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14. ABSTRACT Purpose: To validate biomarkers of recurrence following radical prostatectomy. Scope: Perform genomic profiling of test and validation sets from formalin-fixed samples with long-term follow up data. Major Findings: We have published a manuscript in <i>The American Journal of Pathology</i> describing our set of biomarkers, and presented these data orally at the American Society of Investigative Pathology annual meeting in Washington, DC on April 11, 2011. We have optimized our method for RNA-seq analysis and collected 171 patient samples, of which 108 have passed RNA QC. We are in the process of preparing RNA-seq libraries and anticipate generating large datasets for analysis in the next few months. We have applied our published biomarkers to publicly available datasets and shown that they are predictive. We have demonstrated that four protein biomarkers (BID, SIM2s, FBP1, and RAD23B) are significantly associated with recurrence in prostate cancer. These immunohistochemistry assays may be useful for predicting recurrence in clinical cases.					
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Introduction

Currently, both clinicians and patients are faced with difficult treatment decisions when a diagnosis is made of prostate cancer because it is very difficult to predict whether a patient is likely to progress to aggressive, metastatic disease. Biomarkers that can predict the likely clinical outcome and recurrence for patients after surgical therapy are badly needed in order to aid clinicians in deciding on the appropriate course of treatment. We have developed a panel of biomarkers that is able to predict recurrence following surgery in our initial set of pilot cases. The purpose and scope of this study is to use large sets of ethnically diverse patient samples with long-term, well-defined clinical follow-up data to validate these biomarkers that predict outcome for surgical therapy. Biomarker refinement and validation will be performed on platforms that are amenable to analysis of fixed tissues and ideal for rapid translation into widespread clinical use. Validation studies on separate groups of patients using biopsy specimens will be performed in a clinical laboratory environment at the Winship Cancer Institute at Emory University to speed translation of these biomarkers. By the completion of the work in this proposal, we will have developed and validated a clinical lab test that could be translated to widespread use that would give doctors an idea what is the best course of treatment for patients with prostate cancer and help avoid unnecessary treatments. In the long run, this will result in better patient outcomes and reduced healthcare costs and treatment side effects.

We recently published a panel of ten protein-coding genes and two miRNA genes (RAD23B, FBP1, TNFRSF1A, CCNG2, NOTCH3, ETV1, BID, SIM2, ANXA1, miR-519d, and miR-647) that could be used to separate patients with and without biochemical recurrence ($p < 0.001$), as well as for the subset of 42 Gleason score 7 patients ($p < 0.001$)¹. Importantly, these biomarkers could significantly predict clinical recurrence for Gleason score 7 patients. In March 2010, we received funding for a two-year Prostate Cancer IDEA Award to validate and improve this set of biomarkers on an independent, large set of patient samples. In the first year of the award, we overcame a number of administrative hurdles to obtain the necessary approvals to proceed with the research funded by this award and began collection of patient samples. Because of the delays in the beginning of the project, we requested and received a no-cost extension of the project from a 2-year to a 3-year project. Over the second year of the award, we collected and prepared RNA from 99 patient samples from the Atlanta VA Medical Center, and 72 samples from the Sunnybrook Health Sciences Centre at the University of Toronto. We have validated our new strategy of performing RNA-sequencing on the Illumina HiSeq2000 instrument, constructed tissue microarrays that include 97 samples, and validated antibodies to two protein biomarkers that are associated with outcome.

Our specific aims for this project are as follows:

Aim 1: Perform genomic profiling of 180 prostatectomy samples for both mRNA and miRNA analysis to identify the optimal set of biomarkers of biochemical recurrence.

Aim 2: Test the predictor set of genes from Aim 1 by TaqMan QPCR as an independent method.

Aim 3: Perform a blinded validation on an independent set of 180 samples.

Body

During the course of preparation of 1mm tissue cores for RNA extraction of prostatectomy samples from the Atlanta VA Medical Center (VAMC), we generated a tissue microarray (TMA) that includes cores from 97 VAMC cases with known outcome. The characteristics of these cases are given in **Table 1**. Since five of our candidate biomarkers (RAD23B, SIM2s, Notch3, BID and FBP1) are increased in cases with recurrence and have available commercial antibodies, we tested antibodies to these proteins on our TMAs. Five TMA sections were stained with antibodies to RAD23B, SIM2S, Notch3, BID, and FBP1. Intensity of the various immunohistochemical stains were scored blindly as follows; 0-negative, 1+ (weak), 2+ (intermediate) and 3+ (strong). Representative cores are shown in **Figure 1**.

The expression of these markers was correlated with biochemical recurrence (BCR) using Fisher's exact test with samples divided into categories 0-2+ vs. 3+ for staining and positive vs. negative for BCR. We determined that BID ($p = 0.0005$), SIM2s ($p = 0.007$), RAD23B ($p = 0.05$), and FBP1 ($p = 0.04$) were significantly associated with BCR by Fisher's exact test. NOTCH3 ($p = 0.56$), did not demonstrate statistical significant association with BCR. We also evaluated the staining in survival analysis by Cox Proportional Hazards model and Log Rank test (**Table 2**). Moreover, there was no statistically significant correlation between any of the markers and pathologic stage, Gleason score, patient race, or pre-operative PSA levels. We conclude from these studies that BID, FBP1, RAD23B, and SIM2s may be useful immunohistochemical biomarkers in the prediction of BCR in patients following radical prostatectomy, irrespective of pathologic stage, Gleason score, patient race, or pre-operative PSA levels. We also conclude that BID is the most promising of these five markers. Thus, patients with tumors that demonstrate increased expression of these markers, may benefit from close follow-up following radical prostatectomy. These studies have been presented as a poster abstract entitled "Correlation of Immunohistochemical Expression of Protein-Coding Genes RAD23B, SIM2S, Notch3, BID and FBP with Biochemical Recurrence in Patients Following Radical Prostatectomy for Prostatic Adenocarcinoma", at the United States and Canadian Academy of Pathology's (USCAP) 101st Annual Meeting, March 17-23, 2012 in Vancouver, BC, Canada. We have also applied our published set of biomarkers against a publicly available dataset of prostate cancer gene expression². Applying our predictor to this independent external dataset gave a highly significant p-value ($p = 0.000936$ by log-rank test, **Figure 2**), further validating our biomarkers of recurrence. A manuscript detailing these studies is in preparation for submission.

Progress on Aim 1

We identified 150 cases at the VA hospital between 1990-2000 that could potentially be used for this project. We were able to locate slides and formalin-fixed paraffin embedded (FFPE) blocks for 100 of those cases, and Dr. Oyesiku identified regions of cancer and benign tissue in slides for each of them. These samples were then submitted for processing to obtain 1 mm tissue cores. We prepared RNA from 99 samples of which 79 passed our QC analysis for genomic profiling. We are planning to examine additional cases at Emory and the VA between 2000-2003 to identify samples for use in Aim 3. We have received 72 samples from U. Toronto of which 29 have passed QC. To increase the diversity of our samples and speed the acquisition of them, we have surveyed additional samples at the Atlanta VAMC and identified 154 samples between 2000-2005 of which 57% are African-American. We have also identified potential external resources with an additional 96 RNA samples from FFPE prostatectomy specimens with known long-term outcome if necessary as an alternative.

Platform optimization

Because the Illumina miRNA DASL platform for analysis of miRNA biomarker expression levels has been discontinued, we are likely to use a 48-plex multiplexing method of next generation sequencing on the Illumina HiSeq platform. This should give us deep coverage and high data quality at an acceptable cost.

Since RNA from FFPE samples is typically fragmented, conventional methods for sequencing library preparation that utilize poly-dT hybridization to capture mRNA will not work. We tested two alternative library preparation methods using the Ribominus kit (Ambion) to remove ribosomal RNA, and the Ovation WT Amplification kit for FFPE samples. We have performed pilot RNA-seq analysis on eleven samples (six from Ribominus, and five from Ovation), and determined that the more cost effective option (Ribominus) gives equal or superior sequencing read coverage (**Table 3**).

In addition, we have identified a facility that can perform Illumina RNA-seq at a much lower cost to us at the Southern California Genotyping Consortium (SCGC). The reduced cost of this facility will allow us to perform RNA-seq on close to our originally proposed sample set for DASL analysis of 360 samples. We anticipate that we should be able to afford sequencing of 200-300 samples depending on whether we can multiplex two or three samples per lane on the Illumina Version 3 Flowcells for the HiSeq2000 platform.

A summary of the progress to date is given in **Table 4**. Of the 103 samples that have passed QC, we have prepared sequencing libraries for approximately half of them (library preparation is currently ongoing). We have submitted our first set of 25 samples for RNA-seq analysis to the SCGC and anticipate getting those data in the next few weeks. The data from this first set will determine our depth of coverage and whether we can multiplex two or three samples per lane.

Progress on Aim 2

Development of a TaqMan Low Density Array (TLDA) with assays to our current set of biomarkers is ongoing. Development of a refined TLDA assay will take place once analysis of RNA-seq data from Aim 1 is completed. Initiation of Aim 3 will take place once Aims 1 and 2 are completed.

TABLES

Stage		Gleason Grade	
T1a	1	3+3	12
T1c	4	3+4	53
T2	20	4+3	23
T2a	12	4+4	7
T2b	2	4+5	1
T2c	39	5+3	1
T3a	9		
T3b	7		
T4	2		
Unknown	1		

	Age	PSA
Median	64	6.6
Range	47-74	1.6-72.6

Biochemical Recurrence		Race	
BCR	34	Caucasian	59
No BCR	61	African-American	31
Unknown	2	Unknown	7
Total	97		

Table 1: Clinical characteristics of patients included in the TMA.

p-values	BID	SIM2S	NOTCH3	FBP1	RAD23B
Fisher exact	0.001	0.014	1.000	0.068	0.088
Log-rank	0.040	0.100	0.472	0.138	0.096
Cox PH	0.047	0.107	0.474	0.151	0.106

Table 2: p-values for significance of IHC staining for five candidate biomarkers.

Sample	RB (ribominus) Reads total	OV (ovation) Reads total	RB (ribominus) Reads aligned	OV (ovation) Reads aligned	RB (ribominus) % aligned	OV (ovation) % aligned
0004	6,351,312	10,000,000	3,588,997	3,439,698	56.51%	34.40%
0021	5,665,713	9,633,165	2,816,032	3,460,675	49.70%	35.92%
0028	4,794,196	7,202,909	2,504,281	2,524,433	52.24%	35.05%
0046	6,976,777		3,829,933		54.90%	
0055	8,109,619	9,917,757	4,031,267	3,346,812	49.71%	33.75%
0065	10,000,000	9,962,123	5,955,829	3,558,811	59.56%	35.72%

Table 3: Summary of sequencing reads from pilot analysis of FFPE RNA samples. From these data we concluded that the Ribominus method of library preparation is superior in % of aligned reads and equivalent or superior in total aligned reads, and that RNA-seq analysis of FFPE RNA samples is feasible.

Tissue Microarray prepared	July-August, 2011
TMA stained for five protein biomarkers	Sept-Feb, 2011
Tissue samples identified and cored at U. Toronto	Mar-Oct, 2011
RNA prepared from 99 VAMC samples	Aug-Sept, 2011
RNA QC passed for 67 VAMC samples	October, 2011
Tissue samples received from U. Toronto	October, 2011
RNA-seq library preparation optimization	Oct-Nov, 2011
Pilot RNA-seq data generated	December, 2011
RNA-seq analysis	January, 2011
RNA-seq library preparation	Jan-Mar, 2011

Table 4: Milestones and research progress during the past year.

FIGURES

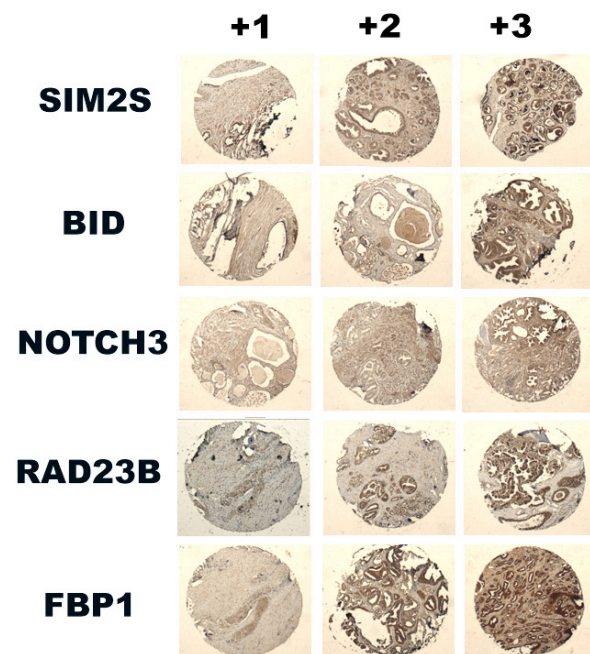


Figure 1: Representative cores from the stained TMA are indicated for each of the five antibodies tested.

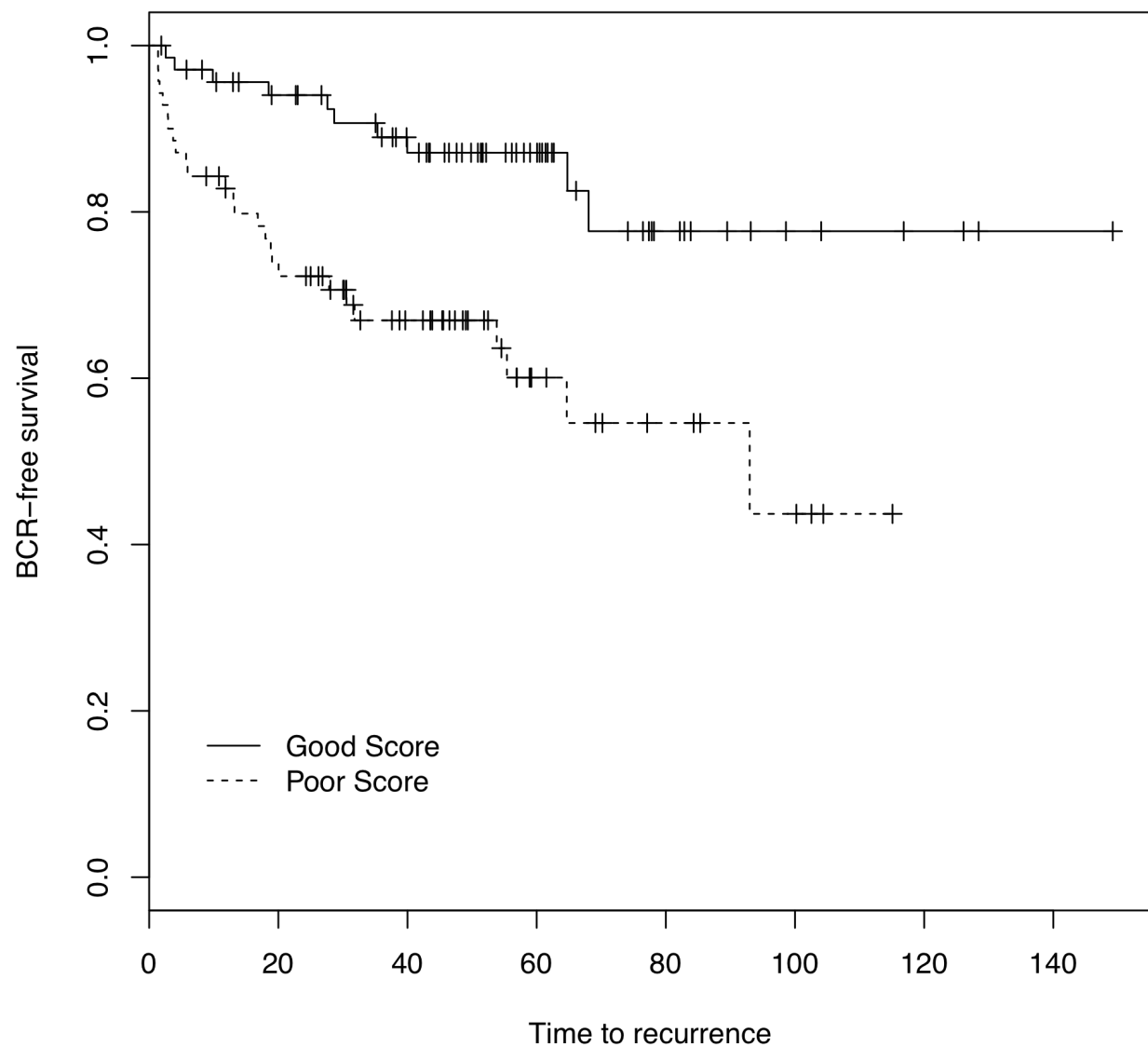


Figure 2: Kaplan-Meier curve of BCR-free survival for 140 cases from Taylor et al² using the set of coefficients from our biomarkers described in Long et al¹.

Key Research Accomplishments

Our key research accomplishments are summarized below:

- Determined that BID ($p = 0.0005$), SIM2s ($p = 0.007$), RAD23B ($p = 0.05$), and FBP1 ($p = 0.04$) were significantly associated with BCR at the protein level using immunohistochemistry analysis of tissue microarrays.
- Demonstrated that RNA-seq analysis of FFPE-derived RNA from archived prostatectomy specimens is feasible.
- Validated our published set of biomarkers on an independent publicly available dataset from Taylor et al².

Reportable Outcomes

Grant Award W81XWH-10-1-0090 renewed	March 15, 2011
Oral Presentation at ASIP 2011 Annual Meeting, MiniSymposium on Cellular and Molecular Advances in Cancer Biology, Washington, DC.	April, 2011
Q Long, BA Johnson, AO Osunkoya, Y-H Lai, W Zhou, M Abramovitz, M Xia, M Bouzyk, R Nam, L Sugar, A Stanimirovic, BR Leyland-Jones, AK Seth, JA Petros, CS Moreno , Protein-coding and MicroRNA Biomarkers of Recurrence of Prostate Cancer Following Radical Prostatectomy, <i>American Journal of Pathology</i> , 179(1) :46-54.	July, 2011
1 year no cost extension granted for W81XWH-10-1-0090	March, 2012

Conclusion

Thus far we have made good progress on our goal to validate biomarkers of recurrence in prostate cancer. We have published a manuscript in *The American Journal of Pathology* describing our set of biomarkers¹, and presented these data orally at the American Society of Investigative Pathology annual meeting in Washington, DC on April 11, 2011. We have optimized our method for RNA-seq analysis and collected 171 patient samples, of which 108 have passed RNA QC. We are in the process of preparing RNA-seq libraries and anticipate generating large datasets for analysis in the next few months. In addition, we have validated our published biomarkers on an independent public dataset and found that they were significant at predicting recurrence free survival.

We have demonstrated that four protein biomarkers (BID, SIM2s, FBP1, and RAD23B) are significantly associated with recurrence in prostate cancer. We conclude from these studies that BID, FBP1, RAD23B, and SIM2s may be useful immunohistochemical biomarkers in the prediction of BCR in patients following radical prostatectomy, irrespective of pathologic stage, Gleason score, patient race, or pre-operative PSA levels. These immunohistochemistry assays may be useful for predicting recurrence in clinical cases. We also conclude that BID is the most promising of these five markers. Thus, patients with tumors that demonstrate increased expression of these markers, may benefit from close follow-up following radical prostatectomy.

References

1. Long Q, Johnson BA, Osunkoya AO, Lai YH, Zhou W, Abramovitz M, Xia M, Bouzyk MB, Nam RK, Sugar L, Stanimirovic A, Williams DJ, Leyland-Jones BR, Seth AK, Petros JA, Moreno CS: Protein-coding and microRNA biomarkers of recurrence of prostate cancer following radical prostatectomy, *Am J Pathol* 2011, **179**:46-54
2. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Socci ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL, Gerald WL: Integrative genomic profiling of human prostate cancer, *Cancer Cell* 2010, **18**:11-22

Appendices

See attached manuscript by Long et al¹.

Biomarkers, Genomics, Proteomics, and Gene Regulation

Protein-Coding and MicroRNA Biomarkers of Recurrence of Prostate Cancer Following Radical Prostatectomy

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An important challenge in prostate cancer research is to develop effective predictors of tumor recurrence following surgery to determine whether immediate adjuvant therapy is warranted. To identify biomarkers predictive of biochemical recurrence, we isolated the RNA from 70 formalin-fixed, paraffin-embedded radical prostatectomy specimens with known long-term outcomes to perform DASL expression profiling with a custom panel that we designed of 522 prostate cancer-relevant genes. We identified a panel of 10 protein-coding genes and two miRNA genes (*RAD23B*, *FBP1*, *TNFRSF1A*, *CCNG2*, *NOTCH3*, *ETV1*, *BID*, *SIM2*, *LETMD1*, *ANXA1*, *miR-519d*, and *miR-647*) that could be used to separate patients with and without biochemical recurrence ($P < 0.001$), as well as for the subset of 42 Gleason score 7 patients ($P < 0.001$). We performed an independent validation analysis on 40 samples and found that the biomarker panel was also significant at prediction of biochemical recurrence for all cases ($P = 0.013$) and for a subset of 19 Gleason score 7 cases ($P = 0.010$), both of which were adjusted for relevant clinical information

including T-stage, prostate-specific antigen, and Gleason score. Importantly, these biomarkers could significantly predict clinical recurrence for Gleason score 7 patients. These biomarkers may increase the accuracy of prognostication following radical prostatectomy using formalin-fixed specimens. (*Am J Pathol* 2011, 179:46–54; DOI: 10.1016/j.ajpath.2011.03.008)

Prostate cancer remains the most common noncutaneous cancer diagnosed for U.S. males, and ranks second among tumor site-specific mortality, with estimates for 2009 at over 192,000 new cases and 27,000 deaths.¹ The majority of patients with prostate cancer are clinically asymptomatic with early-stage, organ-confined disease, and in fact, more than 50% of men who reach the age of 80 develop clinically insignificant prostate cancer. However, a subpopulation of prostate cancer patients progress to highly aggressive, androgen-independent metastatic disease, which is inevitably fatal. One of the important challenges in current prostate cancer research is to develop effective methods to determine whether a patient is likely to progress to aggressive, metastatic disease, to aid clinicians in deciding on the appropriate

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course of treatment. Biomarker assays that could predict progression and metastasis for prostate cancer patients would be of great utility in aiding clinical management of this large patient population. An important challenge in prostate cancer research is to develop effective predictors of tumor recurrence following surgery to determine whether immediate adjuvant therapy is warranted. Thus, biomarkers that could predict the likelihood of success for surgical therapies would be of great clinical significance.

In the past few years, enormous progress has been made in developing technologies to exploit formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples for gene expression analysis. The DASL (cDNA-mediated Annealing, Selection, extension and Ligation) assay is a unique expression profiling platform that is based on massively multiplexed RT-PCR applied in a microarray format, that allows for the determination of expression of RNA isolated from 96 FFPE tumor tissue samples in a high throughput format.^{2,3}

Here, we have identified biomarkers predictive of recurrence by expression profiling archived FFPE tumor samples using both a custom panel of prostate cancer-associated mRNA genes and a panel of microRNA (miRNA) genes. These biomarkers were developed on a training set of 70 patients (29 with biochemical recurrence and 41 controls) and validated on an independent set of 40 samples (13 with biochemical recurrence and 27 controls) and were able to significantly discriminate between patients with and without biochemical recurrence following radical prostatectomy. Moreover, these biomarkers were able to discriminate biochemical recurrence in patients with Gleason score 7, for whom outcome is particularly difficult to predict.

Materials and Methods

Patient Samples

In the initial training set, 70 prostate cancer cases were used (29 with biochemical recurrence and 41 controls without recurrence), 45 patients from Sunnybrook Health Science Center (Toronto, ON), and 25 patients from Emory University (Atlanta, GA). The 45 cases of paraffin-embedded tissue samples from Toronto were drawn from men who underwent radical prostatectomy as the sole treatment for clinically localized prostate cancer between 1998 and 2006. The clinical data include multiple clinicopathological variables such as prostate-specific antigen (PSA) levels, histological grade (Gleason score), tumor stage (pathological stage category, for example; organ confined, pT2; or with extra-prostatic extension, pT3a; or with seminal vesicle invasion, pT3b), and biochemical recurrence rates. For the cases from Emory University, both the training set (25 cases) and validation set (40 cases) FFPE samples were also selected from a screen of over a thousand patients through an institutional review board-approved retrospective study at Emory University of men who had undergone radical prostatectomy between 1990 and 1994. Those who were

included met specific inclusion criteria, had available tissue specimens, had documented long-term follow-up, and consented to participate or were included by institutional review board waiver. The cases were assigned prostate identification numbers to protect their identities. These patients did not receive neoadjuvant or concomitant hormonal therapy. Their demographic, treatment, and long-term clinical outcome data have been collected and recorded in an electronic database. Clinical data recorded include PSA measurements, radiological studies and findings, clinical findings, tissue biopsies, and additional therapies that the subjects may have received. Clinical data associated with the samples used in this study are given in Supplemental Table S1 (available at <http://ajp.amjpathol.org>).

RNA Preparation

Tissue cores (1 mm) were used for RNA preparation rather than sections because of the heterogeneity of samples and the opportunity for obtaining cores with a very high percentage tumor content. H&E-stained slides were reviewed by a board-certified urologic pathologist (A.O.O.) to identify regions of cancer to select corresponding areas for cutting of cores from paraffin blocks. Total RNA was prepared at the Emory Biomarker Service Center from FFPE cores as previously described,⁴ using the Ambion Recoverall MagMax methodology in 96-well format on a MagMax 96 Liquid Handler Robot (Life Technologies, Carlsbad, CA). FFPE RNA was quantitated using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE), and tested for RNA integrity and quality by TaqMan analysis of the RPL13a ribosomal protein on a HT7900 real-time PCR instrument (Applied Biosystems, Foster City, CA). Samples with sufficient yield (>500 ng), A_{260}/A_{280} ratio >1.8, and RPL13a C_T values less than 30 cycles were used for miRNA and DASL profiling.

Custom Prostate Cancer DASL Assay Pool

The DASL assay enables quantitation of expression of up to 1536 probes using RNA isolated from archived FFPE tumor tissue samples in a high throughput format.^{2,3} Data from multiple publicly available gene expression datasets,⁵⁻⁸ along with genes involved in prostate cancer progression based on current understanding of the disease,^{6,9} were distilled to develop a highly predictive set of 522 genes for use in the DASL assay. Due to specific probe design considerations, this panel had three probes for 497 genes, two probes for 20 genes, and a single probe for five genes, two of which were specific to *TMPRSS2-ERG* and *TMPRSS2-ETV1* fusion transcripts. The unique combination of genes was optimized for performance in the DASL assay using stringent criteria that predicts excellent performance of the primer sets. The panel includes genes found to be correlated with Gleason score in Liu et al,¹⁰ Bibikova et al,¹¹ True et al,¹² Lapointe et al,⁷ and/or Singh et al.¹³ It also includes prognostic markers from Dhanasekaran et al¹⁵ and Yu et al,¹⁴ and genes associated with metastasis in Varambally et al.⁶ In addition, a number of genes known from other

studies to be critical in prostate cancer such as *NKX3.1*, *PTEN*, and the androgen receptor are all included in the panel. Other genes that play important roles in the Wnt, Hedgehog, TGF β , Notch, MAPK, and PI3K pathways are also present in this gene set. Finally, primer sets that detect chromosomal translocations in *ERG*,⁹ *ETV1*,¹⁵ and *ETV4*¹⁶ are also included in this panel. The custom prostate cancer panel list of 522 candidate genes (see Supplemental Table S2 at <http://ajp.amjpathol.org>) was submitted to Illumina for synthesis (Illumina, San Diego, CA). The optimal oligonucleotide sequence for each of the 1536 gene probes was determined using an oligonucleotide scoring algorithm. The oligonucleotide pool or DASL Assay Pool was synthesized by Illumina for use with the 96-well Universal Array Matrix.

The DASL Assay

The DASL assay was performed with our 522-gene custom-designed human prostate cancer panel using 200 ng of input RNA at the Emory Biomarker Service Center, Emory University, according to the manufacturer's protocols. Samples, including technical replicates (two, three, or four) were hybridized on Universal Array Matrices, and scanned using the BeadStation 500 Instrument (Illumina). For miRNA DASL assays, the human miRNA v2 DASL panel (Illumina), which allows for the determination of expression of 1146 human miRNAs (>97% coverage of miRBase release 12), was used. These data are available in the Gene Expression Omnibus (GEO) database under accession number GSE26367.

Data Analysis

DASL fluorescent intensities were interpreted in GenomeStudio, quantile normalized, and exported for meta-analysis. Average signal intensity, genes detected (P value = 0.01), background, and noise (SD of background) were analyzed for trends by plate, row, and column. The two endpoints of interest were postoperative biochemical recurrence, defined as two detectable PSA readings (>0.2 ng/mL), and clinical recurrence, defined as evidence of local or metastatic disease. The primary outcome of interest was time to biochemical recurrence following surgery. A local recurrence was defined as recurrence of cancer in the prostatic bed that was detected by either a palpable nodule on digital rectal examination and subsequently verified by a positive biopsy, and/or a positive imaging study [ProstaScint (EUSA Pharma, Langhorne, PA) or computed tomography scan] accompanied by a detectable postoperative PSA result and lack of evidence for metastases. Also, patients whose PSA levels decreased following adjuvant pelvic radiation therapy for elevated postoperative PSA were considered as local recurrence cases. A recurrence with metastases was defined as a positive imaging study indicating presence of a tumor outside of the prostatic bed.

To identify important biomarkers and build and evaluate prediction models for prostate cancer recurrence, we adopted the following strategy. In the training step, the prediction model was built based on the time to biochem-

ical recurrence. Specifically, we first fit a univariate Cox proportional hazard (PH) model for each individual oligonucleotide probe using the training dataset, and a set of important mRNA and miRNA probes were then preselected based on a false discovery rate threshold of 0.30. Next, to identify the optimal prediction score based on the preselected probes, we fit a lasso Cox PH model^{17,18} using the training dataset, where the tuning parameter for lasso was selected using a leave-one-out cross-validation technique.¹⁸ The lasso Cox PH model was fitted first using the set of preselected mRNA probes only and then using the complete set of preselected mRNA and miRNA probes, resulting in an optimal mRNA panel and an optimal combined mRNA/miRNA panel, respectively. Based on each biomarker panel, a final prediction model for recurrence was built to also incorporate relevant clinical biomarkers, namely, T-stage, PSA, and Gleason score, through fitting Cox PH models. For comparison, we also built a prediction model using only clinical information, namely, T-stage, PSA, and Gleason score, through fitting a Cox PH model.

To evaluate and validate the final prediction models obtained from the training phase, 79 samples from 40 patients were used, and replicate samples from the same patient were again averaged to generate a single average signal for each patient. Each prediction model from the training phase was used to generate a predictive score for each subject in the validation dataset, and subjects were subsequently divided into high and low scoring groups using the median predictive score. Kaplan-Meier analysis was performed to compare the time to biochemical recurrence, between high (poor score) and low (good score) risk groups, and the statistical significance was determined using the log-rank test. Similarly, we also evaluated the final model that uses the combined mRNA/miRNA panel for predicting time to clinical recurrence in both training and validation datasets.

Missing data are present in this study, in particular, for clinical recurrence, PSA, and T-stage data. We adopted the available-case approach¹⁹ in our analyses, and the sample sizes used in each step of building and evaluating prediction models may be less than the total sample size.

Results

Custom Prostate DASL Profiling

We performed DASL expression profiling with our custom-designed prostate cancer panel (see the *Materials and Methods* section) and the Illumina DASL microRNA panel on 70 prostatectomy patient samples to identify biomarkers predictive of recurrence. An independent validation profiling experiment was performed on 40 additional samples. miRNA probes were filtered to retain only those that were present on the miRNA microarrays used for both the training and validation sets, reducing the total number of probes examined to 403 miRNA probes. The training set included 29 cases with observed biochemical PSA recurrence (median time to recurrence = 19

Table 1. A Summary of the Clinical Characteristics of the Training and Validation Sets of Patient Samples

	Training set (total)	Training set (no BCR)	Training set (BCR)	Validation set (total)	Validation set (no BCR)	Validation set (BCR)
Number cases	70	41	29	40	27	13
Clinical recurrence	8	0	8	11	0	11
No clinical recurrence	57	41	16	29	27	2
Median time F/U (months)	84	83	81	74	75	73
Median time to BCR (months)	19	NA	19	14	NA	14
Median time no BCR (months)	48	83	19	34.5	56	14
Gleason score (avg $\pm$ SD)	6.9 $\pm$ 0.6	6.7 $\pm$ 0.6	7.0 $\pm$ 0.6	7.0 $\pm$ 0.8	6.8 $\pm$ 0.7	7.4 $\pm$ 1
PSA (avg $\pm$ SD)	9.2 $\pm$ 5.4	8.7 $\pm$ 6.4	9.9 $\pm$ 3.8	12.7 $\pm$ 8.4	12.4 $\pm$ 9.9	13.1 $\pm$ 5.3
Age (avg $\pm$ SD)	61.9 $\pm$ 7.7	61.2 $\pm$ 7.7	62.9 $\pm$ 7.8	63.6 $\pm$ 8.4	63.5 $\pm$ 8.3	64 $\pm$ 8.9

BCR, biochemical recurrence; F/U, follow up; NA, not applicable; PSA, prostate specific antigen; SD, standard deviation.

months), and 41 cases censored, ie, without observed recurrence during follow-up (median follow-up time = 83.0 months). A summary of the clinical characteristics of the training and validation sets of samples is provided in [Table 1](#). The complete dataset for the combined mRNA and miRNA data are provided in Supplemental Table S3 for the training set and Supplemental Table S4 for the validation set (available at <http://ajp.amjpathol.org>).

Integrated DASL Biomarker Analysis

After fitting a univariate Cox proportional hazard model for each individual probe using the training data, a set of 27 important probes were preselected based on a false discovery rate threshold of 0.30 (see Supplemental Table S5 at <http://ajp.amjpathol.org>). Next, to identify the optimal prediction score based on the preselected probes, we fit a lasso Cox proportional hazard model^{17,18} first using the set of 25 preselected mRNA probes only, resulting in a panel of nine protein-coding genes shown in [Table 2](#) (*RAD23B*, *FBP1*, *TNFRSF1A*, *NOTCH3*, *ETV1*, *BID*, *SIM2*, *ANXA1*, and *BCL2*). A final prediction model was then built to include the predictive score based on this panel of nine mRNA biomarkers as well as the relevant clinical biomarkers including T-stage, PSA, and Gleason score, which could be used to predict recurrence following radical prostatectomy. Kaplan-Meier analysis ([Figure 1A](#)) demonstrated that these probes could significantly discriminate patients at higher and lower risk of recurrence by the log-rank test ($P < 0.001$). We next applied the final predictive model developed on the training set to the

validation set, a separate, independent DASL profiling experiment performed on a different day. Kaplan-Meier analysis ([Figure 1B](#)) on this validation set determined that the model could discriminate patients at higher and lower risk of recurrence ($P = 0.010$).

Subsequently, we repeated the above training procedure using the complete set of 27 preselected mRNA and miRNA probes, and we identified an optimal panel of 10 mRNAs and two microRNAs ([Table 3](#)) and built a final prediction model for prostate cancer biochemical recurrence, which again included relevant clinical biomarkers. Kaplan-Meier analysis and the log-rank test determined that this panel could also significantly discriminate patients at higher and lower risk of recurrence both in the training set ($P < 0.001$, [Figure 1C](#)) and in the validation set ($P = 0.013$, [Figure 1D](#)).

Prediction of Cases with a Gleason Score 7

Prediction of recurrence for patients with a Gleason score 7 is particularly difficult. To address this issue, we applied the biomarker panels to the subset of cases in the training and validation sets that had a Gleason score 7. The prediction model based on the nine-mRNA panel was significant at discriminating biochemical recurrence in Gleason score 7 cases in both the training set ($P < 0.001$, [Figure 2A](#)) and the validation set ($P = 0.027$, [Figure 2B](#)). For the prediction model based on the combined panel of 10 mRNAs and two miRNAs in [Table 3](#), the predictive value was again significant for both the training set ($P = < 0.001$, [Figure 2C](#)) and the validation set ($P = 0.010$,

Table 2. Nine-Gene Predictor of Prostate Cancer Recurrence Following Surgery

Symbol	Description	Coefficient	References
<i>RAD23B</i>	RAD23 homolog B	0.152	20, 21
<i>FBP1</i>	Fructose-1,6-bisphosphatase 1	0.310	7, 10, 33
<i>TNFRSF1A</i>	Tumor necrosis factor receptor superfamily, member 1A	-0.560	23, 24
<i>NOTCH3</i>	Notch homolog 3	0.426	25, 26
<i>ETV1</i>	Ets variant gene 1 (ETV1)	0.157	9, 15
<i>BID</i>	BH3 interacting domain death agonist (BID)	0.248	27, 28
<i>SIM2</i>	Single-minded homolog 2	0.043	29-32
<i>ANXA1</i>	Annexin A1	-0.185	33-36
<i>BCL2</i>	B-cell CLL/lymphoma 2	0.028	37-38

Coefficient is derived from the lasso Cox proportion hazards model and was used for computing the predictive score. Positive coefficients indicate a positive association with recurrence, and negative coefficients indicate a negative association with recurrence.

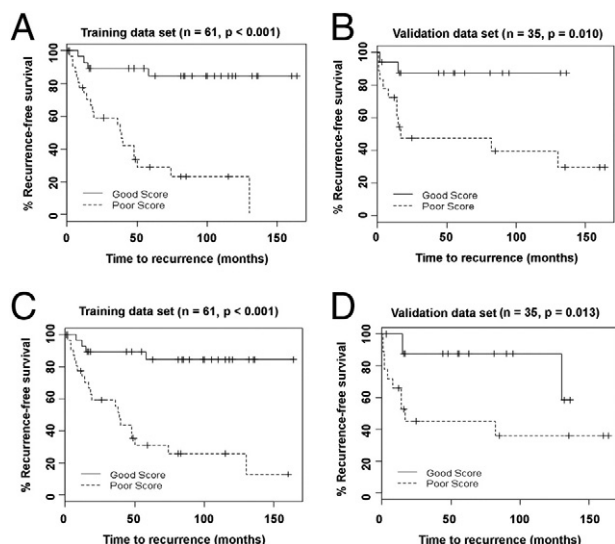


Figure 1. Prediction of biochemical recurrence in all prostate cancer patients using two biomarker panels, adjusted for clinical information. **A:** Kaplan-Meier analysis of the training set patients that were separated based on the mRNA panel described in Table 2. **B:** Kaplan-Meier analysis on the validation cases using the mRNA panel. **C:** Kaplan-Meier analysis of the training set using the combined mRNA and miRNA panel described in Table 3. **D:** Kaplan-Meier analysis of the validation set using the combined mRNA and miRNA panel.

Figure 2D). A summary of the *P* values for predicting biochemical recurrence is given in Table 4. In all cases, the prediction models that use one of the two gene biomarker panels plus clinical information outperforms the prediction model using only clinical information.

Analysis of Clinical Recurrence

Although most patients who have clinical recurrence following prostatectomy also have biochemical recurrence, there is a significant population of patients with biochemical recurrence who do not have clinically significant recurrences observed during their follow-ups. To evaluate our biomarker panel of biochemical recurrence for predicting the clinical recurrence, we tested the prediction model based on the combined mRNA/miRNA panel in the

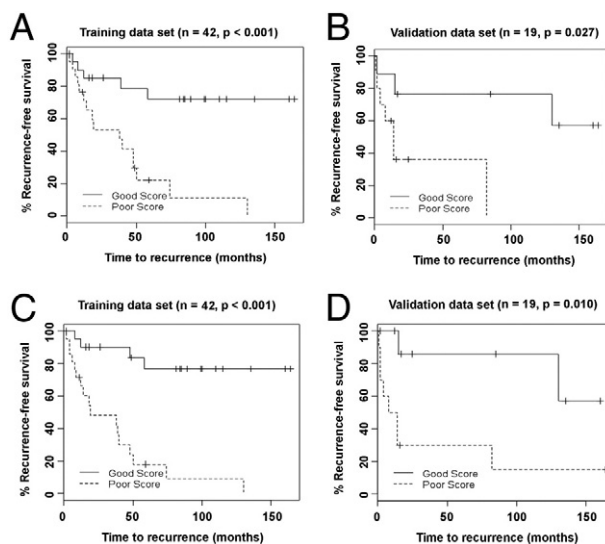


Figure 2. Prediction of biochemical recurrence in prostate cancer patients with a Gleason score 7 using two biomarker panels, adjusted for clinical information. **A:** Kaplan-Meier analysis of the training set of Gleason score 7 cases using the mRNA panel described in Table 2. **B:** Kaplan-Meier analysis of the Gleason score 7 cases in the validation set using the mRNA panel. **C:** Kaplan-Meier analysis of the Gleason score 7 cases in the training set using the combined mRNA and miRNA panel described in Table 3. **D:** Kaplan-Meier analysis of the Gleason score 7 cases in the validation set using the combined mRNA and miRNA panel.

same training and validation samples using their clinical recurrence outcome data. Unfortunately, clinical recurrence data were lacking on some of the samples, and the total number of samples used in the training set was reduced. In the training data, the combined mRNA/miRNA panel was highly significant for predicting clinical recurrence in all patients ($P = 0.002$) as well as in the subset of patients with a Gleason score 7 ($P = 0.004$); in the validation data, it was also significant for predicting recurrence in patients with a Gleason score 7 ($P = 0.023$) and trended toward significance in all patients ($P = 0.078$). A summary of the *P* values for predicting clinical recurrence is given in Table 5. In all cases, the prediction model that uses the combined mRNA and miRNA panel plus the clinical information, again, outperforms the prediction model that uses only the clinical information.

Table 3. Twelve-Gene Predictor of Prostate Cancer Recurrence Following Surgery Using 10 mRNAs and Two microRNAs

Symbol	Description	Coefficient	References
<i>RAD23B</i>	RAD23 homolog B	0.070	20, 21
<i>FBP1</i>	Fructose-1,6-bisphosphatase 1	0.251	7, 10, 22
<i>TNFRSF1A</i>	Tumor necrosis factor receptor superfamily, member 1A	-0.588	23, 24
<i>CCNG2</i>	Cyclin G2	0.008	39–41
<i>hsa-miR-647</i>	hsa-miR-647	-0.318	
<i>LETMD1</i>	LETMD1 domain containing 1	0.063	42–44, 45
<i>NOTCH3</i>	Notch homolog 3	0.367	25, 26
<i>ETV1</i>	ETS variant gene 1 (ETV1)	0.179	9, 15
<i>hsa-miR-519</i>	hsa-miR-519 days	0.551	46
<i>BID</i>	BH3 interacting domain death agonist (BID)	0.128	27, 28
<i>SIM2</i>	Single-minded homolog 2	0.124	29–32
<i>ANXA1</i>	Annexin A1	-0.143	33–36

Coefficient is derived from the lasso Cox proportion hazards model and was used for computing the predictive score. Positive coefficients indicate a positive association with recurrence, and negative coefficients a negative association with recurrence.

Table 4. Summary of *P* Values (Log-Rank Test) of Prediction of Biochemical Recurrence on Training and Validation Sets

Dataset	Combined		Clinical information only
	mRNA panel	mRNA/miRNA panel	
Training			
All cases (<i>n</i> = 61)	<0.001	<0.001	0.096
Gleason score 7 (<i>n</i> = 42)	<0.001	<0.001	0.641
Validation			
All cases (<i>n</i> = 35)	0.010	0.013	0.020
Gleason score 7 (<i>n</i> = 19)	0.027	0.010	0.028

Prediction of biochemical recurrence for the entire dataset and the subset of Gleason score 7 cases was made using two biomarker panels adjusted for T-stage, PSA, and Gleason score, or using clinical information only. Significant *P* values are indicated in bold.

We also performed an analysis to construct a predictive set of biomarkers based on the clinical recurrence data instead of biochemical recurrence. Only three probes passed the initial preselection step for the univariate Cox PH modeling, all corresponding to the *ETV1* gene, which is likely due to the considerably fewer number of clinical recurrences in the training set as well as the smaller total sample size. Furthermore, the prediction model built on this set of gene biomarkers did not perform as well as the models built on biochemical recurrence (data not shown).

Discussion

In the past few years, enormous progress has been made in developing technologies to exploit FFPE tumor tissue samples for gene expression and proteomic analysis. The use of FFPE tissues as a starting material is attractive because this approach should make biomarkers identified in this way much easier to translate into widespread clinical practice. DASL profiling makes it possible to define gene sets using FFPE prostate cancer tissues that could have potential prognostic and predictive value. For example, the DASL assay has been used recently to identify a 16-gene set that correlates with prostate cancer relapse.¹¹ There was no overlap between our panel of 10 mRNA and two miRNA biomarkers described here and the previously described 16-gene panel even though 10 of the genes in the 16-gene panel previously reported

were included in our 522 custom prostate DASL panel. When we analyzed the performance of the probes corresponding to those 10 mRNAs in our dataset, we found that they were not able to significantly discriminate patients at higher or lower risk of recurrence. In this previous study, the gene signature selection and prediction model building were performed in separate steps, and the signature selection was based on the correlation between the gene expression and Gleason score rather than between the gene expression and time to biochemical recurrence; our analytic approach overcomes these limitations. Specifically, our approach of building (training) prediction models takes advantage of recent advancement in regularized regression models for survival outcomes^{17,18}; regularized regression models can achieve simultaneous feature selection and model estimation and avoid model overfitting, leading to better prediction performance. Our use of a preselection step is similar to the recently proposed sure independence screening methods,^{47,48} which have been shown to achieve better performance in the presence of high-dimensional data for survival analysis compared to regularized regression without a preselection step.⁴⁹

Two other recent studies have used DASL profiling to prostate cancer, but have not detected any signature that improved on clinical models in validation sets.^{50,51} Although these studies used large cohorts with long-term follow-up, they examined different panels of mRNA transcripts and did not include probes corresponding to miRNA genes. Moreover, these earlier studies suggested that tumor heterogeneity may play an important role in confounding signature identification. For our study of prostatectomy specimens, we identified the most prominent tumor lesion, and used a tissue core sample from that region to minimize stromal contributions and tumor heterogeneity.

In our 12-gene predictive biomarker panel, nine of the genes are positively associated with recurrence, and three are negatively associated with recurrence. The nine genes positively associated with recurrence included *miR-519d*, Notch homolog 3 (*Notch3*), Fructose-1,6-bisphosphatase 1 (*FBP1*), ETS variant gene 1 (*ETV1*), BH3 interacting domain death agonist (*BID*), Single-Minded homolog 2 (*SIM2*), RAD23 homolog B (*RAD23B*), LETM1 domain containing 1 (*LETMD1*), and Cyclin G2 (*CCNG2*). Little is known about *miR-519d* other than it may be associated with obesity.⁴⁶ NOTCH3 is one of four Notch

Table 5. Summary of *P* Values (Log-Rank Test) of Prediction of Clinical Recurrence on Training and Validation Sets

Dataset	Combined mRNA/ miRNA panel	Clinical information only
Training		
All cases (<i>n</i> = 56)	0.002	0.262
Gleason score 7 (<i>n</i> = 37)	0.004	0.136
Validation		
All cases (<i>n</i> = 35)	0.078	0.193
Gleason score 7 (<i>n</i> = 19)	0.023	0.080

Prediction of clinical recurrence for the entire dataset and the subset of Gleason score 7 cases was made using the combined mRNA/miRNA panel, adjusted for T-stage, PSA, and Gleason score, or using clinical information only. Significant *P* values are indicated in bold.

family receptors in humans, and Notch signaling has been shown to be important for prostate cancer cell growth, migration, and invasion,^{25,26} as well as normal prostate development.^{52,53} FBP1 is expressed in the prostate and is involved in gluconeogenesis.²² The identification of this metabolic enzyme as a biomarker of recurrence is initially surprising, but given the recent identification of isocitrate dehydrogenase 1 (IDH1) mutations in glioblastoma,⁵⁴ and the fact that FBP1 was overexpressed in independent microarray analyses of prostate cancers,^{7,10} the potential of FBP1 as a biomarker should not be underestimated. ETV1 is well established as one of the commonly recurrent translocations found in prostate cancers,^{9,15} and has been used in clinical models of recurrence following prostatectomy.⁵⁵ BID is a proapoptotic protein that binds to BCL2 and potentiates apoptotic responses on cleavage in response to tumor necrosis factor α (TNF α) and other death receptors.^{27,28} SIM2 was identified as a potential biomarker of prostate cancer in 2002²⁹ and later independently confirmed by Halvorsen et al³⁰ and Arredouani et al.³¹ SIM2 functions as a transcription factor that represses the proapoptotic gene *BNIP3*.⁵⁶ RAD23B plays a critical role in DNA damage recognition and nucleotide excision repair,²⁰ as well as inhibiting MDM2-mediated degradation of the p53 tumor suppressor.²¹ *LETMD1* (also known as *HCCR*) is an oncogene that is induced by Wnt⁴² and PI3K/AKT signaling,⁴³ inhibits p53 function,⁴⁴ and is a biomarker for hepatocellular⁵⁷ and breast⁴⁵ cancers. Cyclin G2 is an atypical cyclin that is induced by DNA damage³⁹ in a p53-independent manner, as well as by PI3K/AKT/FOXO signals,⁴⁰ and induces p53-dependent cell cycle arrest.⁴¹

The three genes in the predictive biomarker panel negatively associated with recurrence were *miR-647*, the TNF α receptor (*TNFRSF1A*), and annexin A1 (*ANXA1*). Although little is known about *miR-647*, *TNFRSF1A* (also known as *TNFR1*) mediates proapoptotic responses to TNF α ligand.^{23,24} Annexin A1 expression is reduced in early onset prostate cancer³³ and high-grade prostatic intraepithelial neoplasia.³⁴ *ANXA1* plays important roles in vesicle trafficking and reduced *ANXA1* promotes EMT and metastasis,³⁵ and up-regulates autocrine IL-6 signaling.³⁶ Thus, as a whole, this panel of biomarkers appears to reflect changes in DNA stability, PI3K signaling, p53 activity, apoptosis, and differentiation consistent with more aggressive disease.

Although this study goes beyond a pilot study, enhanced by selection of samples from multiple institutions, the number of specimens tested is still relatively small. Re-analysis of our data using only the Emory samples for the training set did not identify any significant probes, likely due to the substantially smaller sample size. Thus, although the performance of our panel of biomarkers is significant, even for Gleason score 7 patients, future studies beyond the scope of this work will be necessary to perform independent validation on much larger sample sets with greater statistical power. Moreover, it is now feasible to perform DASL assays on virtually the entire genome, in an assay that queries 24,526 transcripts derived from the RefSeq database. Future studies will test

combined mRNA and miRNA biomarker panels, and query the entire genome to determine whether other biomarker panels can achieve even greater success in prediction of biochemical and clinical recurrence of prostate cancer. Planned larger scale validation studies will determine whether these biomarkers are predictive for Gleason score 7 cases, and their utility at predicting clinical as well as biochemical recurrence.

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