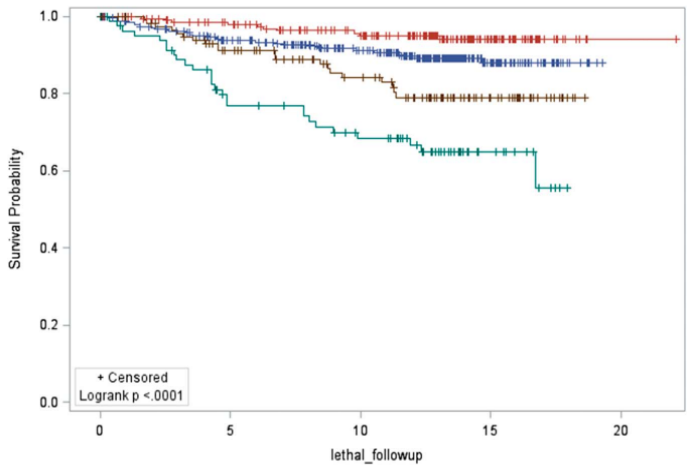

Figure 3: **Kaplan-Meier survival curves of freedom from lethal prostate cancer by PTEN and ERG status**. Blue curve indicates PTEN intact and ERG negative in 363 patients. Red curve indicates PTEN intact and ERG positive in 210 patients. Green curve indicates PTEN loss and ERG negative in 83 patients. Brown curve indicates PTEN loss and ERG positive in 120 patients.

Training and professional development: Nothing to report

Results dissemination to communities of interest: Results from Major Task 1 – Subtask 3 were recently published in *Journal of Urology* (Haney NM, Faisal FA, Lu J, Guedes LB, Reuter VE, Scher HI, Eastham JA, Marchionni L, Joshu C, Gopalan A*, Lotan TL*. PTEN loss with ERG-negative status is associated with lethal disease after radical prostatectomy. *J Urol*. 2020, 203(2):344-350. *Equal Contribution. PMID: 31502941).

SPECIFIC AIM 2 (Dr. Marchionni)

Expected tasks and milestones are summarized below.

Specific Aim 2: Leverage multi-dimensional public domain data to discover genomic features and signaling pathways associated with PTEN loss in ERG-positive and ERG-negative PCa.	**Timeline (Months)**
Major Task 1: Exploratory analysis of genomics datasets	1-6
Subtask 1: Examine gene expression distributions and identify outliers and other potential problems: • Use statistical summaries and visualizations (*e.g.*, principal component analysis, hierarchical clustering) • Apply appropriate transformation to the data if required	1-6
Major Task 2: Classify tumors based on PTEN, ETS, and MKI67 status.	6-24
Subtask 1: Use the EM-algorithm to classify tumors as positive or negative based on the expression levels of PTEN, ETS family members, and MKI67	6-12
Subtask 2: Compare expression-based classification to IHC and in-situ based status from in Specific Aim 1	12-30
Subtask 3: Analysis of PTEN and ETS status in cohorts available from GenomeDX and the public domain • Multivariate models to assess association of PTEN/ETS status based on genes expression dichotomization with metastasis and survival in all cohorts • Correlation of PTEN/ETS status based on genes expression dichotomization with proliferation in all cohorts	12-24
Major Task 3: Comprehensive meta-analysis of differential gene expression programs modulated by PTEN and ETS status in prostate cancer and characterization of their biological and clinical correlates	12-30
Subtask 1: Use generalized linear model to identify genes differentially expressed and differentially modulated by PTEN and ETS in prostate cancer	12-24
Subtask 2: Identification of relevant biological processes and signaling pathways associated with PTEN/ETS molecular signatures in prostate cancer	18-30
Subtask 3: Development and validation of predictive models based on associated with PTEN/ETS molecular signatures in prostate cancer	24-36
Milestone #2: Co-author manuscript on comprehensive meta-analysis of genes and signaling pathways associated with PTEN/ETS status in prostate cancer	24-36
Milestone #3: Co-author manuscript on prognostic values of PTEN/ETS molecular signatures in prostate cancer	24-36

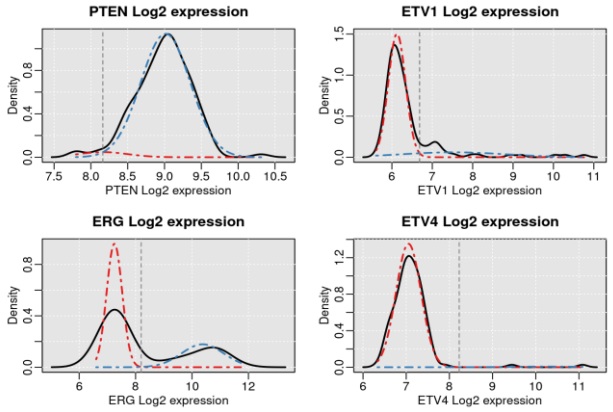

Figure 4: Gene expression distributions for PTEN, ERG, ETV1, and ETV4 in the MSKCC cohort. The underlying distributions from the EM-algorithm are shown in red and blue. ERG and ETV1 expressions are clearly bimodal.

Progress on Major Task 1 – Subtask 1: We have performed exploratory data analysis on all clinically annotated prostate cancer datasets available from the public domain and through the collaboration with GenomeDX. We used statistical summaries and data visualizations techniques (*e.g.*, principal component analysis, hierarchical clustering) to identify outliers and unwanted sources of variation in the data, applying appropriate pre-processing procedures and transformations as required.

Progress on Major Task 2 – Subtask 1: We have used the EM-algorithm to classify tumors as positive or negative based on the expression levels of PTEN, ETS family members, and MKI67. Overall, ERG gene expression proved to be bimodal in all datasets analyzed, with nearly perfect concordance with results from IHC and

CNV status. On the contrary, PTEN classification based on EM-classification of gene expression proved more challenging, with some degree of variation between datasets (an example in **Figure 4** for the MSKCC cohort).

Table 3. Comparison between ERG status and PTEN status based on IHC and EM-algorithm classification. Analyses were performed in the MSKCCC, the HPFS/HPS, and the Natural History cohorts.

	MSKCCC		HPFS/PHS		Natural History	
	ERG	**PTEN**	**ERG**	**PTEN**	**ERG**	**PTEN**
Sensitivity	0.97	0.98	0.47	0.87	0.92	0.01
Specificity	0.84	0.08	0.94	0.24	0.98	1.00
Positive Predictive Value	0.82	0.70	0.88	0.79	0.97	1.00
Negative Predictive Value	0.97	0.67	0.64	0.36	0.95	0.37
Prevalence	0.42	0.69	0.50	0.77	0.41	0.63
Detection Rate	0.41	0.68	0.24	0.67	0.38	0.01
Detection Prevalence	0.50	0.96	0.27	0.84	0.39	0.01
Balanced Accuracy	0.91	0.53	0.71	0.56	0.95	0.51
Overall Accuracy	0.90	0.70	0.71	0.72	0.96	0.38
Kappa	0.89	0.69	0.41	0.12	0.90	0.01

Progress on Major Task 2 – Subtasks 2: We compared results between IHC based assessment of PTEN and ERG expression with classification obtained based on gene expression using the EM algorithm. We performed this analysis on the MSKCCC, the HPFS/HPS, and the Natural History cohorts. For this analysis, IHC status was used as the gold-standard and cross-tabulated with the prediction based on the EM-algorithm classification the ERG and PTEN gene expression levels. Overall, the concordance between IHC and EM-predictions was much higher for ERG status than for PTEN status (**Table 3**). Based on these findings, we decided to develop a more robust, multigene signature for PTEN classification using expression levels.

Progress on Major Task 2 – Subtasks 3: This analysis produced a list of differentially expressed genes associated with ERG and PTEN status. These lists accounted for a core set of genes shared across the different datasets, as well as for genes differentially expressed only in each individual dataset considered. For this reason we therefore decided to focus on genes and pathways identified in a metanalysis in conjunction with the development of a prognostic signature (see below, section **Progress on Major Task 3 – Subtasks 3)**.

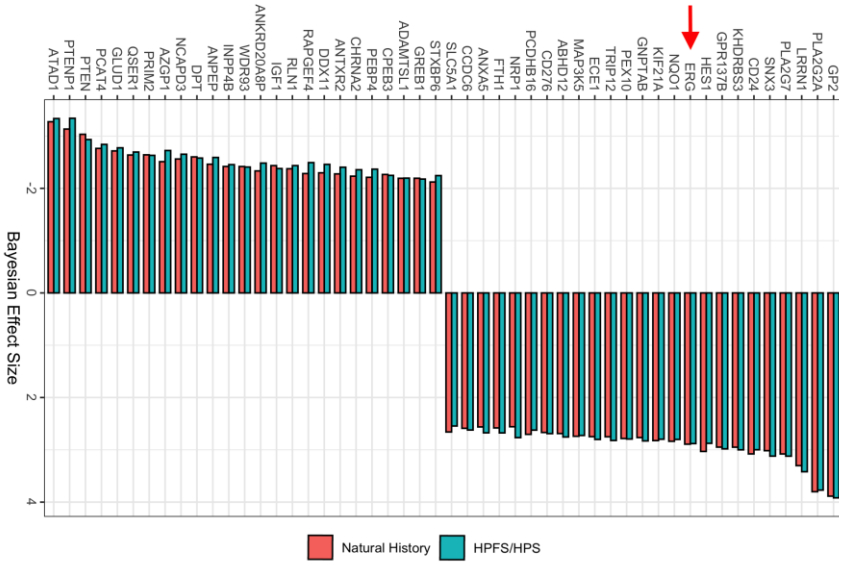

Figure 5. PTEN signature from meta-analysis. PTEN signature obtained by multi-level model for cross-study detection of differential gene expression based on IHC calls on Natural History and HPFS cohorts. Figure shows the effect size of each cohort. ERG is one of the most upregulated genes associated with PTEN loss (red arrow).

Progress on Major Task 3 – Subtask 1: During the first two years of project, we have developed and characterized in depth a consensus signature for PTEN loss using a meta-analytic approach. In the third year of the project, we have investigated the association of this signature with ERG status. This analysis has revealed that the ERG gene itself is among the top upregulated genes in our PTEN loss sigature (**Figure 5**).

Based on this observation, we have therefore hypothesized that our PTEN signature could be heavily influenced by the ERG rearrangement, since this gene encodes a transcription factor. In order to test this hypothesis, we have therefore repeated the meta-analysis by splitting the samples by ERG status and then by fitting two separate Bayesian hierarchical models for differential expression by PTEN status. In the samples with ERG rearrangement, we observed a signature similar to the overall PTEN consensus signature we previously developed in year 2. On the contrary, in the samples without ERG rearrangement, we could not find any significant differences between samples with PTEN loss and PTEN intact.

This finding was surprising, given that PTEN is a powerful tumor suppressor capable of triggering important molecular and functional changes. We speculated that this result could be caused by two reasons: 1) PTEN loss in the absence of ERG rearrangement, does not impact the cell in any significant way; or 2) The absence of ERG

rearrangement generates a high level of heterogeneity that makes it hard to estimate difference between PTEN-null and PTEN intact samples. The first hypothesis, however, is highly unlikely, given the fact that it is well-established that PTEN loss triggers deep changes in cellular metabolism and growth. Therefore, we performed experiments to test if the second hypothesis was true.

In order to test if tumors without ERG rearrangement presented overall higher heterogeneity levels than tumors with it, we stratified the samples based on their PTEN and ERG status. We used the divergence framework available through the R/Bioconductor package 'divergence. Using individual genes (for transcriptomic data) as features of interest, the normal samples were used to estimate baseline profiles and then the divergence was computed for the tumor samples in TCGA and HPFS cohorts. A similar analysis was conducted for the methylation and genomic mutation data from TCGA, using individual CpGs and mutations/copy-number-variation as the features of interest. A random sampling based on the size of the smallest group was extracted from the resulting binary coding to compute the average hamming distances between pairs of samples, this step was performed with 1000 bootstraps.

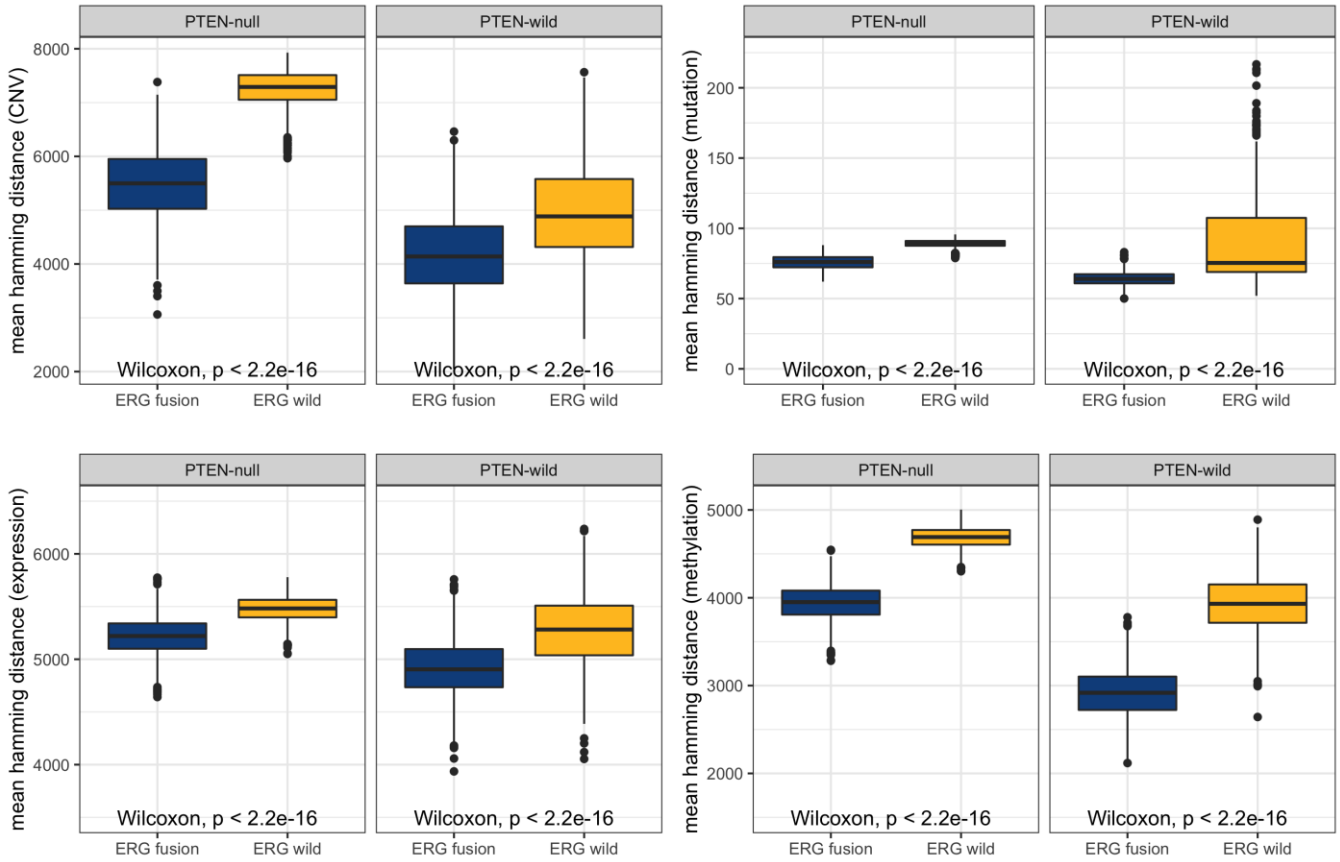

Figure 6. Heterogeneity analysis in ERG positive and negative tumors. Average hamming distance based on 1000 bootstraps intra-samples between each group, showing that samples in absence of ERG rearrangement (ERG wild) presented higher levels of heterogeneity (higher distances) than samples with rearrangement (ERG fusion). **Top left)** Hamming distance based on CNV in TCGA; **Top right)** Hamming distance based on mutation in TCGA; **Bottom left)** Hamming distance based on divergence expression levels in TCGA and **Bottom right)** Hamming distance based on divergence methylation levels in TCGA.

For all molecular data types and for both cohorts, we observed that the intra-group distances between the ERG positive samples (*i.e.*, those with ERG rearrangement) were always significantly higher than between ERG negative tumors, thus confirming our hypothesis (**Figure 6**).

Progress on Major Task 3 – Subtask 2: In our analysis of biological processes and signaling pathways associated with PTEN/ETS molecular signatures in prostate cancer, we saw a strong enrichment in immune related pathways upon PTEN loss (see **Figure 7**). This finding was particularly surprising given that PTEN is itself a key positive regulator of innate immune response. Disruption of PTEN expression has been previously reported to lead to decreased innate immune response. Remarkably, despite the loss of PTEN being associated

with higher expression of the immune checkpoint gene programmed death ligand-1 (PD-L1) in several cancer types this is not true in PCa. So far, current immunotherapeutic interventions, such as PD-1 blockade, in PCa have not been successful. One of the possible reasons is the lack of PD-L1 expression. Therefore, alternative targets must be considered for immunotherapy in PCa. One alternative target is the checkpoint molecule B7-H3 (CD276), whose expression has already been associated with PCa progression and worse prognosis and has been suggested as a target for immunotherapy. CD276 was one of the most concordant up-regulated genes in our signature (**Figure 5**) suggesting that its expression is associated with PTEN loss. The positive enrichment of MHC class II antigen presentation, neutrophil degranulation, vesicle-mediated transport, and FC receptor pathway-related genes suggests that PTEN-null tumors may be immunogenic. This observation has potential implications in the context of precision medicine since immune responsive tumors are more likely to respond to immunotherapies.

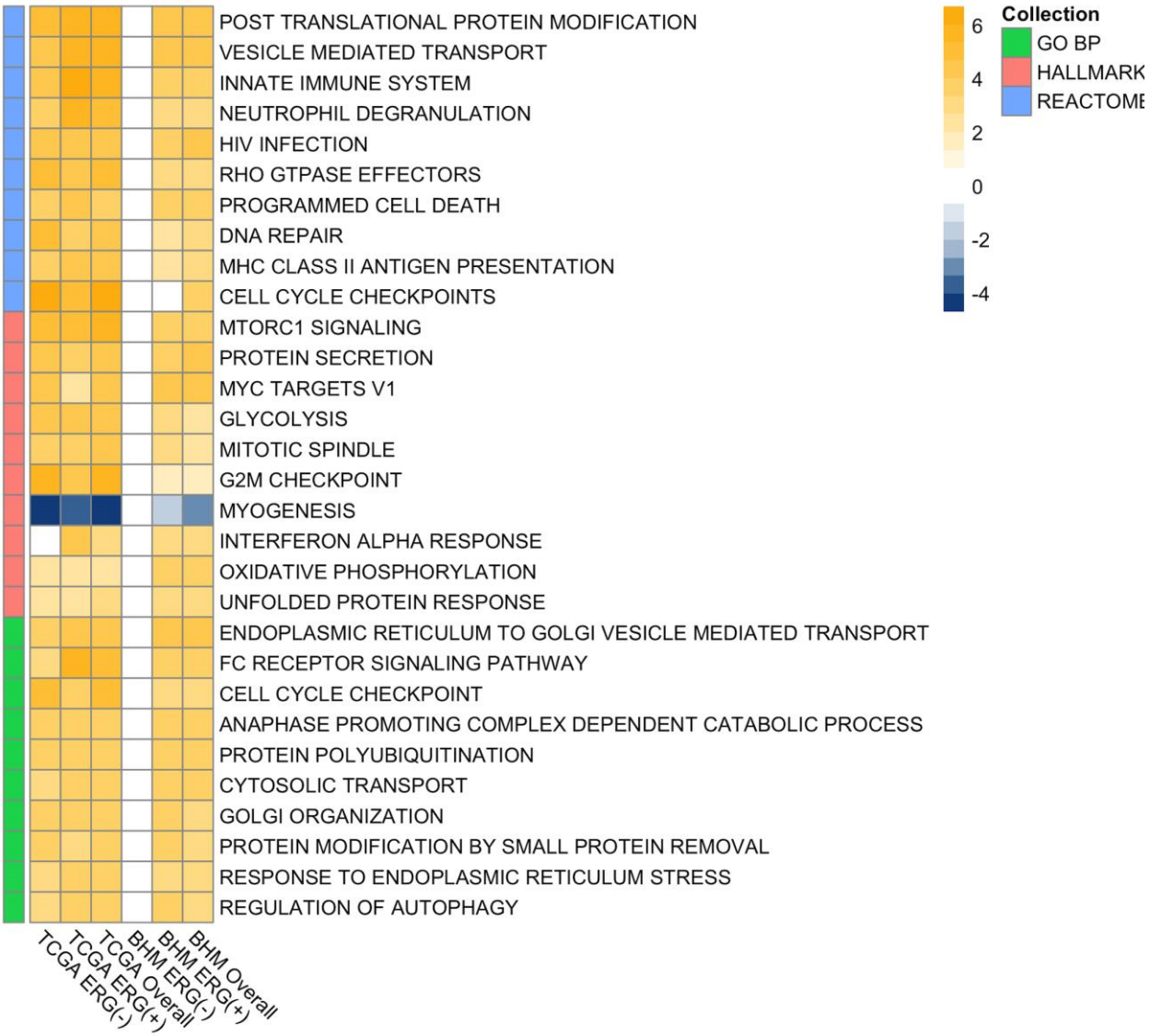

Figure 7. Top enriched gene sets enriched across PTEN-null and PTEN-intact in the TCGA and meta-analysis (BHM) cohorts stratified by ERG status and overall. Heatmap of mean-centered $\log_2$ signed p-values (normalized enriched score multiplied by $\log_{10}$ of p-value) showing the top 10 enriched gene sets of each collection (ranked by signed p-value).

Progress on Major Task 2 and 3 – Subtask 3: During the third year of research, however, we have generated a prognostic gene expression signature for prostate cancer progression using a combination of gene expression data from the public domain, as detailed below. To this end, a total of 674 primary prostate cancer samples (from 3 distinct studies) were used for discovery of the gene signature, while an independent cohort of 248 samples was used for validation and signature performance assessment (see **Table 4**).

Table 4. Collected data sets showing the number of samples and the number of metastasis cases. 3 datasets were used for training and one data set (GSE116918) was used as an independent validation cohort.

GEO accession	GPL	Number of samples (metastasis cases)	Training/Testing
GSE55935	GPL10558	44(8)	Training
GSE51066	GPL5188	85(51)	Training
GSE46691	GPL5188	545(212)	Training
GSE116918	GPL25318	248(22)	**Testing**

First, we have performed a large scale differential expression analysis of gene expression data from different microarray platforms. We have identified 49 up-regulated and 26 down-regulated genes in prostate cancer metastasis cases. We have then further optimized this signature using a ”forward search” process reducing the original list to just 14 up-regulated and 17 down-regulated genes. Finally, we have combined the gene expression levels for these genes into a meta-score for use in subsequent analyses, including multivariable Cox proportional hazard model analyses with other clinical and pathological variables (Age, PSA, T-stage ,and Gleason grade).

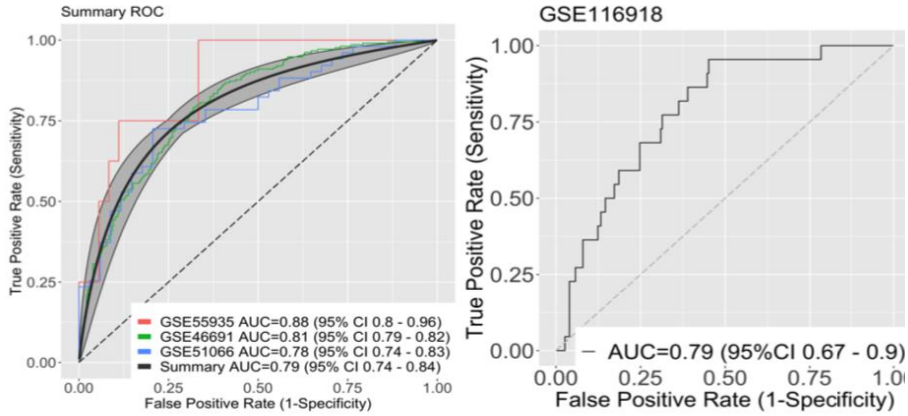

Figure 8. Performance of the prognostic signature. ROC curves in the 3 training data sets with a summary ROC curve of all data sets combined (Left) and ROC curve in the independent testing data set (Right).

To assess the performance of our signature, we measured the area under the receiver operating characteristic curve (AUC). In the training the AUC ranged from 0.78 to 0.88 (**Figure 8**, right panel), while in the testing cohort the AUC was 0.79 (**Figure 8**, left panel), confirming the prognostic value of the signature.We performed Kaplan-Meier analyses in the testing cohort. Patients with higher signature meta-score had worse metastasis-free survival than those with lower score (p-value < 0.0001, see **Figure 9**). Additionally, we also performed survival analyses using individual gene expression levels rather than the signature meta-score. In this analysis, 7 out of 14 up-regulated genes (TMSB10, IQGAP3, CST2, STC2, FOXH1, PTDSS1, HES6) were significantly associated with lower survival, while 8 out of the 17 down-regulated genes (AZGP1, NT5DC1, KCTD14, PTPRN2, UFM1, CCK, KIAA1210, POTEG) were significantly associated with better survival when highly-expressed.

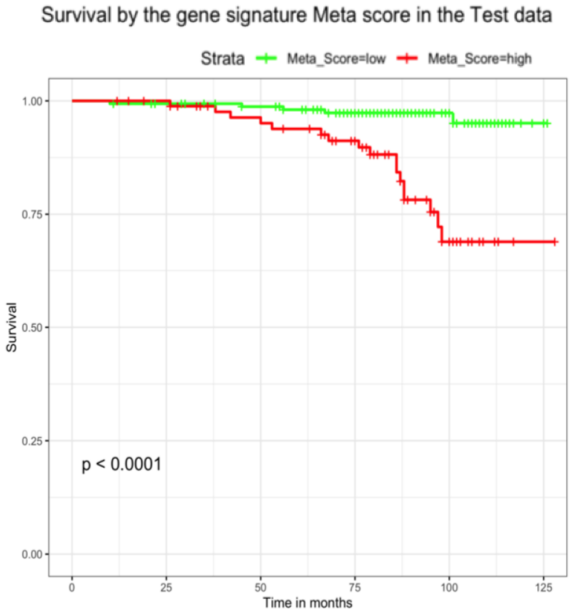

Figure 9. Survival analysis in the testing cohort. Kaplan-Meier curves based on signature meta-score. Patients with low score have a better metastasis-free survival than those with a high score (p-value < 0.0001).

Most importantly, the signature meta-score was the only significant variable in the multivariable Cox regression analysis performed in the testing cohort. The model included

the meta-score together with age, PSA (prostate specific antigen), Gleason grade and T-stage, with a hazard ratio of 5.67 (95% CI : 2.02 - 15.9, see **Figure 10**)

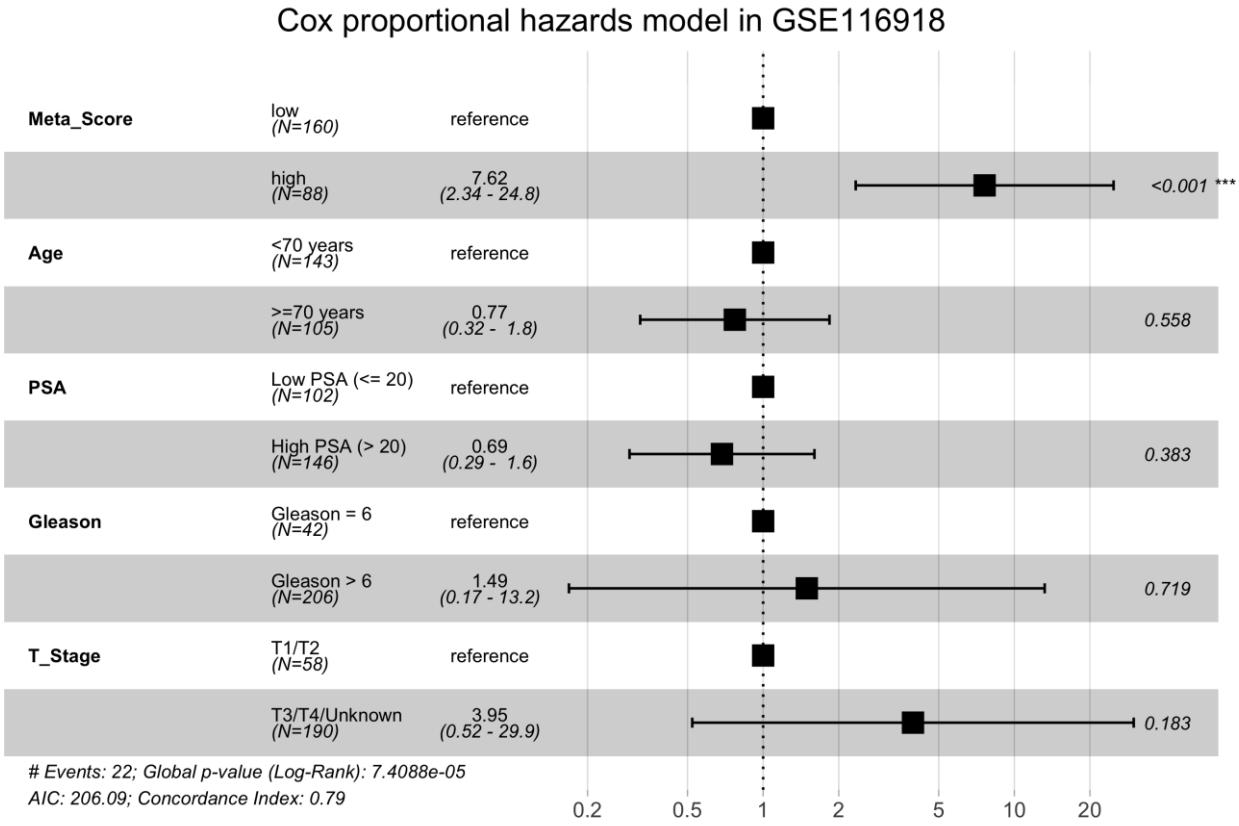

Figure 10. Forest plot for Cox proportional hazards model results in the testing cohort. The signature meta-score is the only significant variable, outperforming other clinical and pathological variables.

Collectively, these analyses show the importance of integrating gene expression data from multiple studies to identify accurate and consistent prognostic signatures. We are currently integrating this signature with PTEN and ERG classification obtained by the EM-algorithm, as previously described.

Training and professional development: Nothing to Report.

Results dissemination to communities of interest: Results from Major Task 1,2,3 were recently published in the following bioRxiv pre-print article: "Transcriptional landscape of PTEN loss in primary prostate cancer", by Eddie Luidy Imada, Diego Fernando Sanchez, Wikum Dinalankara, Thiago Vidotto, Ericka M Ebot, Svitlana Tyekucheva, Gloria Regina Franco, Lorelei Mucci, Massimo Loda, Edward M Schaeffer, Tamara Lotan, Luigi Marchionni doi: https://doi.org/10.1101/2020.10.08.332049. This article is currently under review in Modern Pathology.

SPECIFIC AIM 3 (Drs. Lotan and Marchionni)

Expected tasks and milestones are summarized below.

Specific Aim 3: Discover and validate gene regulatory and expression signatures associated with PTEN loss on genetically homogeneous ERG-positive and ERG-negative backgrounds.	**Timeline (Months)**
Major Task 1: Select 40 FFPE tumors from Johns Hopkins Surgical Pathology archives (20 ERG-positive and 20 ERG-negative, ETV1-negative). Within each group 10 have heterogeneous PTEN loss, 5 have homogeneous PTEN loss and 5 have intact PTEN by IHC	1-12
Subtask 1: Immunostaining 100 index tumors from Gleason 3+4=7 radical prostatectomies	1-6
Subtask 2: Score staining and select cases	4-8
Subtask 2: Punch blocks and prepare RNA for CAGE	8-12

Major Task 2: Perform CAGE analysis of the tumors resulting from Major Task 1 of Specific Aim3. Technology assessment and troubleshooting in collaboration with Dr. Carninci (RIKEN, Japan)	6-24
Subtask 1: CAGE library preparation, quality assessment, and sequencing • Performed at the Next Generation Sequencing Center (NGSC, Dr. Yegnasubramanian)	6-18
Major Task 3: Bioinformatics analysis of CAGE data generated in Major Task 2 of Specific Aim 3. Technology assessment and troubleshooting in collaboration with Dr. Carninci (RIKEN, Japan)	12-36
Subtask 1: CAGE short reads quality evaluation and alignment to the reference genome • Performed using NGSC computing cluster (Dr. Wheelan)	12-24
Subtask 2: Quantification of expressed genomic regions using CAGE tags • Performed using the School of Public Health (SPH) High Performance Computing Cluster (HPCC)	18-30
Subtask 3: Classification of expressed genomic regions, identification of active enhancers, promoters, and transcript • Performed using SPH HPCC	24-30
Subtask 4: Gene expression regulatory network reconstruction and analysis • Performed using SPH HPCC	24-36
Milestone #4: Co-author manuscript on CAGE analysis of PTEN/ETS status in prostate cancer	30-36

Progress on Major Task 1 – Subtask 1-3 (Dr. Lotan): These activities have been successfully completed.

Progress on Major Task 2 – Subtasks 1 (Dr. Marchionni): During years 1 and 2 of the proposal, we have tested CAGE and nanoCAGE sequencing protocols using high quality RNA obtained from several prostate cancer cell lines. These protocols were optimized for an Illumina mySeq instrument. In year 3 of the proposal, we have focused on optimizing the protocols for RNA samples prepared from tissue specimens. We also worked on developing optimal multiplexing protocols, in order to take advantage of the higher sequencing throughput of the Illumina HiSeq2500 instrument. To this end, we have obtained RNA from 12 tumor samples, prepared the nanoCAGE libraries, and then performed sequencing, as detailed below.

Tumor samples from Major Task 1 were multiplexed and the nanoCAGE protocol was used to the prepare the pooled libraries for sequencing. Before processing the samples on the Illumina HiSeq2500 instrument, we also performed after a successful mini-run on a mySeq instrument. For an unknown reason, however, the sequencer analytical pipeline failed to demultiplex the sequenced samples. We therefore extensively reviewed the experiments and performed an in depth troubleshooting. The quality control analysis in the whole dataset revealed that although the overall sequence quality was good (> 30 Phred Score), there was a high level of duplicated reads (82.6% and 64.2% for R1 and R2, respectively).

We therefore attempted to analyze the sequencing data using an alternative pipeline. Specifically, we tried to process the libraries using the TagDust2 software, which also failed in demultiplexing the libraries. Next, we also aligned the reads to the human genome (hg38) to check if the sequences obtained were originating from the tumor RNA or from the sequencing kit by-products. In this analysis, only about ~6% of the reads aligned uniquely to the human genome, and around ~17% aligned to multiple loci, indicating that most of the sequences obtained were not originating from the human RNA from the tumors. Finally, we tried to align the sequences to the PhiX genome since this DNA was used during the library preparation to increase the library complexity. This analysis revealed that around ~46% of the reads aligned to the PhiX genome, highlighting potential problems during library preparation and/or sequencing (*e.g.*, incorrect primer loading in the Illumina HiSeq2500). Unfortunately, due the COVID-19 pandemic in year 4, the development and troubleshooting of the nanoCAGE libraries had to be halted and this task could not be completed. We, however, were still able to complete our goals with an alternative strategy (see Major Task 3 bellow)

Progress on Major Task 3 – Subtasks 1, 2, 3, and 4 (Dr. Marchionni)

In year 1 and 2 of the project, we have created a comprehensive atlas of gene expression based on recent annotations from the FANTOM consortium based on CAGE-sequencing data (CAGE Associated Transcriptome,

referred as FANTOM-CAT) and the recount2 database. This resource – called FC-R2 – accounts for gene expression summaries for over 109,000 genes across over 70,000 human samples. It encompasses expression information for dozens of thousands lncRNAs genes, including enhancers and promoters. This resource was used as an alternative venue for the study of lncRNAs due to the shortcomings of Major Task 2 – Subtask 1. It enabled us to explore enhancers, promoters and other lncRNAs that have not been explored in this context before.

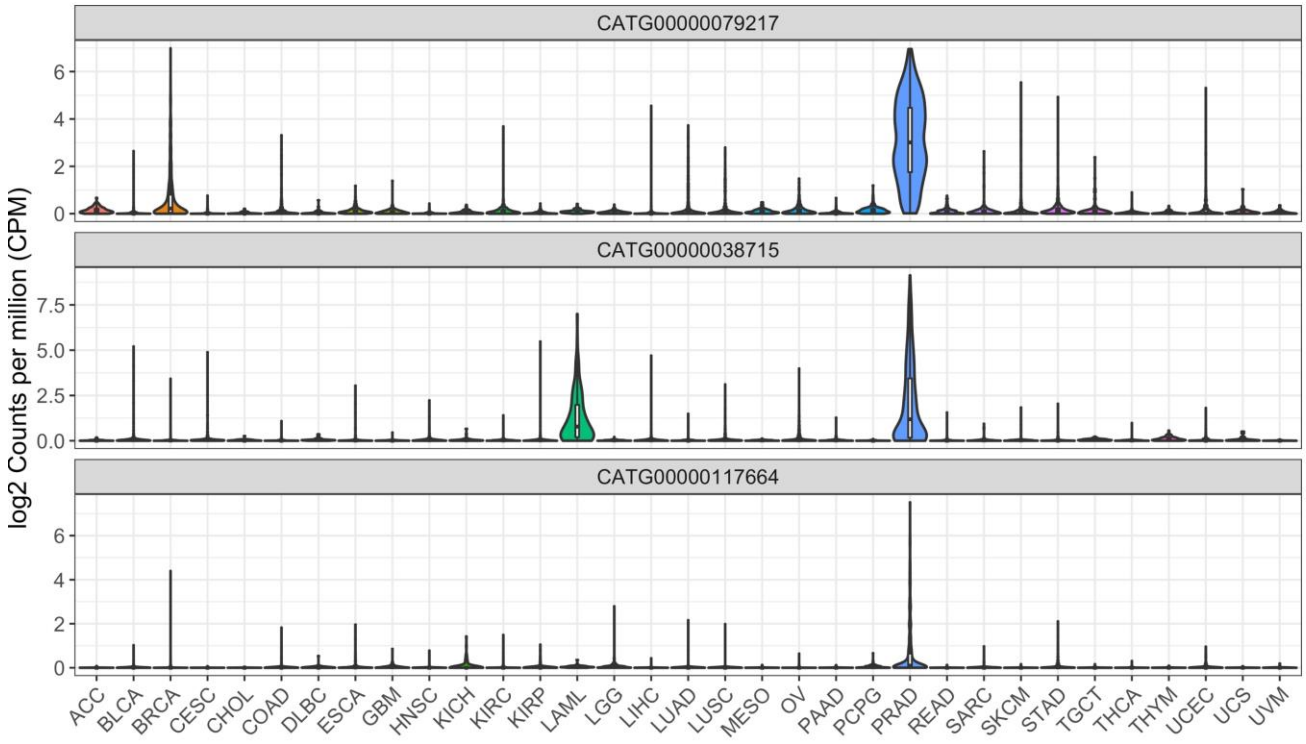

Figure 11 - Expression profiles of novel FANTOM-CAT genes CATG00000038715, CATG00000079217 and CATG00000117664 across 33 cancer types. Violin-plots shows expression (log2 CPM+1) distribution.

In year 3 of the project, we have leveraged the FC-R2 resource and we have performed differential expression analysis between PTEN-null and PTEN-intact samples (see Aim 2 – Major task 3 – subtask 2). In this analysis, we found 264 lnRNAs, including enhancers and promoters, associated with PTEN in PCa, with around half of them not previously reported in association with PCa and were only annotated in the FANTOM-CAT meta-assembly. The FANTOM consortium has recently characterized hundreds of lncRNAs via molecular phenotyping, however none of the lncRNAs associated with PTEN-loss was included in their study, and therefore they still lack an assigned function. In this their study it was shown that the expression levels of genes in the same topological domain are highly correlated only in tissue types in which these genes play a functional role. For this reason, we characterized our novel PTEN associated lncRNAs by analyzing the expression correlation with nearby genes across all cancer types in TCGA.

Among the FANTOM-CAT exclusive genes with the highest fold change in close proximity with coding genes, were CATG00000038715, CATG00000079217 and CATG00000117664. These genes were positioned in the same loci as the genes encoding for CYP4F2, FBXL7, and GPR158, respectively. These lncRNAs genes were mostly expressed in PCa as opposed to other cancer types in TCGA, which might suggest their function are associated with PCa progression (**Figure 11**). All these genes were shown to be highly correlated with their respective "local" coding gene. For example, CATG00000038715 is near CYP4F2 and CYP4F11, which encodes members of the cytochrome P450 enzyme superfamily, and the expression levels of CATG00000038715 and CYP4F2 were found highly correlated almost exclusively in PCa ($R=0.91$, $p < 2.2e-16$) suggesting that CATG00000038715 function might be associated with CYP4F2 in a highly specific manner in PCa (**Figure 12**).

Moreover, all of the coding genes mentioned above (i.e. CYP4F2, FBXL7, and GPR158) are involved in immune response, corroborating with the results of the pathways analysis.

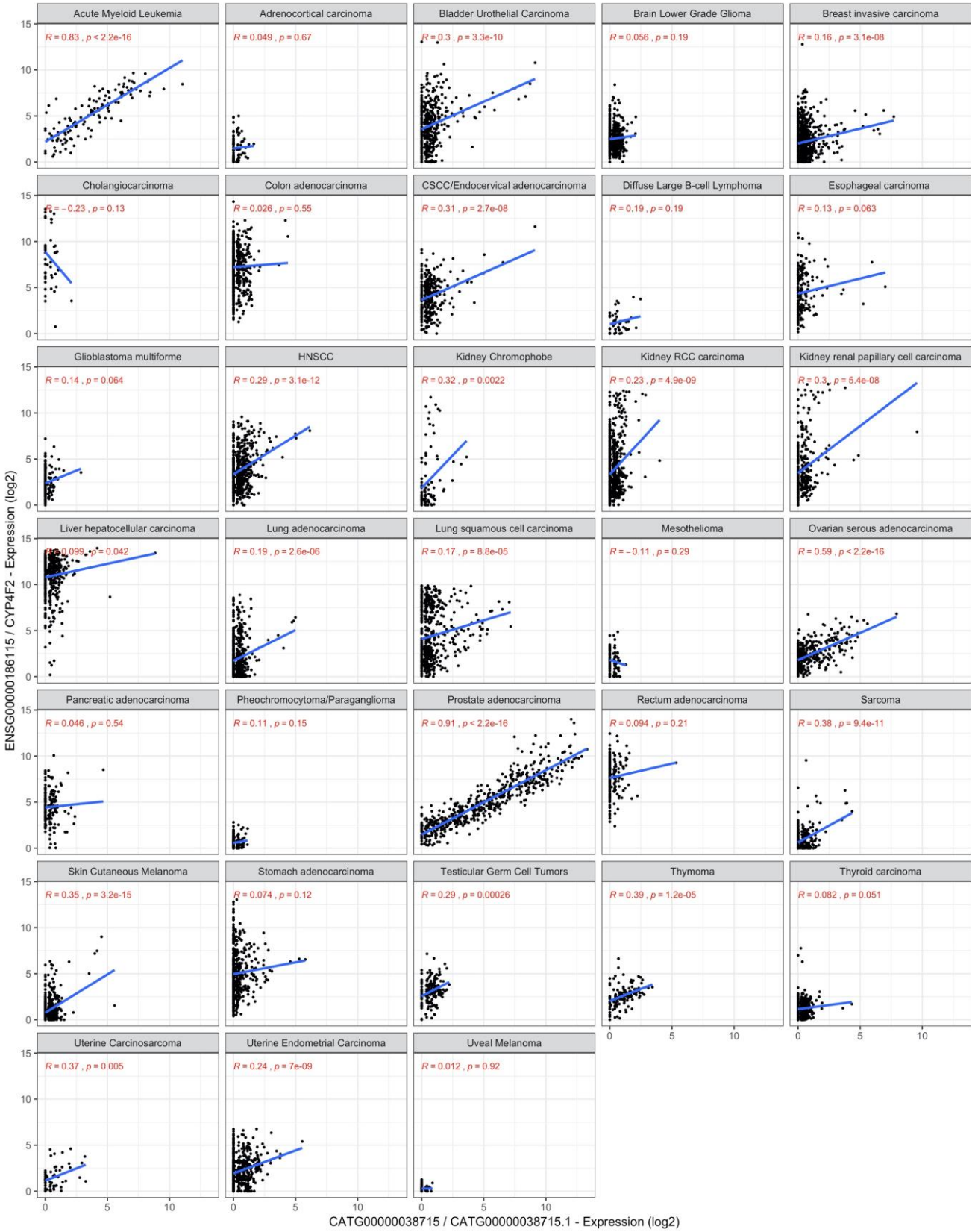

Figure 12 - Person correlation of the unknown gene CATG00000038715 and CYP4F2 across cancer types. CATG00000038715 and CYP4F2 expression are shown to be highly correlated in prostate cancer. Moreover, CATG00000038715 expression is shown to be highly specific to prostate cancer. With exception of leukemia cells, none of the other tumors expressed high levels of CATG00000038715.

Training and professional development: Nothing to Report.

Results dissemination to communities of interest: : Results from Major Task 2 – Subtasks 2, 3, and 4 were recently published in Genome Research: "Recounting the FANTOM Cage Associated Transcriptome", by Eddie-Luidy Imada, Diego Fernando Sanchez, Leonardo Collado-Torres, Christopher Wilks, Tejasvi Matam, Wikum Dinalankara, Aleksey Stupnikov, Francisco Lobo-Pereira, Chi-Wai Yip, Kayoko Yasuzawa, Naoto Kondo, Masayoshi Itoh, Harukazu Suzuki, Takeya Kasukawa, Chung-Chau Hon, Michiel JL de Hoon, Jay W Shin, Piero Carninci, FANTOM consortium, Andrew E Jaffe, Jeffrey T Leek, Alexander Favorov, Gloria R Franco, Ben Langmead, and Luigi Marchionni. doi: https://doi.org/ 10.1101/gr.254656.119

4. IMPACT

Impact on prostate cancer research

We have successfully classified ERG status in all available datasets analyzed. Furthermore, we have successfully reproduced in an independent cohort our previous findings indicating that PTEN loss is associated with a worst prognosis in ERG/ETS-negative patients.

We have successfully applied highly validated IHC and in situ hybridization assays to determine PTEN and ETS status in 2 additional cohorts (MSKCC and JHU) with accompanying gene expression data for future analysis. Association of PTEN with Ki-67 proliferation index has been performed and analyzed for two datasets.

We have developed a consensus molecular signatures of PTEN loss in prostate, showing that PTEN-loss were associated with immune response pathways and biological processes. We have also revealed that ERG negative samples show a higher level of heterogeneity as compared with the ERG positive group, which can be associated with the worst prognosis observed in the former group.

We have generated a comprehensive catalog of expression of coding and non-coding genes using the FANTOM-CAT annotation and the recount2 atlas. Using this resource we have identified hundreds of lncRNAs associated with PTEN and ERG status and investigated potential roles for the top differentially expressed ones.

This project will add significantly to prostate cancer research by further refinement and validation of this prognostic biomarker as we develop expression signatures in the next reporting periods.

Impact on other disciplines: The implementation of the FC-R2 gene expression atlas based on recount2 gene expression summary and the FANTOM-CAT meta-transcriptome will provide a useful resource for studying enhancer and promoter expression in other fields beyond prostate cancer research.

Impact on technology transfer: Nothing to Report.

Impact on society beyond science: Nothing to Report.

5. CHANGES/PROBLEMS

The major change in the research has been the fact that we could not get the CAGE and the nanoCAGE protocols to work properly. For these reason we have developed a bioinformatics pipeline that enables to quantify promoter and enhancer expression from standard RNA-sequencing data. We have then used this pipeline to implement recount2-FANTOM-CAT gene expression atlas. This resource represent a comprehensive compendium of gene expression across the human transcriptome containing over 109,000 genes, greatly expanding the features available for our analyses, by including distinct classes of coding and non-coding genes, such as messenger RNAs, intergenic lncRNAs, and expressed divergent promoters and enhancers. Using this resource we were able to analyze promoter and enhancer expression in PTEN and ERG prostate cancer tumors, ultimately attaining the scientific goals for which the use of CAGE and nanoCAGE were originally proposed.

6. PRODUCTS

As results of the research activities supported on this award the following manuscripts were published:

1. *"Recounting the FANTOM CAGE-Associated Transcriptome."* Eddie Luidy Imada, Diego Fernando Sanchez, Leonardo Collado-Torres, et al. Genome Res. 2020 Jul; 30(7): 1073–1081. doi: 10.1101/gr.254656.119. PMCID: PMC7397872
2. *Functional annotation of human long noncoding RNAs via molecular phenotyping."* Jordan A. Ramilowski, Chi Wai Yip, Saumya Agrawal,et al. Genome Res. 2020 Jul; 30(7): 1060–1072. doi: 10.1101/gr.254219.119 - Correction in: Genome Res. 2020 Sep; 30(9): 13771. PMCID: PMC7397864
3. *"PTEN Loss with ERG Negative Status is Associated with Lethal Disease after Radical Prostatectomy*". Haney NM, Faisal FA, Lu J, et al. J Urol. 2020 Feb;203(2):344-350. doi: 10.1097/JU.0000000000000533. Epub 2019 Sep 10. PMID: 31502941.

As results of the research activities supported on this award the following pre-print were published:

1. *"Transcriptional landscape of PTEN loss in primary prostate cancer"* by Eddie Luidy Imada, Diego Fernando Sanchez, Wikum Dinalankara, et al. Preprint in biorXiv doi: https://doi.org/10.1101/2020.10.08.332049.

As results of the research activities supported on this award the following resources were made available:

1. F2-RC gene expression atlas: http://marchionnilab.org/fcr2.html

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Luigi Marchionni
Project role:	Initiating Principle Investigator
Researcher Identifier:	0000-0002-7336-8071 (ORCID)
Institution:	Johns Hopkins University
Nearest person month worked:	4 (rounded to 4)
Contribution to Project:	Dr. Marchionni coordinated the project, provided supervision of research activities provided by the fellows, and directly performed the analyses
Funding Support:	NA

Name:	Tamara Lotan
Project role:	Partnering Principle investigator
Researcher Identifier:	0000-0002-0494-9067 (ORCID)
Institution:	Johns Hopkins University
Nearest person month worked:	1
Contribution to Project:	Dr. Lotan coordinated the project, provided supervision of research activities provided by the fellows, and directly performed the analyses
Funding Support:	NA

Name:	Anne Jedlicka
Project role:	Co-investigator
Researcher Identifier:	NA
Institution:	Johns Hopkins University

Nearest person month worked:	1
Contribution to Project:	Dr. Anne Jedlicka coordinated the experiments with CAGE
Funding Support:	NA

Name:	Amanda Dziedzic
Project role:	Research specialist
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	1
Contribution to Project:	Ms. Amanda Dziedzic performed the experiments with CAGE

Name:	Wikum Dinalankara
Project role:	Post-doctoral fellow
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	5 (rounded to 5)
Johns **Contribution to Project:**	Dr. Dinalankara performed bioinformatics and statistical analyses under Dr. Marchionni supervision
Funding Support:	NA

Name:	Eddie Luidy-Imada
Project role:	Post-doctoral fellow
Researcher Identifier:	0000-0001-9527-3703 (ORCID)
Institution:	Johns Hopkins University
Nearest person month worked:	12
Contribution to Project:	Dr. Luidy-Imada performed bioinformatics and statistical analyses under Dr. Marchionni supervision
Funding Support:	NA

Name:	Diego Sanchez Martinez
Project role:	Graduate Student
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	8
Contribution to Project:	Dr. Sanchez Martinez performed bioinformatics and statistical analyses under Dr. Marchionni supervision for developing the prognostic signature.
Funding Support:	NA

Name:	Lotte Mulder
Project role:	Student
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	2
Contribution to Project:	Ms. Mulder performed bioinformatics and statistical analyses under Dr. Marchionni supervision for developing the prognostic signature.
Funding Support:	NA

Name:	Daniella Salles
Project role:	Post-doctoral fellow
Researcher Identifier:	
Institution:	Johns Hopkins University
Nearest person month worked:	1
Contribution to Project:	Dr. Salles performed laboratory analyses under Dr. Lotan supervision
Funding Support:	NA

Name:	Ericka M. Ebot
Project role:	Co-investigator
Researcher Identifier:	NA
Institution:	Harvard T.H. Chan School of Public Health

Nearest person month worked:	1 (rounded to 1)
Contribution to Project:	Dr. Ebot provided analytical support for the PHS/HPHS cohorts
Funding Support:	NA

Name:	Kaushal Asrani
Project role:	Postdoctoral fellow
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	5
Contribution to Project:	Dr. Asrani performed data collection and interpretation supervised by Dr. Lotan
Funding Support:	NA

Name:	Rafael Guerrero-Preston
Project role:	
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	1
Contribution to Project:	
Funding Support:	NA

Name:	Lanlan Ji
Project role:	
Researcher Identifier:	NA
Institution:	Johns Hopkins University
Nearest person month worked:	2
Contribution to Project:	
Funding Support:	NA

Change in active other support

Dr. Marchionni:

- No longer supported by 1U54RR023561-01A1 (Ford)
- No longer supported by KKESH (Eberhart) – this award is completed
- No longer supported by W81XWH-12-PCRP-TIA – this award is completed
- No longer supported by R01CA163594 (Sidransky) – this award is completed
- PC141474 (Tomlins and Schaeffer) – this award is completed
- R21 AI124776-01 (Romerio) – this award is completed
- 1R01CA211695-01A1 (Hurley) – this award is completed
- R01 PA-13-302 (Marchionni) – this award is now active and moved from pending
- R01CA206027 (Sidransky/Hoque) – this award is now active and moved from pending
- R01CA208709 (Sidransky/Hoque) – this award is now active and moved from pending
- W81XWH-16-PCRP-IDA (Lupold) – this award is now active and moved from pending
- U01CA231776 (Marchionni/Tran/Hann) – this award is now active and moved from pending
- R01CA235681 (Hahn) – this award is now active and moved from pending
- W81XWH-19-1-0292 (Lotan) – this award is now active and moved from pending

Dr. Lotan:

- New Award W81XWH-19-1-0292 (Lotan[PI], Title: Epigenomic Landscape of Primary Prostate Cancer in African-American Men, 10% effort)

- New Award W81-XWH-19-1-0781 (Asrani[PI], Title: mTORC1 Regulates MiTF Expression and Lysosomal Biogenesis, 2% effort)
- New Award R01 CA238218 (Pienta [PI], Title: Dissecting the prostate cancer diaspora, 1% effort)
- New Award PC180810 (Luo [PI], Title: Genetic and genomic determinants of homologous recombination repair deficiency as treatment selection markers for lethal prostate cancer, 5% effort)
- New Award PC180375 (Isaacs[PI], Title: Discovery and Functional Analyses of Susceptibility Genes for Lethal Prostate Cancer, 5% effort)
- No longer supported by W81XWH-15-1-0661, R01CA211695, RSG-17-160-01-CSM.

Other organizations were involved
Organization Name: Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA
Organization Name: Memorial Sloan Kettering Cancer Center, New York, NY, USA

Partner's contribution to the project
Collaboration: Dr. Ericka Ebot (Harvard) provided analytical support for the PHS/HPHS cohorts (<1 person/month effort).

Dr. Anu Gopalan (MSKCC) is a pathologist who created the MSKCC TMAs described above and she has participated in Ki-67 scoring and data analysis of these materials after providing them to us (<1 person/month effort).

8. SPECIAL REPORTING REQUIREMENTS

This project (W81XWH-16-1-0739) is a collaborative award with Dr. Tamara Lotan (Partnering PI, award).

9. APPENDICES

Manuscripts are attached below.

PTEN Loss with *ERG* Negative Status is Associated with Lethal Disease after Radical Prostatectomy

Nora M. Haney, Farzana A. Faisal,* Jiayun Lu, Liana B. Guedes, Victor E. Reuter, Howard I. Scher,† James A. Eastham, Luigi Marchionni, Corinne Joshu, Anuradha Gopalan‡ and Tamara L. Lotan‡

From the Departments of Urology (NMH, FAF, TLL), Pathology (LBG, TLL) and Oncology (LM, TLL) and Center for Computational Genomics (LM), Johns Hopkins University School of Medicine and Department of Epidemiology, Johns Hopkins University Bloomberg School of Public Health (JL, CJ), Baltimore, Maryland, and Departments of Pathology (VER, AG), Genitourinary Oncology (HIS) and Urology (JAE), Memorial Sloan Kettering Cancer Center, New York, New York

Abbreviations and Acronyms

AA = African American
ADT = androgen deprivation therapy
AR = androgen receptor
BCR = biochemical recurrence
EA = European American
ERG = ETS-related gene
FISH = fluorescence in situ hybridization
GG = Grade Group
IHC = immunohistochemistry
PCa = prostate cancer
PTEN = phosphatase and tensin homolog
RP = radical prostatectomy
TMA = tissue microarray

Purpose: Few groups have investigated the combined effects of *PTEN* loss and *ERG* expression on the outcomes of metastasis of or death from prostate cancer in surgically treated patients. We examined the association of *PTEN*/*ERG* status with lethal prostate cancer in patients treated with radical prostatectomy.

Materials and Methods: Included in analysis were 791 patients with clinically localized prostate cancer treated with radical prostatectomy at a single institution. Genetically validated immunohistochemistry assays for *PTEN* and *ERG* were performed on tissue microarrays. Multivariable Cox proportional hazard models were used to assess the association of *PTEN*/*ERG* status with lethal prostate cancer (defined as metastasis or prostate cancer specific death), adjusting for patient age, race, pathological grade and stage, and surgical margin status.

Results: Median followup in the cohort was 12.8 years. Of 791 cases 203 (25%) demonstrated *PTEN* loss and 330 of 776 (43%) were *ERG* positive. On multivariable analysis *PTEN* loss (HR 1.9, 95% CI 1.2–3.0, p=0.012) but not *ERG* expression (HR 0.6, 95% CI 0.4–1.1, p=0.11) was associated with an increased risk of lethal prostate cancer. The association of *PTEN* loss with lethal disease only remained among men with *ERG* negative tumors (HR 2.3, 95% CI 1.3–4.1, p=0.005) and not *ERG* positive tumors (HR 1.1, 95% CI 0.6–2.1, p=0.81).

Conclusions: *PTEN* loss is associated with an increased risk of lethal prostate cancer after radical prostatectomy and this risk is most pronounced in the subgroup of patients with *ERG* negative tumors. This work corroborates the use of *PTEN* and *ERG* status for risk stratification in surgically treated patients.

Key words: prostatic neoplasms, prostatectomy, mortality, PTEN phosphohydrolase, oncogene proteins

Accepted for publication September 1, 2019.

Supported by CDMRP (Congressionally Directed Medical Research Programs) Prostate Cancer Research Program Awards W81XWH-12-PCRP-TIA (HIS, TLL, AG, VER) and W81XWH-16-1-0737 (TLL, LM, AG), and NCI (National Cancer Institute) Cancer Center Support Grant 5P30CA006973.

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† Financial interest and/or other relationship with Asterias Biotherapeutics, Ambry Genetics, Konica Minolta, Amgen, ESSA Pharma, Janssen, OncLive Insights, Menarini Silicon, Physicians Education Resource, Sanofi Aventis, WCG Oncology, Epic Sciences, Illumina, Innocrin Pharma and ThermoFisher.

‡ Equal study contribution.

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PHOSPHATASE and tensin homolog is a commonly deleted tumor suppressor in PCa. Its loss results in unopposed activity of PI3K and up-regulation of the oncogenic Akt/mTOR signaling pathways.[1] *PTEN* deletion frequently occurs as focal and subclonal events in primary prostate tumors but homogeneous and heterogeneous loss can be reliably detected by genetically validated IHC.[2–4]

PTEN loss is associated with adverse pathological features at RP and an increased risk of BCR after RP.[5–8] Few studies have been done to examine the association of *PTEN* loss with more clinically meaningful outcomes in surgically treated patients, such as metastasis or death.[3,9] Others have been limited to outcomes in conservatively managed cohorts.[10–12]

PTEN loss commonly occurs in tumors with *ERG* gene rearrangements. Fusion of *ERG* (an *ETS* family transcription factor) with the androgen regulated gene *TMPRSS2* is the most common genomic rearrangement found in PCa, occurring in about 50% of patients with PCa who are of European descent.[13] *TMPRSS2-ERG* rearrangements by translocation or deletion in tumor cells subsequently put *ERG* expression under the control of an androgen regulated promoter. While *ERG* rearrangement alone does not predict poor prognosis in surgical cohorts,[14] animal studies suggest that *ERG* rearrangement and *PTEN* loss may work synergistically in tumor progression.[12,15] However, retrospective clinical studies conflict.

Initial studies showed that *PTEN* loss in an *ERG* positive background increased the risk of BCR after surgery[16,17] and yet larger studies have shown that *PTEN* loss predicts BCR regardless of *ERG* status.[2,18] When death from PCa is the primary outcome, *PTEN* loss with *ERG* negative status is associated with worse survival.[3,11,12] Data on a population based, prospective cohort showed that *PTEN* loss was associated with lethal progression after surgery only when *ERG* status was negative.[3] Reid et al performed FISH assays revealing that *PTEN* deletion without *ERG* rearrangement predicted cancer specific death in a conservatively managed cohort.[11] To our knowledge only 1 study has been done to examine *PTEN*/*ERG* status by IHC in a large RP cohort uniformly treated at a single institution.[9] This study showed that while *PTEN* loss predicted metastasis and PCa specific death after RP, *ERG* status did not provide any additional benefit.

Given the paucity of studies and conflicting results, we investigated the combined effects of *PTEN* and *ERG* status on long-term oncologic outcomes in a large, surgically treated cohort from a single institution. Using automated and genetically validated IHC we examined the association of *PTEN* and *ERG* status with lethal PCa after RP.

MATERIALS AND METHODS

Study Population and Tissue Microarray Construction

Institutional Review Board approval was obtained from the 2 participating institutions, namely Memorial Sloan Kettering Cancer Center and the Johns Hopkins Medical Institutions (IRB No. NA 00091198). The cohort consisted of men treated with RP of localized PCa between 1985 and 2003 at Memorial Sloan Kettering Cancer Center.[19] Those who received neoadjuvant or adjuvant ADT, or radiation therapy were excluded from study. Only patients with available slides, blocks and followup information were included in the final cohort, which included 915 RP specimens with a total of 2,745 tumor cores in TMA sets.

H&E slides of the RP specimens were reviewed and slides containing tumor were marked and matched with corresponding paraffin blocks. Tissue cores (0.6 mm) were punched out in triplicate from randomly selected locations in marked tumor areas and mounted in blank recipient blocks using an automated tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin). Samples were from the largest tumor focus in most cases. Separate tumor foci were punched only when there were small tumor foci with no dominant nodule. TMAs were tested with validated IHC to determine *PTEN*/*ERG* status.

Clinicopathological and long-term followup information was available on all patients in the final cohort. The primary outcome was lethal PCa, defined as distant metastasis detected on imaging or PCa specific death. This composite definition of lethal PCa was chosen since metastasis-free survival is a strong surrogate for survival in patients with localized PCa.[20]

Immunohistochemistry Assays and Scoring

PTEN and *ERG* IHC were performed in a CLIA (Clinical Laboratory Improvement Amendments) accredited laboratory on the Ventana Discovery Ultra platform (Ventana Medical Systems, Tucson, Arizona) using previously validated protocols.[2–4,21] Briefly, these assays use rabbit antihuman *PTEN* antibody (Clone D4.3 XP, Cell Signaling Technology®) or rabbit antihuman *ERG* antibody (EPR3864). After staining all TMAs were scanned at 20× magnification using an Aperio® device and segmented into TMAJ (http://tmaj.pathology.jhmi.edu/) for scoring.

PTEN and *ERG* protein status was blindly scored by trained urological pathologists (LBG and TLL). *PTEN* was scored as homogeneous *PTEN* loss if all tumor glands sampled in a given case showed cytoplasmic and nuclear *PTEN* loss compared to surrounding internal control benign glands in stroma. *PTEN* was scored as heterogeneous *PTEN* loss if some but not all tumor tissue sampled in a given case showed *PTEN* loss. *PTEN* was scored as intact if all tumor tissue sampled showed *PTEN*. *ERG* was scored as positive if any tumor glands showed nuclear *ERG* expression. *ERG* was scored as negative if no sampled tumor gland showed *ERG* expression.

Statistical Analysis

Clinicopathological characteristics of the *PTEN*/*ERG* status subgroups were compared using the Wilcoxon or Kruskal-Wallis test for continuous variables and the chi-square test for categorical variables. Univariable and multivariable Cox proportional hazard regression models were constructed to estimate the HR and 95% CI of lethal PCa. Patient age and race, pathological grade and stage, and surgical margin status were included in the multivariable model. We used the Kaplan-Meier method to examine the risk of lethal PCa stratified by *PTEN* and *ERG* status.

All tests were 2-sided with $p < 0.05$ considered statistically significant. Analyses were done with SAS®, version 9.4.

RESULTS

Tumor was present on TMA slides in 915 cases, of which 791 (86%) had interpretable staining results with adequate *PTEN* control staining. Of the 791 cases with *PTEN* staining data 776 (93%) had informative staining results for *ERG*. *PTEN* loss was present in 203 of 791 cases (26%), of which 96 (12%) and 107 (14%) showed homogeneous and heterogenous *PTEN* loss, respectively. *ERG* expression was present in 330 of 776 cases (43%). *PTEN* loss was more common among *ERG* positive than *ERG* negative cases (120 of 330 or 36% vs 83 of 446 or 19%, $p < 0.001$). *PTEN* loss and *ERG* expression were more prevalent in EA men than in AA men with *PTEN* loss in 27% of EA vs 9% of AA men ($p = 0.003$) and *ERG* expression in 44% of EA vs 21% of AA men ($p < 0.001$).

On IHC *PTEN* loss was associated with adverse pathological features at RP (table 1). Of the 192 tumors with *PTEN* loss 76 (approximately 38%) were GG 3-5 compared to 108 of 577 GG 3-5 tumors (18%) with intact *PTEN* ($p < 0.001$). Similarly, 98 of 203 *PTEN* loss tumors (48%) were nonorgan confined while 29% with *PTEN* intact demonstrated extraprostatic disease ($p < 0.001$).

Median followup was 12.8 years. Lethal PCa events developed in 92 of the 776 patients (12%) with complete *PTEN* and *ERG* results. On multivariable analysis *PTEN* loss was significantly associated with an increased risk of lethal PCa (HR 1.98, 95% CI 1.15–3.04, $p = 0.012$, table 2). *ERG* expression did not predict lethal PCa on multivariable analysis (HR 0.64, 95% CI 0.36–1.11, $p = 0.113$). Table 2 and the figure show the association of joint categories of *PTEN* loss and *ERG* status with lethal PCa. Compared to cases with *PTEN* intact and *ERG* negative status, *PTEN* loss with *ERG* negative status was the only subgroup significantly associated with an increased risk of lethal progression on univariable analysis (HR 3.76, 95% CI 2.27–6.21, $p < 0.001$) and multivariable analysis (HR 2.31, 95% CI 1.29–4.14, $p = 0.005$, log rank $p < 0.001$). *ERG* positive cases with *PTEN* loss carried a higher risk of lethal disease on univariable analysis (HR 1.84, 95% CI 1.06–3.18, $p = 0.030$).

Table 1. *Clinicopathological characteristics of patients stratified by* PTEN *and* ERG *status*

	PTEN (791 pts)			*ERG* (776 pts)			*PTEN/ERG* (776 pts)				
	Loss	Intact	p Value*	Neg	Pos	p Value*	*PTEN* Intact/*ERG* Neg	*PTEN* Intact/*ERG* Pos	*PTEN* Loss/*ERG* Neg	*PTEN* Loss/*ERG* Pos	p Value*
No. pts	203	588	—	466	330	—	363	210	83	120	—
Median age at RP	62.60	61.32	0.026	62.33	60.36	0.002	62.08	59.55	63.33	61.43	<0.001
No. race (%):			0.016			0.003					0.001
European American	189 (93.1)	512 (87.1)		387 (86.8)	301 (91.2)		309 (85.1)	190 (90.5)	78 (94.0)	111 (92.5)	
African American	5 (2.5)	49 (8.3)		42 (9.4)	11 (3.3)		40 (11.0)	8 (3.8)	2 (2.4)	3 (2.5)	
Other	5 (2.5)	15 (2.6)		9 (2.0)	10 (3.0)		9 (2.5)	5 (2.4)	0	5 (4.2)	
No. Grade Group (%):			<0.001			<0.001					<0.001
1	33 (16.3)	216 (36.7)		119 (26.7)	122 (37.0)		108 (29.8)	100 (47.6)	11 (13.3)	22 (18.3)	
2	83 (40.9)	253 (43.0)		191 (42.8)	141 (42.7)		163 (44.9)	86 (41.0)	28 (33.7)	55 (45.8)	
3	44 (21.7)	56 (9.5)		65 (14.6)	34 (10.3)		46 (12.7)	9 (4.3)	19 (22.9)	25 (20.8)	
4	19 (9.4)	32 (5.4)		39 (8.7)	11 (3.3)		26 (7.2)	5 (2.4)	13 (15.7)	6 (5.0)	
5	13 (6.4)	20 (3.4)		24 (5.4)	9 (2.7)		16 (4.4)	4 (1.9)	8 (9.6)	5 (4.2)	
No. stage (%):			<0.001			0.066					<0.001
T2	105 (51.7)	417 (70.9)		291 (65.3)	219 (66.4)		253 (69.7)	152 (72.4)	38 (45.8)	67 (55.8)	
T3	87 (42.9)	156 (26.5)		135 (30.3)	106 (32.1)		99 (27.3)	55 (26.2)	36 (43.4)	51 (42.5)	
T4	11 (5.4)	15 (2.6)		20 (4.5)	5 (1.5)		11 (3.0)	3 (1.4)	9 (10.8)	2 (1.7)	
No. margin (%):			0.230			0.737					0.695
Neg	122 (60.1)	381 (64.8)		285 (63.9)	207 (62.7)		236 (65.0)	134 (63.8)	49 (59.0)	73 (60.8)	
Pos	81 (39.9)	207 (35.2)		161 (36.1)	123 (37.3)		127 (35.0)	76 (36.2)	34 (41.0)	47 (39.2)	
No. *PTEN* loss (%):		—	—	—	—	—	—	—	—	—	—
Heterogenous	96 (47.3)										
Homogeneous	107 (52.7)										

* Wilcoxon test or Kruskal-Wallis test for continuous variables and chi-square test for categorical variables.

Table 2. *Univariable and multivariable Cox proportional hazard models for lethal prostate cancer*

			Univariable		Multivariable*	
	No. Cases	No. Controls	HR (95% CI)	p Value	HR (95% CI)	p Value
PTEN:						
Intact	46	542	Referent	—	Referent	—
Loss	47	156	3.25 (2.16–4.88)	<0.001	1.87 (1.15–3.04)	0.012
ERG:						
Neg	62	384	Referent	—	Referent	—
Pos	30	300	0.64 (0.41–0.99)	0.043	0.64 (0.36–1.11)	0.113
PTEN/*ERG*:						
PTEN intact/*ERG* neg	35	328	Referent	—	Referent	—
PTEN intact/*ERG* pos	10	200	0.47 (0.23–0.96)	0.037	0.48 (0.18–1.26)	0.136
PTEN loss/*ERG* neg	27	56	3.76 (2.27–6.21)	<0.001	2.31 (1.29–4.14)	0.005
PTEN loss/*ERG* pos	20	100	1.84 (1.06–3.18)	0.030	1.09 (0.56–2.12)	0.809

* Adjusted for age at RP, race, grade group, stage and surgical margin status.

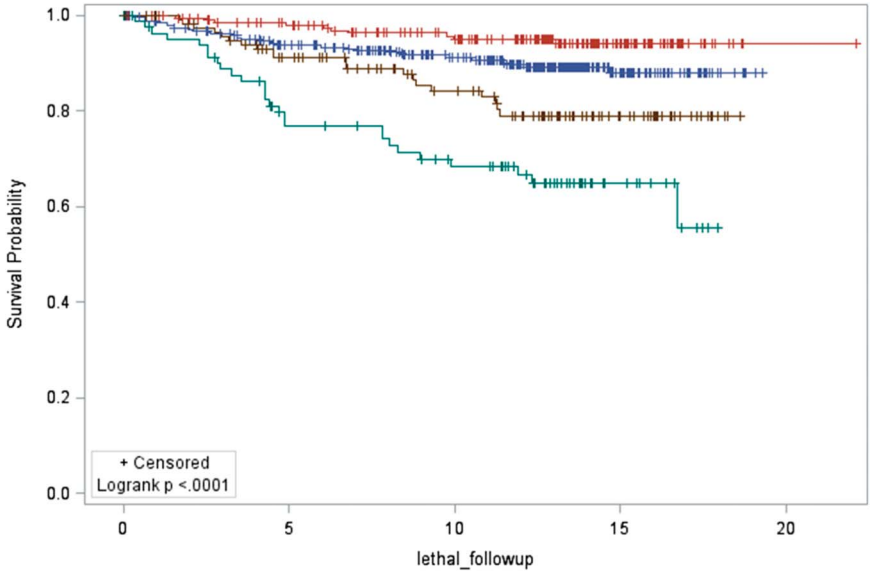
However, this was not significant on multivariable analysis (HR 1.09, 95% CI 0.56–2.12, p = 0.809).

DISCUSSION

Long-term studies demonstrating an association between *PTEN* loss and clinically meaningful outcomes such as metastasis or cancer specific death have been lacking. Moreover, studies of the modifying effect of *ERG* status on *PTEN* loss have conflicted. Using FISH techniques in large cohorts to determine *PTEN*/*ERG* status is time-consuming and technically challenging. Using automated and validated IHC, we found that *PTEN* loss was significantly associated with an approximately twofold increased risk of lethal PCa in a large cohort treated with RP and followed long-term at a single institution. This risk was only significant in the subgroup of patients with *PTEN* loss and with *ERG* negative tumors. Patients with *PTEN* loss but *ERG* positive tumors were not at increased risk for lethal progression. These findings support the clinical usefulness of automated and inexpensive IHC assays for *PTEN* and *ERG* for risk stratification and treatment in post-RP cases.

At this institution we routinely perform *PTEN* and *ERG* IHC testing in GG 1 biopsies. Loss of *PTEN*, particularly when *ERG* is negative, is a relative contraindication to active surveillance. Given these results, we plan to incorporate *PTEN*/*ERG* testing in the RP setting to guide postoperative management.

Our group genetically validated automated IHC for *PTEN* detection to study *PTEN* loss.[2–4] There is high correlation of automated IHC with FISH. Intact *PTEN* immunostaining is 91% specific for the absence of *PTEN* deletion by FISH, and 97% and



Kaplan-Meier survival curves of freedom from lethal PCa by *PTEN* and *ERG* status. Blue curve indicates *PTEN* intact and *ERG* negative in 363 patients. Red curve indicates *PTEN* intact and *ERG* positive in 210 patients. Green curve indicates *PTEN* loss and *ERG* negative in 83 patients. Brown curve indicates *PTEN* loss and *ERG* positive in 120 patients.

65% sensitive for the detection of homozygous and hemizygous deletion by FISH, respectively.[4] The effects of fixation technique and duration, tissue processing type and slide or block age are largely negligible.[22] Interobserver variability is minimal with κ values consistently above 0.9.[3] IHC also provides significant cost and time savings compared to FISH.

Several studies have demonstrated the prognostic role of *PTEN* in predicting upgrading at surveillance biopsy, discontinuation of active surveillance, adverse pathology at RP and BCR after surgery.[5–8,23–25] *PTEN* loss strongly correlates with unfavorable histological features, including intraductal carcinoma, cribriform Gleason pattern 4 and stromogenic PCa.[26]

However, only a few studies have been done to investigate the effect of *PTEN* loss stratified by *ERG* status on metastasis and death outcomes. Reid et al found that *PTEN* deletion without *ERG* rearrangements by FISH increased the risk of cancer specific death in a conservatively managed cohort, although they could not reproduce this finding in a larger cohort.[11] In a surgically treated cohort Ahearn et al used IHC to determine that *PTEN* loss and *ERG* negative tumors were associated with lethal progression.[3] However, data were collected from national population based studies including patients treated for more than 20 years at multiple institutions with self-reporting relied on for followup. Leapman et al analyzed 424 cases treated with RP at a single institution and found that *ERG* status did not add to the c-index of the CAPRA-S (Cancer of the Prostate Risk Assessment Post-Surgical) score and *PTEN*.[9] However, it was not explicitly examined whether cases with *PTEN* loss had worse outcomes when *ERG* was negative compared to those that were *ERG* positive.

It is unclear why some previous studies have shown that *PTEN* loss with *ERG* positive status was associated with the highest risk of BCR after surgery.[16,17,27] Long-term followup has shown that *ERG* negative *PTEN* loss is the subgroup at increased risk for lethal progression.[3,11,12] One important caveat is that due to the frequency of *PTEN* loss among *ERG* positive tumors there are substantially more *ERG* positive tumors with *PTEN* loss than *ERG* negative tumors with *PTEN* loss. Thus, smaller studies were almost certainly underpowered to compare the effects of *PTEN* loss on *ERG* positive and *ERG* negative backgrounds while larger studies revealed no effect of *ERG* status on the association of *PTEN* loss with BCR.

Furthermore, BCR is a different outcome than metastasis or death. Since salvage radiation and ADT are generally introduced after BCR, biomarkers predictive of a response to radiation therapy or ADT may be associated with metastasis and death but not with BCR. Thus, while there may be a lack of interaction between *PTEN* and *ERG* for an association with BCR, this interaction may be seen in cohorts with longer followup (perhaps those in which ADT is introduced early) for an association with metastasis and death. Clearly additional trials are necessary to formally test this hypothetical interaction of *PTEN*/*ERG* with radiation therapy and/or ADT after BCR.

Preclinical studies have been done to examine the influence of *PTEN*/*ERG* status on androgen signaling. *PTEN* loss has been demonstrated to down-regulate AR and AR driven gene transcription.[28,29] Murine models have shown that in the absence of *ERG* expression *PTEN* negative tumors demonstrate diminished AR signatures compared to *PTEN* positive tumors but these signatures are restored to almost normal in the presence of *ERG* expression.[15] Similarly, Blee et al found that tumors in mice with *PTEN* deletions and *TP53* mutations but without *ERG* expression lost AR expression and were resistant to enzalutamide while the same tumors with *ERG* expression maintained AR expression and were sensitive to enzalutamide.[30] They further described the reliance of tumors with *PTEN* and p53 loss (and lacking *ERG* expression) on a separate RB/E2F1 pathway, which could be chemotherapeutically targeted with a CDK4/6 inhibitor such as palbociclib, known for use in breast cancer. It is possible that this androgen independence among *ERG* negative tumors with *PTEN* loss modulates tumor progression and contributes to subsequent metastasis, castrate resistance and PCa specific mortality.

Study limitations include patient selection since only 74 men were nonEA. Therefore, the findings may not be generalizable to AA or other minority men in whom *PTEN* loss and *ERG* rearrangements are significantly less common. Additionally, relevant clinical and pathological information were missing in this patient cohort, including the preoperative prostate specific antigen level and pathological node status. Overall the number of lethal events in our cohort was not high at 92. This raises the potential for overfitting our multivariable model and yet this study remains one of the largest data sets of surgically treated tumors with available *PTEN* and *ERG* status.

As tumors with *PTEN* loss without *ERG* rearrangement were associated with poor prognosis in this cohort, there is the possibility that these 2 subtypes also share specific adverse morphological or histological features.[30] Additionally, other molecular subtypes could be mutually exclusive with *ERG* expression and, thus, contribute to lethal outcomes

in patients with *ERG* negative tumors. Further research can be done to explore the genomic background and molecular underpinnings of the aggressive behavior of *PTEN* loss/*ERG* negative tumors.

Lastly, risk stratification tools, such as the CAPRA-Sm which incorporate clinicopathological parameters after RP, still remain valuable prognostic tools. However, molecular and genomic tests are becoming increasingly available to providers. Additional studies are required to compare *PTEN*/*ERG* IHC tests to commercially available gene panel assays in predictive models. For example, initial studies have suggested that *PTEN* loss performs similarly to the cell cycle proliferation score.[9] However, studies comparing *PTEN* to the OncotypeDx® test as well as to Decipher® are warranted since *PTEN* IHC testing is considerably less expensive than RNA based tests.

CONCLUSIONS

Using a highly validated and automated IHC assay we found that *PTEN* loss was associated with an increased risk of lethal PCa in surgically treated patients. This risk remained significant only in the subgroup of patients with *ERG* negative tumors. This work corroborates the combined use of *PTEN* and *ERG* IHC assays as prognostic tools for risk stratification and treatment management after RP.

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EDITORIAL COMMENT

The authors report that loss of *PTEN* expression on IHC using a CLIA certified assay correlated with death from PCa and the prediction was strongest in *ERG* negative tumors. As they note, this finding is somewhat controversial since *PTEN* loss was previously associated with biochemical recurrence in *ERG* positive tumors (reference 27 in article). It is likely that the stratification based on *ERG* status was due to arbitrary differences in other prognostic features such as age and grade between *ERG* positive and *ERG* negative tumors in the cohort, which has been observed previously.[1]

The most important feature of this study is the association of *PTEN* status with hard outcomes, namely metastasis and death from PCa. Similar findings were reported recently using an immunofluorescent based IHC assay.[2] The findings also make biological sense since *PTEN* signaling pathway alterations are common in metastatic PCa, implying that they are selected for during progression.

Developing prognostic biomarkers in PCa has proved challenging, primarily because the Gleason GG is so powerful. Given the repeated association of *PTEN* loss with adverse outcomes, mostly recurrence after surgery but also adverse pathological features and progression in patients on surveillance, this study adds substantially to data arguing that *PTEN* should be used routinely as a tissue based biomarker when assessing PCa biopsies and RPs. It finally might be time for molecular biomarkers that provide prediction of hard outcomes independent of grade and stage, like *PTEN* and *AZGP1*,[3] to be moved into clinical practice.

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