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14. ABSTRACT Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. We have completed the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping. We have set up a structure for reviewing and approving biomarker proposals based on sound scientific principles and strong preliminary data. We have devised and tested a centralized distribution mechanism at Stanford University of collating and shipping TMAs to participating sites, We have found shortcomings with the BLISS system and STMAD for histological image capture and storage for pathological review and have developed a much improved, highly efficient system using a Leica scanner and Path.XL image analysis software suite. We also have made significant progress in testing TACOMA, an automater TMA scoring algorithm. We have completed staining of the TMAs for H & E, High Molecular Weight Keratin, p27, ERG, SPKINKI, Ki67 (MIBI), MUCI, Survivin and PTEN FISH. Over the next year, we will expand our database to add more tested TMAS Biomarkers, perform QA/QC to ensure high quality, and evaluate their performance for predicting recurrence. We will further refine TACOMA algorithm to facilitate the scoring of TMA stains. We will work with investigators to write papers reporting tested TMA Biomarkers.

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TMA, Recurrence, biomarkers, Case-Cohort design, antibody

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Validation of Biomarkers for Prostate Cancer Prognosis

Progress Report

Synergy Award: W81XWH-11-1-0381

Co-PIs: Ziding Feng & James D. Brooks

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Introduction

The most significant challenge in managing localized prostate cancer is the decision of whether or not it needs to be treated. Nearly ½ of prostate cancers diagnosed in the U.S. fall into the low or very low risk category and have little likelihood of causing death. However, it is well known that a significant fraction of low risk cases are misclassified and actually have occult high-risk features or are destined to progress to high-risk disease. Therefore a critical need in localized prostate cancer is the development of biomarkers that predict occult or incipient aggressive disease in the low-risk population.

To address this challenge, we formed the multi-institutional Canary Tissue Microarray Project. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases. Funding from the Department of Defense allowed us to complete construction of the TMAs as well as the necessary infrastructure and begin testing biomarker candidates. With this infrastructure in place, we now have a robust validation platform for testing prostate cancer biomarkers. Based on our success, this resource will be a source for future biomarker validation studies even after the DOD funding has ceased.

The DOD has catalyzed the formation of the infrastructure to support this project and we have now completed or are near completion of several biomarkers. Staining has been completed, slides have now been analyzed and statistical analyses are underway. I requested and received an extension of my half of the award because of my move from Seattle to MD Anderson Cancer Center in Houston. Actually, this will be very beneficial for this project since the next phase for several of the biomarkers is the statistical analysis of the data. My team is actively working on data analyses and communicating back and forth with lab and clinical collaborators and we expect over the next year will complete several projects and should lead to several publications. This will serve as critical preliminary data for us to continue this resource and apply for competitive funding.

Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.

1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

The multi-institutional TMAs have been constructed at all sites. The final TMA cohort is 1326 patients with only 31 patients excluded due to data error. We are in the process of updating follow-up on the TMAs since several years of additional follow-up have been accumulated since the cases were first selected. Patients have been selected at random from the pool of patients who had undergone radical prostatectomy at each of the sites, with special attention to selecting patients with features typical of low-intermediate risk patients seen in contemporary urologic practices. Details of patient selection, statistical considerations, and TMA construction are summarized in our publication in Advances in Anatomic Pathology published earlier this year and appended to last year's report. In addition to this cohort, a separate TMA has been constructed from 220 patients who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death.

Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed several stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving while some aspects continue to be developed:

1) The Data Transfer Agreement (DTA) was completed between FHCRC and MDACC so the study data could be freely shared and communicated between FHCRC and MDACC. MDACC has established new database to warehouse the study data, receiving and archiving assay data from different labs/groups submitted to this project.

2) We have concluded that TACOMA algorithm as it currently stands, it inadequate for automatic imaging reading. The main reason is that it still requires pathologists to sketch the boundary for cancer cell region. Though Dr. Tim Randolph will continue collaborating with Dr. Richard Levenson to add that functionality by another new software, it wouldn't be available in the life length of this project period to reduce pathologist reading time.

3) Data management and data analysis: We have performed data analyses for all biomarkers whose data has been submitted to MDACC. The details of the findings are summarized below.

1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy

In our ongoing monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (http://www.canaryfoundation.org). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have now completed staining for many of the biomarkers listed in our proposal and are expanding to novel biomarkers discovered since our application.

The primary objective is to correlate these two biomarkers with survival endpoints. Three survival endpoints were of interest: recurrence-free survival (RFS, where event was defined as any recurrence or metastasis or prostate cancer death), disease-specific survival (DSS, where event was defined as metastasis or prostate cancer death), and overall survival (OS, where event was defined as death of any cause).

Completed biomarkers:

1) ERG: Immunohistochemistry for the ERG protein has been completed, scored and is being analyzed by the DMCC. Preliminary data show that ERG staining does not provide prognostic information either on univariate or multivariate analysis. (Table 1) A manuscript is quite far along and will be submitted in the next few months.

Table 1. Summary of multivariate Cox proportional hazard model results by survival endpoint. Backwards elimination procedure was used to identify the final model for each endpoint. Hazard ratio higher than 1 means worse prognosis. Conclusions:

1. Being SPINK1 negative was significantly associated with worse RFS after adjusting for margin, SVinv status, Gleason score, and pre-op PSA.
2. ERG or SPINK1 was not significantly associated with DSS or OS based on this dataset.

Endpoint	Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
RFS (N = 674, E = 306)	SPINK1	Neg vs. Pos	2.84	1.17	6.90	0.02
	Margin	Pos vs. Neg	1.78	1.41	2.24	<0.0001
	SVinv	Yes vs. No	2.37	1.63	3.43	<0.0001
	Gleason	3+4 vs. <= 6	1.46	1.10	1.95	0.009
		4+3 vs. <= 6	2.09	1.49	2.93	<.0001
		8-10 vs. <= 6	1.82	1.26	2.65	0.002
	Log(pre-op PSA)	1 unit increase	1.56	1.31	1.86	<.0001
DSS (N = 929, E = 46)	Gleason	3+4 vs. <= 6	2.69	1.11	6.49	0.03
		4+3 vs. <= 6	3.67	1.34	10.07	0.01
		8-10 vs. <= 6	6.27	2.41	16.31	0.0002
	Log(pre-op PSA)	1 unit increase	1.80	1.23	2.64	0.003
OS (N = 940, E = 58)	Gleason	3+4 vs. <= 6	0.88	0.44	1.73	0.71
		4+3 vs. <= 6	1.11	0.44	2.77	0.82
		8-10 vs. <= 6	3.25	1.70	6.24	0.0004
	Age	1 unit increase	1.06	1.02	1.10	0.006

N = total number of patients, E = number of events

2) SPINK1: As reported previously, SPINK1 positive tumors constitute a minority of prostate cancer – in the Canary TMA only 6% of cases. In addition, positive staining is confined to the ERG-fusion negative cases, with 2 exceptions in our dataset. However, unlike previous data, SPINK1 high level expression appears to be correlated with favorable outcome in that it is associated with higher recurrence free survival RFS in a preliminary analysis (Table 1). We will be reporting the ERG and SPINK1 results in a single manuscript in the next few months.

3) PTEN FISH: In collaboration with Dr. Jeremy Squire at Queens University, Ontario, Canada, we have used a multiprobe FISH assay to interrogate copy number alterations (allelic loss) at the PTEN locus. In our series, homozygous deletion of PTEN was found in 9% of cases and heterozygous allelic loss was found in an additional 9% of cases. PTEN loss was associated with adverse pathology including extracapsular extension, seminal vesicle invasion and lymph node spread. In addition, allelic loss events of any type were associated with poorer RFS. Finally, tumors with homozygous deletion appear to have more aggressive features than those with hemizygous deletion or no

structureal alterations at the PTEN locus (Table 2). A manuscript has been accepted by "The Prostate".

Table 2 Summary of logistic regression model results correlating PTEN with ECE, SV, and Gleason score.

Endpoint	Parameter	Comparison	Odds Ratio	95% LCL	95% UCL	Pairwise P-value	Overall P-value
Extra-Capsular Invasion (yes, no)	PTEN	Homo vs. No Del	3.45	1.97	6.06	<0.0001	<0.0001
		Hemi vs. No Del	1.49	0.79	2.70	0.20	
	PTEN	Any Del vs. No Del	2.32	1.51	3.57	<0.0001	
Seminal Vesicle Invasion (yes, no)	PTEN	Homo vs. No Del	4.33	1.87	9.43	0.0003	0.002
		Hemi vs. No Del	2.49	0.89	6.07	0.06	
	PTEN	Any Del vs. No Del	3.39	1.70	6.62	0.0004	
Gleason (<=6, 7, >=8)	PTEN	Homo vs. No Del	3.37	1.83	6.19	<0.0001	<0.0001
		Hemi vs. No Del	1.54	0.82	2.89	0.23	
	PTEN	Any Del vs. No Del	2.32	1.55	3.48	<0.0001	

4) ERG IHC and PTEN IHC: In collaboration with Tamara Lotan at Johns Hopkins, we completed IHC staining for PTEN on our TMAs. There was excellent agreement between the PTEN IHC results and PTEN FISH. IHC has the advantage of working in a larger number of cores than FISH so we were able to carry out a more complete evaluation of the cohort. Again, PTEN loss was associated with adverse outcome. Moreover, PTEN loss was associated with poor outcome to a much greater degree in the ERG fusion negative cases as opposed to the ERG positive cases. This work will be presented at several up-coming international meetings. A manuscript has been completed and is being revised for submission in the next month.

5) Ki67: Ki67staining has been used as a measure of proliferative index and has been shown to be prognostic in several tumor types including prostate cancer. However, since prostate cancer has a low proliferative index (PI), and there is considerable inter-observer variation of interpretation of Ki67 stains, we decided to use an automated imaging process to score Ki67 staining. We used the Aperio system to quantify stained and unstained nuclei in regions of prostate cancer across 1000+ samples on our TMA. Ki67 PI was significantly associated with adverse pathologic features and RFS in univariate and multivariate analysis. High Ki67 PI was also associated with overall survival and prostate cancer specific survival in this cohort. It appears to be an excellent prognostic biomarker (Table 3 & Figure 1). A manuscript has been drafted and final comments are being assembled. It should be submitted within the next 1

month.

Table 3. Summary of multivariate Cox proportional hazard model results using weighted average Ki-67 score or maximum Ki-67 score for RFS. Conclusions:

1. **High Ki-67 score (>=5%) for both weighted average and maximum score were significantly associated with worse RFS after adjusting for pre-op PSA, margin status, SV invasion status, and Gleason score.**

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
Ki-67 Weighted Average Score	**>=5% vs. <5%**	1.63	1.24	2.15	0.0005
Log(Pre-op PSA)	**1 unit increase**	1.62	1.36	1.93	<.0001
Margin Status	**Pos vs. Neg**	1.66	1.32	2.10	<.0001
SV Invasion	**Yes vs. No**	2.28	1.58	3.28	<.0001
Gleason	**3+4 vs. <=6**	1.33	1.00	1.75	0.05
	4+3 vs. <=6	1.78	1.28	2.49	0.0007
	8-10 vs. <=6	1.62	1.13	2.33	0.01
Ki-67 Maximum Score	**>=5% vs. <5%**	1.39	1.10	1.76	0.01
Log(Pre-op PSA)	**1 unit increase**	1.58	1.33	1.89	<.0001
Margin Status	**Pos vs. Neg**	1.67	1.32	2.10	<.0001
SV Invasion	**Yes vs. No**	2.33	1.62	3.36	<.0001
Gleason	**3+4 vs. <=6**	1.34	1.02	1.77	0.04
	4+3 vs. <=6	1.85	1.32	2.57	0.0003
	8-10 vs. <=6	1.66	1.16	2.38	0.01

Figure 1: K-M curve for Ki-67.

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6) AZGP1: AZGP1 has been shown to be prognostic in several datasets and was originally described by the Brooks group in 2004. We have performed both IHC and RNA ISH for AZGP1 and the TMAs have been scored. An initial analysis has been completed by my group and is currently being revised. A preliminary look at the data shows that AZGP1 positive IHC staining is correlated with a lower risk of RFS (Table 4 & Figure 2). When this analysis is completed, we expect a manuscript to be submitted before the end of the calendar year.

Table 4. Multivariate Cox proportional hazard model for RFS. RFS event is defined as any recurrence, metastasis, or prostate cancer death. Hazard ratio higher than 1 means worse prognosis. Conclusions:

1. **Negative or weak AZGP1 IHC staining was significantly associated with worse RFS after adjusting for pre-surgery PSA, margin status, SVI, ECE, and Gleason score.**
2. **Negative or weak AZGP1 CISH staining was significantly associated with worse RFS after adjusting for pre-surgery PSA, margin status, SVI, and Gleason score.**

Model	Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
1 (Total #Pts = 835, #Events = 382	AZGP1 IHC	Negative/Weak vs. Moderate/Strong	1.39	1.13	1.71	0.002
	Log(PSA)	1 unit increase	1.43	1.21	1.68	<.0001
	Margin	Pos vs. Neg	1.62	1.31	2.02	<.0001
	SVI	Pos vs. Neg	2.20	1.58	3.06	<.0001
	ECE	Pos vs. Neg	1.26	1.01	1.58	0.04
	Gleason	3+4 vs. <=6	1.19	0.93	1.52	0.16
		4+3 vs. <=6	1.99	1.47	2.69	<.0001
		8-10 vs. <=6	1.43	1.02	1.99	0.04
2 (Total #Pts = 811, #Events = 377)	AZGP1 CISH	Negative/Weak vs. Moderate/Strong	1.28	1.04	1.58	0.02
	Log(PSA)	1 unit increase	1.46	1.24	1.73	<.0001
	Margin	Pos vs. Neg	1.71	1.39	2.12	<.0001
	SVI	Pos vs. Neg	2.26	1.62	3.15	<.0001
	Gleason	3+4 vs. <=6	1.22	0.96	1.57	0.11
		4+3 vs. <=6	2.12	1.57	2.86	<.0001
		8-10 vs. <=6	1.60	1.15	2.23	0.006

Figure 2: K-M curve for AZGP1 IHC

7) Ongoing studies: Dr. Brooks' team has completed staining and pathologist reads for CD38, p63, CD10, and Muc1. We have also completed a project in image analysis of H & E slides with Gustavo Ayala at University of Texas with assay data just sent to Dr. Feng 1/30/2015. Finally, we have completed an analysis of a radical modification of the Gleason scoring system with Jesse McKenney at the Cleveland clinic. Each of these projects needs to be analyzed by the DMCC now that the data have been acquired. In addition, we have ongoing pathologist reads going for ARG2, p27 (using the Aperio system) SMAD7 and Trichrome stain for stromal desmoplastic reaction. Once these are completed they too will be sent to MDACC and analyzed by my team. We also have 4 additional projects approved and are about to cut new sections for these projects. We expect the next 2 years to be highly productive.

New analysis results recently completed for Dr. Gustavo Ayala on stroma index number/percent predicting recurrence (Table 5):

Conclusion:

1. We identified optimal cutoff points for stroma index number and percent that separate patients with respect to 5-year RFS status and RFS. We found that that higher stroma index number was associated with higher chance of RFS event within 5 years, but lower stroma index percent was associated with higher chance of event. RFS event is defined as any recurrence, mets, or prostate cancer death.
2. Since we wanted to identify optimal cutoff points, we did not perform cross-validation within training set. These cutoff points need to be validated using an independent data set.

Table 5. Summary of stroma index number and percent dichotomized using optimal cutoff point identified using RPA. Notice that higher stroma index number was associated with higher chance of RFS event within 5 years, but lower stroma index percent was associated with higher chance of event. RFS event is defined as any recurrence, mets, or prostate cancer death. Sensitivity and specificity are highlighted in the table.

	Recurrence/Mets/Ca Death by year 5 post-op			
	No		Yes	
	N	%	N	%
Stroma Index Num				
<221.6	59	10.97	70	16.59
>=221.6	479	89.03	352	83.41
Stroma Index Pct				
<25.75%	335	62.27	228	54.03
>=25.75%	203	37.73	194	45.97

Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. In all of the biomarkers we have tested thus far, we have interrogated each for its correlation with Gleason score. In general, most of them are correlated, although not completely. While these do not address the intent of this Aim, we are not disappointed since it does appear that *these biomarkers are supplying prognostic information that is independent of Gleason score.* The intent of Aim 2, on the other hand, was to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease. As we get through our candidate prognostic markers (listed above and in the queue) we will refocus on biomarkers that predict Gleason grade. This could be useful in characterizing biopsy samples to predict upgrading.

However, this clinical question might become less relevant in the future since several tools have been developed that already predict up-grading. For example the OncotypeDx assay has been calibrated and already validated precisely for this purpose. In addition, multiparametric MRI shows good correlation with grade in that only the high-grade lesions are visible, while the low grade lesions are not. As the clinical practice

evolves, we will decide whether we wish to continue to pursue development of IHC biomarkers that predict Gleason score

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists, both in their correlations with Gleason score and in their complementary property with Gleason score.

Key Research Accomplishments

- Provided statistical expertise in biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Data receiving, reconcile data questions, and archiving at MDACC.
- Received final clinical data that will be used for analysis of biomarker performance to the MD Anderson DMCC.
- Established and tested the data analysis pipeline for anticipated additional biomarker data.
- Evaluated TACOMA imaging analysis algorithm using Survivin, CD117, and ERG data and concluded that it is inadequate for automated imaging analysis as it stands along.
- Completion of analysis of PTEN FISH and a manuscript accepted.
- Completion of analysis of Ki67 PI and imminent submission of a manuscript.
- Completion of analysis of ERG IHC and PTEN IHC and presentation at international meetings and imminent submission of a manuscript.
- Ongoing analysis of ERG and SPINK with a manuscript near completion.
- Ongoing analysis of AZGP1 with a manuscript expected soon.
- Ongoing analysis of image analysis with Gustavo Ayala.
- Ongoing analysis of a modified Gleason grading system with Jesse McKenney, as well as confirmation in an additional validation set.
- Ongoing analysis of Muc1, p63, CD10 and CD38. We expect all of these, regardless of outcome (prognostic or not) will be submitted as separate publications.
- Significant preliminary data from this collaboration that will position us well for the next phase of funding.

Reportable Outcomes

1) Publications referencing this grant:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22:** 183-187, 2012.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Ziding Feng, Lawrence D. True and James D. Brooks: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. *Advances in Anatomic Pathology* **20:** 39-44, 2013.

J James D. Brooks: Managing localized prostate cancer in the era of prostate specific antigen testing. *Cancer* **119:** 3906-3909, 2013.

Zuxiong Chen, Zulfiqar G. Gulzar, Catherine A. St. Hill, Bruce Walcheck, James D. Brooks: Increased expression of *GCNT1* is associated with altered *O*-glycosylation of PSA, PAP and MUC1 in human prostate cancers. *Prostate* **74:** 1059-1067, 2014.

Troyer D, Jamaspishvili T, Wei W, Feng Z, Good J, Hawley S, Fazli L, McKenney J, Simko J, Hurtado-Coll A, Carroll P, Gleave M, Lance R, Lin D, Nelson P, Thompson I, True L, Brooks J, Squire J. A multicenter study shos PTEN deletion is strongly associated with seminal vesicle involvement and extracapsular extension in localized prostate cancer. The Prostate. In press.

Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard by for evaluation of prognostic biomarkers. We have completed all phases of pipeline construction and continue to refine our work-flow to improve functionality as we work with the resource. We now have tested several biomarkers and confirmed that they are prognostic. We will complete analysis of the biomarkers in the context of the clinical data over the next year and plan several publications. In addition, we will continue to carry out analysis of new biomarkers and solicit applications for biomarkers inside and outside our research group. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.