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14. ABSTRACT The Prostate Cancer Pathology Resource Network (which has since been renamed the Prostate Cancer Biorepository Network or PCBN) is a collaboration between the Johns Hopkins School of Medicine (JHU) and the New York University School of Medicine (NYU). The PCBN has developed a biorepository with high quality, well annotated specimens that can be used by prostate cancer researchers. The specimens in the PCBN include prostatectomy tissues (frozen, paraffin embedded, and tissue microarrays (TMAs), serum, plasma, buffy coat, prostatic fluid, and derived specimens (DNA and RNA); these specimens are linked to clinical and outcome data and supported by an informatics infrastructure. The PCBN is currently made accessible to outside researchers through a website. The PCBN has been open to researchers since July 1 2011. Since that time it has been publicized at national and international meetings, surveyed prostate cancer researchers across the country for their tissue and biospecimen needs, finalized policies and procedures for access to tissue, finalized SOPs for most common processes, conducted biospecimen research on DNA and RNA best practices and presented these at international meetings, sponsored a successful workshop on biospecimen and biomarker issues for advanced prostate cancer, significantly increased the number of TMAs and specimens available, and received 35 requests for biospecimens from researchers in the US and abroad, of which 24 have been fulfilled and 11 more in process. Accrual of specimens from men with advanced or metastatic disease is an increasing focus of the PCBN.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4 - 8
Key Research Accomplishments.....	8 - 9
Reportable Outcomes.....	9
Conclusion.....	9 - 10
References.....	none
Appendices.....	11 – 32
Appendix 1: Workshop draft agenda	12 - 13
Appendix 2: Biospecimen science manuscript submitted	14 - 32

INTRODUCTION

The Prostate Cancer Biorepository Network (PCBN) is a collaboration between the Johns Hopkins School of Medicine (JHU), the New York University School of Medicine (NYU), and the Department of Defense (DOD).

The PCBN is organized with a **Coordinating Center** (JHU – led by Bruce Trock, Ph.D.), and **Network Sites** at NYU (led by Jonathan Melamed, M.D. and Peng Lee, M.D.) and JHU (led by Angelo De Marzo, M.D. and George Netto, M.D.). **The goal of the PCBN** is to develop a biorepository with high quality, well-annotated specimens obtained in a systematic, reproducible fashion using optimized and standardized protocols, and an infrastructure to facilitate the growth of the resource and its wide usage by the prostate cancer research community. The specimens in the PCBN include tissues from prostatectomies, serum, plasma, buffy coat, prostatic fluid, derived specimens such as DNA and RNA, linked to clinical and outcome data, and supported by an informatics infrastructure. A website has been established to make the PCBN accessible to the prostate cancer research community: <http://prostatebiorepository.org>.

BODY

During the first 2 years of funding of the PCBN the major focus was on infrastructure development and refinement, with development of governance policies, informatics (including the website), standard operating procedures (SOPs), specimen accrual, and marketing being the major focus areas. These have been described in previous Annual Reports. We briefly describe below how developments to date satisfy the 6 Performance Metrics described in the original Program Announcement. During the 3rd year of operation emphasis has been on biospecimen accrual, usage by the research community, and biospecimen science. Progress in these areas will be the main focus of this report.

Performance Metrics

The criteria for evaluating the Network comprised the following 6 Performance Metrics:

- The Network Coordinating Center must develop standard operating procedures for biospecimen collection methods and post-collection processing.
 - *These have been developed and are posted on the website; a manuscript describing the SOPs for DNA and RNA has been submitted.*
- The Network Coordinating Center must demonstrate sufficient data quality control and assurance through documentation that standard operating procedures are being followed for biospecimen annotation (e.g., patient history and demographic, clinical history, treatment, pathology, and outcome such as disease progression, recurrence, and prostate specific antigen (PSA) levels and/or other biochemical status).
 - *Appropriate annotation has been provided with all specimen requests, and we have demonstrated the ability to successfully link biospecimens to clinical/pathology data.*
- The Network Coordinating Center must demonstrate sufficient and ongoing efforts to harmonize the biorepository informatics system with the informatics systems of other national biorepositories, including caBIG.
 - *Although we initially demonstrated that we could map data elements to caTISSUE and can automatically export data to a caTISSUE-accessible format, uncertainties about the future of*

caBIG and the recommendations of one of our External Advisors convinced us not to pursue this pathway. Given that the initial 3 year funding of the Network was to support a pilot effort, we have used our existing informatics systems that use common data elements used by many other centers, and will make the development of a harmonized flexible informatics system a goal in our application for renewal of PCBN funding.

- Each Pathology Resource Network Site must contribute biospecimens from a minimum of 50 patients per year, with the expectation that biospecimen contribution will exceed the minimum requirement. Biospecimens from ethnic minority populations should match or exceed the existing ethnic minority patient population available to the Pathology Resource Network Site.
 - *Each year both sites have contributed over 500 newly accrued specimens, with specimens obtained as frozen tissue, formalin fixed paraffin embedded tissue, TMAs, body fluids (serum, plasma, buffy coat, prostatic fluid, seminal vesicle fluid), and derivatives (DNA, RNA). Minority patient representation at each institution is relatively low (<15% of prostate cancer patients), and is exceeded by representation in the PCBN; notably a TMA has been constructed comprised of specimens from African American men matched to Caucasian men.*
- Each Pathology Resource Network Site must submit quality data and reports in a timely manner as outlined by the Coordinating Center.
 - *Reports have been satisfactory.*
- Network Coordinating Center must demonstrate sufficient activity with the prostate cancer research community through ongoing documentation of Letters of Intent for utilization of biorepository specimens, to include the number of requests received, approved, or rejected, and the types of specimens distributed.
 - *Requests for specimens and letters of support continue to increase; usage will be described in detail below.*

Progress

1. Biospecimen Accrual:

One of the strengths of both the NYU and JHU sites is the large number and variety of biospecimens available prospectively due to large patient volumes. In particular, both teams have extensive experience building and sharing biospecimens in the form of TMAs. Other specimens include fixed tissue (radical prostatectomy, TURP, suprapubic prostatectomy), snap frozen tissue (radical prostatectomy, seminal vesicles), body fluids (serum, plasma, buffy coat, prostatic fluid; most can be matched to tumor and benign tissue), and derived specimens (DNA, RNA, protein).

It is notable that the NYU site contributed a substantial number of biospecimens despite hospital closures resulting from Superstorm Sandy; the 3 hospitals that constitute the NYU site were closed from 2-9 months following the hurricane.

The table below shows the total specimens newly accrued to the PCBN during the last 12 months:

Specimen Category	Last 12 months (JHU / NYU)
Total cases accrued	942 (716 / 226)
Frozen tissue cases	209 (121 / 88)
Seminal vesicle cases	710 (622 / 88)
Prostatic fluid cases	586 (585 / 1)
Seminal fluid cases	70 (0 / 70)
Metastatic cases	30 (19 / 11)
Rapid autopsy cases	4 (2 / 2)

Autopsy and Advanced Disease Specimens. NYU has been approved by the IRB to start a warm autopsy program at both NYU and VA hospitals. Dr. Melamed has arranged for the necessary permissions and personnel to perform autopsies on an on-call basis. Currently, 7 men with advanced metastatic disease have signed consent agreeing to rapid autopsy in the event of their death.

A similar program at JHU has now been re-activated under the aegis of the PCBN. Two rapid autopsies have been completed at JHU during this year, with successful harvest of soft tissue and bone metastatic sites. The rapid autopsy program is being revised with input from Dr. Kenneth Pienta, who recently joined JHU as Director of Research for the Brady Urological Institute, and who formerly directed a highly successful rapid autopsy program at University of Michigan. The previous rapid autopsy program at JHU (prior to the funding of the PCBN) had collected tissue from 33 autopsies. These specimens are being catalogued, with plans to develop TMAs for PCBN. In addition to the 2 rapid autopsies performed this year, metastatic lymph node tissue has been obtained from 19 prostatectomy cases.

We are still determining the status and availability of a large collection of serum samples at JHU (assembled and maintained by Dr. Mario Eisenberger) from men with biochemical recurrence or metastatic disease; serial samples are available for some men. Many of these can be matched to prostatectomy tissue. Most of these samples have not undergone any freeze-thaw cycles. There has been substantial delay because the samples were not accrued as part of a single IRB-approved process. Rather, they were from a number of clinical trials, each of which had their own IRB-approved protocol. All of those trials have ended and the protocols have been terminated. We are working with Dr. Eisenberger and the JHU IRB to determine the most appropriate way to transfer ownership of the samples to the PCBN, and whether this entails new human subjects issues. Furthermore, the samples are not annotated in a single unified database, but instead, in a number of small trial-specific databases. Some of these were developed by programmers no longer at JHU. We are trying to determine the extent of the collection and the programming effort that will be required to assemble a single

unified database. Development of such a database will be included in our renewal application to the DOD if the value of these specimens can be confirmed (i.e. data availability and quality, sample volumes and integrity).

New TMAs. 2 new TMAs have been constructed. One TMA used prostatectomy tissue and varied the age of the tissue block and storage conditions; this will be used to evaluate the impact of such pre-analytical variation on *in situ* methodologies. The other TMA was constructed with primary and metastatic tumor tissue as well as matched benign tissue from one recent Rapid Autopsy case.

Derived Specimens. In the past 12 months the following specimens have been extracted from frozen tissue at JHU:

- DNA (102 samples from 44 cases)
- RNA (60 samples from 28 cases)
- Protein (77 samples from 29 cases)

Almost all samples also have matched tumor and benign tissue available. These samples were extracted following a comprehensive quality control process to determine the optimal protocol.

2. Usage by the Research Community

Usage continues to increase, and we continue to receive queries and requests from researchers who have not previously contacted the PCBN. Usage is summarized below:

- 74 queries received and responded to
- 35 applications for tissue received from 32 individual investigators. Of these 35 requests:
 - 24 requests completed – samples shipped to investigators
 - 2 requests – sample shipment on hold per investigator request
 - 1 request preparing samples for shipment
 - 4 requests MTA in process
 - 1 request under review
 - 3 requests in pre-review

In addition to these requests for specimens, we provided letters of support to 9 investigators applying for DOD or NIH grants.

Importantly, one investigator has published a high impact paper in PNAS using a TMA provided by the PCBN; support from the PCBN was cited:

Hurley PJ, et al. Secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) is down regulated in aggressive prostate cancers and is prognostic for poor clinical outcome. Proc Natl Acad Sci 2012; 109:14977-82.

Marketing. We have continued to exhibit the PCBN at national meetings. In 2013 PCBN exhibit booths were included at the AACR meeting, American Urological Association meeting, and the ASCO Genitourinary Cancer Symposium. In addition, in the most recent program announcements about opportunities for prostate cancer research awards from the DOD PCRP, the PCBN was specifically highlighted and applicants were encouraged to request biospecimens from the PCBN.

In addition, a podium presentation on the PCBN was presented at the recent NCI-sponsored symposium “Federally Supported Cancer Biospecimen Resources Available to the Research Community” held during the AACR meeting in Washington DC in April 2013.

The PCBN sponsored a one day Workshop in conjunction with the ASCO Genitourinary Cancer Symposium in Orlando FL in February. The title was “Validating Tissue Biomarkers in Primary and Metastatic Prostate Cancer.” The workshop featured 9 internationally regarded speakers from academia and industry and was attended by 50 prostate cancer scientists from around the country. There was a great deal of productive discussion, including problems of sampling metastatic tissue, pros and cons of rapid autopsy programs, addressing tumor heterogeneity, and neuroendocrine tumors arising after targeted therapy. The workshop agenda is attached as Appendix 1. *The Workshop was a deliverable required in the original Program Announcement.*

3. Biospecimen Science

We have been actively conducting studies to test standard operating procedure developed for derivative extraction from frozen tissue. These studies have demonstrated that DNA and RNA quality, and 7 RNA and protein biomarkers were comparable for derivatives from standard open prostatectomy compared to robotic assisted laparoscopic prostatectomy. A manuscript describing the comparison of derivatives from open vs. robotic assisted laparoscopic prostatectomy has been submitted, and is included as Appendix 2. The title of the manuscript is “Biobanking of derivatives from radical retropubic and robot–assisted laparoscopic prostatectomy tissues as part of the Prostate Cancer Biorepository Network (PCBN).” This research was also presented as a poster at the Society of Basic Urological Research conference in November 2012 in Miami Beach, FL.

Addressing changes in key personnel

Dr. De Marzo returned to Johns Hopkins in a full-time capacity in June 2013. He will continue as Co-Principal Investigator for the PCBN. Sadly, Patricia Kolmer, R.N. passed away in May after a long illness. Her responsibilities have been subsumed by Helen Fedor and Medha Darshan, and will be taken over by a Clinical Coordinator (TBN) during the 1 year No Cost Extension period.

KEY RESEARCH ACCOMPLISHMENTS

During the first two years of the PCBN most accomplishments related to infrastructure development. With the prostate cancer biorepository now being fully operational we cite the following milestones:

- a. Continued prospective collection of high quality biospecimens, including tissues now totaling nearly 3000 prostate cancer cases and nearly 700 frozen tissue cases since inception.
- b. Poster presentation and manuscript submission describing biospecimen science on optimization of DNA and RNA quality performed by PCBN scientists.
- c. PCBN support cited in a manuscript published in a high impact journal (PNAS).
- d. Conduct of a workshop addressing biospecimen and biomarker issues for advanced prostate cancer.

REPORTABLE OUTCOMES

Marketing by PCBN booth exhibitions at AACR, AUA, and GU ASCO.

Establishment of Rapid Autopsy programs at both NYU and JHU, with 4 autopsies performed during this reporting period.

Submission of manuscript describing DNA and RNA optimization procedures and lack of influence of surgical technique.

Successful conduct of Workshop as required by the Program Announcement

CONCLUSIONS

Our previous Annual Report documented 5 goals for the 3rd year of operation: (1) Our primary goal is to increase usage, (2) In concert with this goal we intend to increase accrual of high demand samples, i.e. metastatic and high risk disease tissues, derivatives, and specimens from African Americans, (3) A major goal is to conduct the Workshop, (4) Conduct biospecimen science studies to evaluate how variation in fixation parameters and ischemia affect a range of commonly used biomarkers, using our newly constructed Fixation and Ischemia TMAs, (5) Write a manuscript describing the development of the PCBN and lessons learned.

The first 3 goals have clearly been achieved. Biospecimen science studies for Goal #4 are nearly completed and results will be available soon. However, as described above, other biospecimen science efforts were also completed, reported at a national meeting, and submitted for publication. The manuscript, while not entirely addressing goal 5 still presents some of the development of the PCBN and lessons learned, which will be more thoroughly discussed in a manuscript currently in development.

Current status of biorepository

The biorepository continues to function as normal and continues to receive requests. New TMAs are being designed (e.g. a TMA of men with intermediate and high risk disease treated by surgery and followed for development of metastasis and castration resistance). At Johns Hopkins we have re-activated our rapid autopsy program using funds from the current budget because our External Advisory Board has recommended increased focus on obtaining samples of metastatic tumor tissue.

Currently there is a projected balance of unspent funds at the time the grant is scheduled to terminate in June 2013. The main reason for the unspent funds is personnel changes. Several personnel left the university and either were not replaced or replaced at a lower level of effort. In addition, one member of the team had missed considerable time due to a serious illness and recently passed away. Because there has been considerable uncertainty over the last 2 years about continued funding of the PCRP within the Department of Defense we have attempted to be frugal and have taken advantage of institutional resources, anticipating that we might have a balance and would require a no cost extension. Because we were charged in the original program announcement with finding ways to become self-sufficient in the event that DOD funding could not continue, we thought that having funds to carry us through a period of no cost extension would provide time for us to seek other sources of funds, such as through other grant mechanisms (which have a considerable lag time between application and award) and philanthropic sources.

Plans for No Cost Extension period

We plan to continue operation during this period, with increased focus on rapid autopsy and bone marrow biopsy for accrual of tissues and blood samples from men with metastatic and advanced disease. This is a very labor-intensive effort that was not emphasized in the original Program Announcement, but which has become evident as a major unmet need in the research community. Additional TMAs are planned for construction, such as (1) a “heterogeneity TMA” which will sample multiple tumor nodules from each patient to allow evaluation of heterogeneity in biomarker expression and prognostic impact; (2) patients who span the range of Gleason scores (with adequate numbers in each Gleason category) with long-term follow-up; (3) expansion of our race disparity TMA to include specimens from other institutions with different source populations; (4) primary tumor specimens from men who developed metastatic disease with different treatment response.

Furthermore, we will submit a renewal application to continue and expand the current biorepository in response to the PCRP FY13 Program Announcement. We have agreement from two other institutions to participate as Network Sites, and planning for that application is already underway. Because an award would be unlikely to be made before September 2014, we would use the unspent funds to continue operation while awaiting a funding decision on our application.

Finally, PCBN has been selected as a participating site in the Movember Global Action Plan (GAP) Unique TMA project, to construct high quality TMAs from high demand specimens collected from multiple institutions in multiple countries. We have proposed to be the central TMA construction facility.

APPENDICES

	<u>Pages</u>
Appendix 1: Agenda for PCBN Workshop	12 - 13
Appendix 2: Biospecimen science manuscript submitted	14 - 32

Validating Tissue Biomarkers in Primary and Metastatic Prostate Cancer

Prostate Cancer Biorepository Network 
Johns Hopkins School of Medicine and New York University School of Medicine

February 13, 2013
Rosen Shingle Creek Hotel
Suwannee 16 Room
Orlando, Florida

AGENDA

- 11:00-11:15 Introduction to goals and focus of workshop
Bruce Trock (Johns Hopkins School of Medicine)
- 11:15-11:35 Translating whole genome sequencing data into clinical biomarkers
Himisha Beltran (Weill Medical College, Cornell University)
- 11:35-11:45 Discussion
- 11:45-12:05 Impact of pre-analytical variables on protein biomarkers
Veronique Neumeister (Yale University School of Medicine)
- 12:05-12:15 Discussion
- 12:15-1:15 LUNCH (provided)
- 1:15-1:35 Optimizing derivatives from limited biopsy material
Phillip Febbo (University of California San Francisco School of Medicine)
- 1:35-1:45 Discussion
- 1:45-2:05 Phenotypic heterogeneity of prostate cancer metastases
Robert Vessella (University of Washington School of Medicine)
- 2:05-2:15 Discussion
- 2:15-2:35 Intra-tumoral subclonal heterogeneity
Elaine Mardis (Washington University School of Medicine)
- 2:35-2:45 Discussion
- 2:45-3:05 Study design for biomarkers of targeted therapy
Stephen Hewitt (National Cancer Institute)

3:05-3:15	Discussion
3:15-3:30	BREAK
3:30-3:50	High throughput <i>in situ</i> hybridization analysis Yuling Luo (Advanced Cell Diagnostics, Inc.)
3:50-4:00	Discussion
4:00-4:20	Sample acquisition in the metastatic setting Kenneth Pienta (University of Michigan School of Medicine)
4:20-4:30	Discussion
4:30-5:00	Summary and discussion of potential collaborative efforts for metastatic disease Angelo De Marzo (Johns Hopkins, and Predictive Biosciences, Inc.)

Biobanking of Derivatives from Radical Retropubic and Robot-Assisted Laparoscopic Prostatectomy Tissues as Part of the Prostate Cancer Biorepository Network

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Running title: Biobanking of DNA and RNA Derivatives from RRP and RALP

Abstract

Background: The goal of the Prostate Cancer Biorepository Network (PCBN) is to develop a biorepository with high-quality, well-annotated specimens obtained in a systematic, reproducible fashion using optimized and

standardized protocols, and an infrastructure to facilitate the growth of the resource and its wide usage by the prostate cancer research community. An emerging area of concern in the field of prostate cancer biobanking is an apparent shift in the proportion of surgical procedures performed for prostate cancer treatment from radical retropubic prostatectomy (RRP) to robot-assisted laparoscopic prostatectomy (RALP). Our study aimed to determine the potential impact of the RALP procedure on the detection of known prostate cancer biomarkers, and the subsequent suitability of RALP-derived specimens for prostate cancer biomarker studies.

Methods: DNA and RNA were extracted from RRP and RALP specimens. Quality assessment was conducted using spectrophotometric analysis and RNA was analyzed for RNA integrity number (RIN) and by real-time reverse-transcription PCR (qRT-PCR) for racemase, hepsin, ERG, TMPRSS2-ERG gene fusions and the microRNAs *miR-26a*, *miR-26b*, *miR-141* and *miR-221*.

Results: We demonstrate that extraction of derivatives from frozen tissues from RRP and RALP specimens yields samples of equally high quality as assessed by spectrophotometric and RIN analysis. Likewise, expression levels of genes analyzed by qRT-PCR did not differ between RRP and RALP-derived tissues.

Conclusions: Our studies analyzing the differential expression of tumor-associated biomarkers in RRP versus RALP-derived specimens indicate that samples obtained from RALP specimens are suitable for prostate cancer biomarker studies – an important finding given the current shift in surgical procedures for prostate cancer treatment.

Keywords: Biobanking, radical prostatectomy, DNA, RNA, biomarker, biorepository, prostate cancer

Introduction

The importance of biorepositories and biospecimen science has gained a great deal of recent attention (1), including in prostate cancer biobanking (2-6). This is particularly important in prostate cancer research as a result the marked heterogeneity in clinical behavior of prostate cancer, which ranges from a relatively indolent localized cancer to a widely metastatic lethal form. Emphasis has been placed on the development of standard procedures for the entire biospecimen lifecycle: ranging from institutional review board reviews and the patient consent process to sample collection, processing and storage, to standardization of sample distribution.

The Prostate Cancer Biorepository Network (PCBN) is a collaboration between the Johns Hopkins University School of Medicine (JHU) and the New York University School of Medicine (NYU), and its infrastructural support is funded by the Department of Defense (DOD) Congressionally Directed Medical Research Program. The goal of the PCBN is to develop a biorepository with high-quality, well-annotated specimens obtained in a systematic, reproducible fashion using optimized and standardized protocols, and an infrastructure to facilitate the growth of the resource and its wide usage by the prostate cancer research community. The PCBN is a public bioresource that provides tissue and other biospecimens to all prostate cancer investigators through an application process (<http://www.prostatebiorepository.org/>). One specific focus of the PCBN is to characterize critical parameters in the biospecimen “life cycle” that influence the molecular integrity of research tissues and downstream derivatives of these tissues. As part of our focus on developing standard operating procedure (SOP) protocols for DNA and RNA extraction from prostatectomy tissues, we aimed to scrutinize the potential use of robot-assisted laparoscopic prostatectomy (RALP) specimens for prostate cancer biomarker studies. An emerging area of concern in the field of prostate cancer tissue and derivative biobanking is an apparent shift in the proportion of surgical procedures performed for prostate cancer treatment from radical retropubic prostatectomy (RRP) to RALP (7-9). Hesitation towards the use of RALP-derived biospecimens in prostate cancer research studies is driven by the fact that RALP specimens undergo a longer period of intra-operative warm ischemia time. This can be at least double to triple the period of warm ischemia time that RRP specimens undergo (6, 10). Previous studies have demonstrated that the quality and integrity of DNA and RNA in prostatectomy specimens are not compromised during RALP (6, 10, 11). In immunohistochemistry (IHC) studies, Best *et al.* (10) reported no alterations in tissue architecture or expression of p63, E-cadherin, and the cytokeratin stain AE1/AE3 between RRP and RALP-derived tissues. In contrast, Ricciardelli *et al.* (6) reported significant increases in protein expression by IHC for kallikrein 3 (KLK3, PSA), kallikrein 4 (KLK4), cytokeratins 8/18 (CK8/18), and cytokeratins 5/14 (CK5/14) in RALP tissues and a significant reduction in kallikrein 2 (KLK2) and kallikrein 14 (KLK14) protein expression by IHC in RALP compared to RRP tissues. No differences were observed in IHC for androgen receptor (AR), chondroitin 0-sulfate, ghrelin, Ki67, proteinase activator receptor (PAR2), proliferating cell nuclear antigen (PCNA), p63, vascular endothelial growth factor (VEGF-C), versican and Y-box binding protein (YB1) in RRP versus RALP-derived tissues. In quantitative PCR (qPCR) experiments with RNA derived from prostate cancer samples from RRP versus RALP tissues, Ricciardelli *et al.* (6) observed no significant difference in the expression of AR, PSA, KLK2, KLK4, or hypoxia-induced factor (HIF1A).

Unlike previous studies, in the present study, we specifically assessed prostate cancer biomarkers that are known to be differentially expressed in tumor versus benign tissues (racemase, hepsin, ERG, and the microRNAs *miR-26a*, *miR-26b*, *miR-141* and *miR-221*) (12-17), as well as the presence of TMPRSS2-ERG gene fusions (18), to determine the suitability of RALP-derived biospecimens for prostate cancer biomarker research. To our knowledge, this is also the first study to analyze changes in microRNA levels in relation to surgical procedure.

Materials and Methods

Tissue Collection. This study was conducted under a protocol approved by The Johns Hopkins University School of Medicine institutional review board (IRB). All prostate samples described in this study were derived from patients treated at the Johns Hopkins Hospital in Baltimore, MD. Prostatectomy specimens acquired by the harvesting team were immediately transported from the surgical suite to the surgical pathology department without delay and for immediate preparation. The tissue harvesting process from prostate removal to snap freezing of tissue is typically performed in less than 60 minutes time. All specimens included in the studies herein were collected between 2003 and 2011.

Specimen Preparation. All of the prostatectomy specimens are handled in a manner that does not compromise clinical evaluation of histopathological data necessary for clinical management, by following the basic principles outlined recently by Samaratunga *et al.* (19). A detailed description of harvesting of tumor and benign frozen tissues from prostatectomy specimens can be found in the Supplemental Methods. The remaining prostate tissue is formalin-fixed and processed into quadrants in a manner that preserves the 3 dimensional orientation of all tissues (11) for further processing and placement into individual cassettes for histopathological diagnosis.

Histological Characterization and Sample Selection. A detailed description of histological characterization and sample selection can be found in the Supplemental Methods. Frozen sections from matched tumor and benign pairs were obtained from 10 RRP and 10 RALP specimens. Specimens comprised Gleason scores 6, 7, 8, and 9 and pathological (P) stages from T2N0MX to T3BN0MX (Table 1).

DNA and RNA Extraction and Quantification. DNA was isolated from prostate tissue samples using the DNeasy Blood & Tissue kit (Qiagen). DNA quantification and 260:280 ratios were obtained by Nanodrop (Thermo Fisher Scientific Inc.). RNA was isolated from tissue sections using Trizol (Invitrogen). RNA quantification and 260:280 ratios were obtained by Nanodrop and RNA integrity number (RIN) was assessed by 2100 bioanalyzer (Agilent Technologies). Additional information regarding PCBN SOPs for DNA and RNA extraction can be found at <http://www.prostatebiorepository.org/protocols>.

Quantitative Real-time PCR. RNA was treated with DNase I (RNase -free) (Ambion) followed by cDNA synthesis using the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamers. qRT-PCR was carried out using iQ SYBR Green Supermix (Biorad) for 18S, GAPDH, β -globin, hepsin, racemase and ERG. To determine the relative amount of TMPRSS-ERG rearrangement, a primer/probe mix (IDT) specific to the fusion was used to carry out qRT-PCR using TaqMan Universal PCR Mix (Applied Biosystems). PCR primers (Invitrogen) and TMPRSS-ERG primer/probe (IDT) sequences are shown in Supplementary Table S1. The fold differences in expression levels of hepsin, racemase, and ERG in tumor samples were determined using the $\Delta\Delta C_t$ method, relative to GAPDH and to the matched benign tissue. *miR-221*, *miR-141* as well as mature *miR-26a* and *miR-26b*, levels were measured by Taqman assay (Applied Biosystems) according to manufacturer's instructions, and normalized to U6 expression.

Statistical Analysis. Differences between RRP and RALP specimens were evaluated by two-tailed, unpaired t test or Wilcoxon rank sum test for continuous variables, and chi-square test for categorical variables.

Results

Sample quality assessment. Each DNA or RNA sample extracted for the PCBN undergoes a series of quality control (QC) assays before inclusion in the biorepository. This includes DNA/RNA quantification and assessment of 260:280 ratios using the Nanodrop as well as a series of real-time PCR assays for housekeeping genes (18S and β -globin for DNA samples, 18S and GAPDH for RNA samples). We have not observed any difference in the sample quantity, 260:280 ratio, or Ct values for housekeeping genes in real-time PCR assays for DNA or RNA samples collected from RRP versus RALP (data not shown).

Fig. 1A shows a representative example of DNA samples contained in the PCBN repository. Gel electrophoresis of DNA samples shows one strong, high molecular weight band and no evidence of DNA degradation. RIN analysis of 103 RNA samples contained within the biorepository indicated a mean RIN of 7.7 $\pm$ 1.8 and a median RIN of 8.3; however the majority of samples have a RIN $>$ 8 (Fig. 1B). We found no correlation between low RIN values and the age of the sample (Supplementary Fig. S1).

We next analyzed the RIN distribution for samples collected from RRP versus RALP. As shown in Fig. 2, the median RIN did not differ significantly for RRP vs. RALP specimens, at 8.3 (interquartile range 7.3-8.8) and 8.5 (interquartile range 7.7-8.6) respectively, $p=0.923$ (Wilcoxon rank sum test).

Comparative biomarker expression between RRP and RALP by real-time PCR. The clinical and pathologic characteristics of the RRP and RALP cohorts used for comparative biomarker expression by real-time PCR are given in Table 1. Patient's samples were varied by Gleason score (ranging from 6-9), pathological stage, and patient age (49-70 years). Patient age was significantly higher in the RALP cohort, which is reflective of the skewed proportion of RALP surgeries being performed in older men (Table 1). Patient samples were also intentionally varied for sample age (with samples collected between 2006 and 2011) to prevent any bias in experimental results due to age of the specimen. As such, there was no significant difference in sample age between RRP and RALP specimens (Table 1). The proportion of Gleason score 7-10 patients was the same in RRP and RALP patients (80% vs. 80%), $p=1.0$, but RRP patients exhibited a higher proportion with T3 stage (70% vs. 50%), although the difference was not statistically significant, $p=0.650$, reflecting the small sample size.

Analysis of racemase and hepsin expression levels in tumor relative to matched benign tissues (fold increase) showed a similar distribution for samples derived from RRP and RALP (Fig. 3). As expected, racemase and hepsin expression levels were higher in tumor samples versus benign. There was no significant difference in the fold increase in racemase or hepsin expression levels in RRP versus RALP specimens ($p = 0.899$ and $p = 0.849$, respectively, Fig. 3).

The proto-oncogene ERG (ETS related gene) is known to be over-expressed in a subset of prostate cancers due to the presence of TMPRSS2-ERG gene fusions (18). As shown in Fig. 4, increased ERG expression was

observed in tumor samples relative to matched benign samples in a subset of the samples collected from both RRP and RALP. Overall, there was no significant difference in ERG expression between samples collected from RRP versus RALP ($p = 0.845$, Fig. 4). Importantly, ERG over-expression was exclusively limited to tumor samples in which TMPRSS2-ERG gene fusions were also detected (Fig. 4).

Finally, we assessed expression of 4 different microRNAs, *miR-26a*, *miR-26b*, *miR-141*, and *miR-221* in RRP versus RALP specimens. Each of these microRNAs has been previously reported to show differential expression in prostate cancer versus benign (15-17, 20-22). It should be noted that high circulating levels of *miR-141* have also been previously demonstrated as a serum biomarker of prostate cancer, and specifically for patients with advanced disease (23-28). In the present study, we assayed *miR-141* levels in prostate cancer at the tissue level.

As shown in Fig. 5, both *miR-26a* and *miR-221* were found to be consistently downregulated in tumor tissues compared to matched benign in both RRP and RALP-derived samples. There was no significant difference in expression levels of *miR-26a* and *miR-221* between RRP and RALP ($p = 0.957$ and $p = 0.135$, respectively, Fig. 5). There were also apparent trends towards downregulation of *miR-26b* and upregulation of *miR-141* in tumor samples relative to matched benign in both RRP and RALP-derived samples and there was no significant difference in expression levels of *miR-26b* and *miR-141* in RRP versus RALP ($p = 0.057$ and $p = 0.305$, respectively, Fig. 5). However, half of the RALP samples showed greater *miR-26b* expression in tumor relative to benign, and 4 of ten of the RRP specimens showed downregulation of *miR-141* in tumor relative to benign. We questioned whether the degree of downregulation or upregulation of the microRNAs was associated with tumor grade for any of the microRNAs analyzed. Interestingly, for RALP samples, *miR-141* was significantly upregulated in higher grade tumors (Gleason 7-9) versus low grade tumors (Gleason 6) ($p = 0.010$, Supplementary Fig. S2). The same trend was observed for RRP samples, however this did not reach statistical significance ($p = 0.506$). There was no correlation between *miR-26a*, *miR-26b*, or *miR-221* expression and tumor grade (Supplementary Fig. S2).

Discussion

Herein we describe the establishment of SOP protocols for DNA and RNA extraction from frozen prostate tissues as part of the PCBN biorepository. We demonstrate that samples extracted using these protocols are of high quality as assessed by a number of QC measures including quantification and determination of 260:280 ratio by Nanodrop, RIN analysis, and a series of quantitative real-time PCR assays for standard housekeeping genes.

Hesitation towards the use of RALP-derived biospecimens in prostate cancer research studies is centered on the fact that RALP specimens undergo a longer period of intra-operative warm ischemia time. Interestingly, previous gene expression microarray studies on prostatectomy specimens exposed to longer periods of warm ischemia have demonstrated little variability in global gene expression patterns (29, 30). As part of the growing trend towards RALP surgeries as opposed to RRP surgeries at our own institution and others, we aimed to

assess the impact of the RALP procedure on known biomarkers of human prostate cancer, namely racemase, hepsin, ERG, TMPRSS2-ERG fusions, and prostate-cancer associated microRNAs. Our results demonstrate that RALP specimens performed equally to RRP specimens in terms of comparable RIN values for RNA samples extracted from each sample type (Fig. 2), similar gene expression levels in tumor versus benign for racemase and hepsin (Fig. 3), correlation between ERG overexpression and the presence of TMPRSS2-ERG gene fusions (Fig. 4), and consistent downregulation of the microRNAs *miR-26a* and *miR-221* (Fig. 5).

Of note, for our microRNA studies, whereas the overall trend for downregulation of *miR-26a*, *miR-26b*, and *miR-221* and upregulation of *miR-141* was apparent in all RRP and RALP specimens, half of the RALP showed greater *miR-26b* expression in tumor relative to benign, and 4 of ten of the RRP specimens showed downregulation of *miR-141* in tumor relative to benign (Fig. 5). It is possible that since we observed upregulation of *miR-26b* in a fraction of the RALP specimens, that expression of this microRNA may be induced by warm ischemia. Alternatively, this finding for *miR-26b* may be consistent with a previously published study where a trend for downregulation of *miR-26b* was found in 18 matched benign and primary prostate cancer specimens, however without reaching statistical significance (15). Therefore, downregulation of *miR-26b* may not occur in every primary prostate cancer and might vary from patient to patient. In the same study, *miR-26a* was found to be significantly and consistently downregulated in most tumor specimens versus matched benign tissues (15), which is consistent with the findings in both RRP and RALP specimens in the present study.

As previously mentioned, increased levels of *miR-141* have been consistently demonstrated in the serum of prostate cancer patients, and particularly in patients with advanced disease. This association between increased levels of *miR-141* and advanced disease has also been found at the tissue level. For example, *miR-141* was found to be upregulated both in primary tumors from radical prostatectomies as compared to benign prostatic hyperplasia (BPH) from prostatectomy and in castration-resistant prostate cancer (CRPC) from transurethral resection of the prostate (TURP) specimens as compared to BPH from TURP specimens (17). Another study reported that intra-tumoral expression of *miR-141* is predictive of a reduced relapse-free interval (21). In the present study, where we aimed to assess *miR-141* at the tissue level, and found that *miR-141* is overexpressed in tumor versus benign tissues in a subset of prostate cancer patients (Fig. 5), and that overexpression of *miR-141* was significantly correlated with higher Gleason score in RALP specimens and showed a similar trend in RRP specimens (Supplementary Fig. S2). This finding indicates that the increased serum levels of *miR-141* in advanced prostate cancer patients may also be apparent at the tissue level in the primary tumor.

In all, our study demonstrates the potential applicability of RALP-derived samples in prostate cancer biomarker studies – an important assessment given the apparent shift in surgical procedure for prostate cancer treatment from RRP to RALP.

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Figure Legends

Figure 1. Assessment of nucleic acid quality as part of SOP development. (A) Agarose gel of DNA samples extracted from frozen prostate tissue specimens (B) RIN of RNA samples extracted from frozen prostate tissues (Agilent Bioanalyzer). Mean RIN = 7.7 +/- 1.8, median RIN = 8.3.

Figure 2. Distribution of RIN numbers obtained for RNA extracted from radical retropubic prostatectomy (RRP, n=83) versus robot-assisted laparoscopic prostatectomy (RALP, n=20) specimens. Median RIN for RRP vs. RALP specimens was 8.3 (interquartile range 7.3-8.8) and 8.5 (interquartile range 7.7-8.6) respectively, p=0.923 (Wilcoxon rank sum test).

Figure 3. Fold difference in racemase and hepsin expression in tumor as compared to benign among tumor/benign RNA pairs from RRP and RALP.

Figure 4. Fold difference in ERG expression in tumor samples as compared to benign among RNA pairs from RRP and RALP. Samples shown in grey represent cases where the tumor sample was found to be positive for TMPRSS-ERG gene fusions via qPCR.

Figure 5. Fold difference in *miR-26a*, *miR-26b*, *miR-141*, and *miR-221* expression in tumor samples relative to benign among RNA pairs from RRP and RALP as assessed by TaqMan® Small RNA Assay.

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Table 1. Clinical and pathologic parameters of patient samples.

10 RRP Cases			
Age of Specimen (weeks)	Gleason Score; Tertiary Gleason Grade	Pathologic Stage	Patient Age
382	3+3	T2N0MX	55
194	4+5; 3	T3AN0MX	49
191	4+5	T3AN1MX	57
170	3+4	T2N0MX	53
170	4+4	T3AN0MX	59
162	4+5	T3AN0MX	55
143	4+3	T3AN0MX	52
124	4+5; 3	T3BN0MX	64
115	4+4	T3AN0MX	60
105	3+3	T2N0MX	53
10 RALP Cases			
370	3+3	T2N0MX	69
191	3+4	T2N0MX	57
190	5+4; 3	T3AN0MX	70
185	3+4	T2N0MX	54
179	4+3	T3BN0MX	67
174	3+4	T2N0MX	62
133	4+5	T3AN0MX	62
128	3+4	T2N0MX	66
115	3+3	T3ANXMX	59
106	3+3	T3AN0MX	60
	RRP	RALP	P value
Mean age of patient	55.7	62.6	0.005
Mean age of specimen	176 weeks	177 weeks	0.966

Supplementary Table S1. qRT-PCR primers

18S	Forward: 5'-GATGGTAGTCGCCGTGCC-3' Reverse: 5'-GCCTGCTGCCTTCCTTGG-3'
GAPDH	Forward: 5'-CGCTCTCTGCTCCTCCTGTT-3' Reverse: 5'-CCATGGTGTCTGAGCGATGT-3'
β-globin	Forward: 5'-GTGCACCTGACTCCTGAGGAGA-3' Reverse: 5'-CCTTGATACCAACCTGCCCAG-3'
Hepsin	Forward : 5'-AGAGGAGAACAGCAACGATATTGC-3' Reverse: 5'-CCCGTCACGGTACAGATCTTG-3'
Racemase	Forward: 5'-AATGTAGAAAATGAGGAAATGCC-3' Reverse: 5'-AGTTTGGAATGTGCTTAGAGGG-3'
ERG	Forward: 5'-CGCAGAGTTATCGTGCCAGCAGAT-3' Reverse; 5'-CCATATTCTTTCACCGCCCACTCC-3'
TMPRSS-ERG	Forward:5'-CAGGAGGCGGAGGCGGA-3' Reverse: 5'-GGGCTCATCTTGGAAGTCTG-3' Probe: FAM-CGGGGAGCGCCGCCTGGAGCG-BHQ1

Figure 1

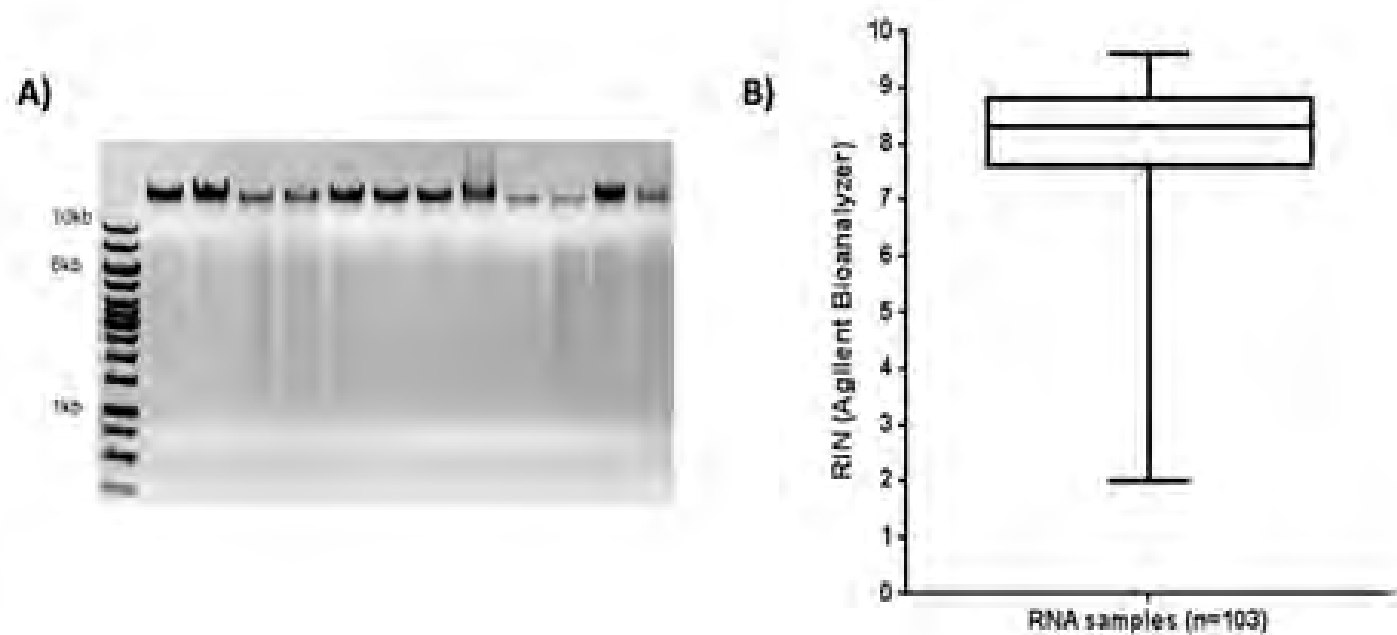
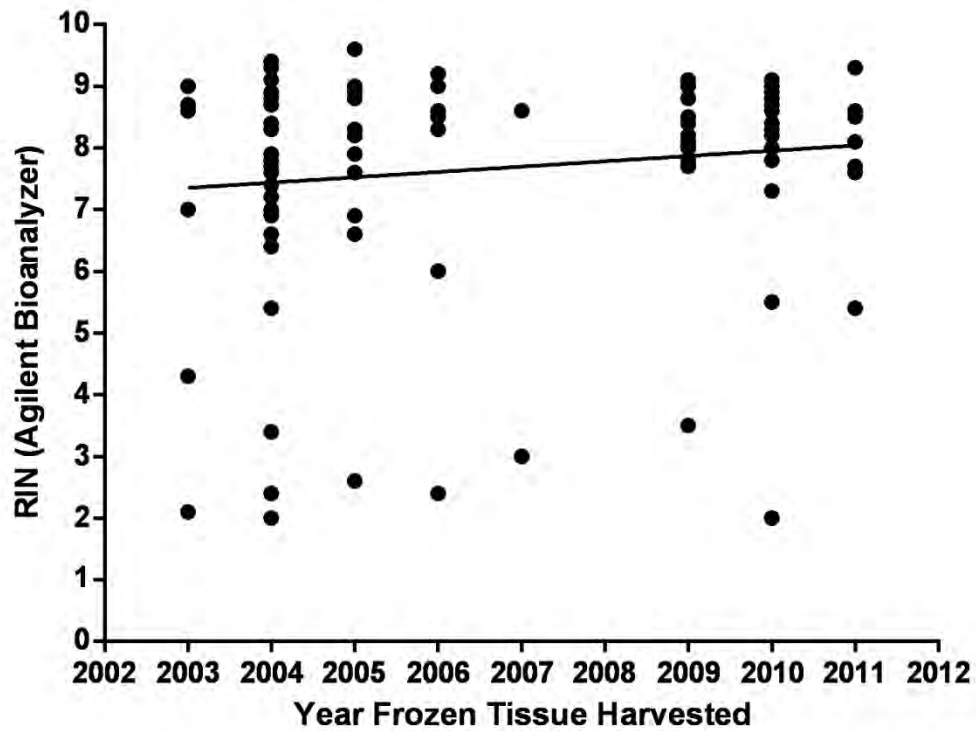


Figure 1. Assessment of nucleic acid quality as part of SOP development. (A) Agarose gel of DNA samples extracted from frozen prostate tissue specimens (B) RIN of RNA samples extracted from frozen prostate tissues (Agilent Bioanalyzer). Mean RIN = 7.7 $\pm$ 1.8, median RIN = 8.3.

Supplementary Figure S1



Supplementary Figure S1. Assessment of RIN value as a factor of year of tissue collection.

Figure 2

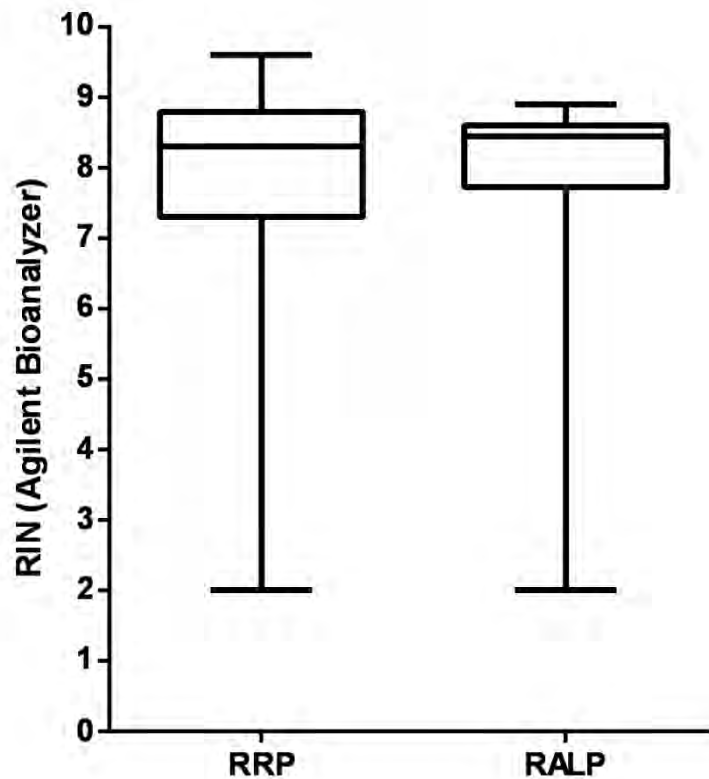


Figure 2. Distribution of RIN numbers obtained for RNA extracted from radical retropubic prostatectomy (RRP, n=83) versus robot-assisted laparoscopic prostatectomy (RALP, n=20) specimens. Median RIN for RRP vs. RALP specimens was 8.3 (interquartile range 7.3-8.8) and 8.5 (interquartile range 7.7-8.6) respectively, $p=0.923$ (Wilcoxon rank sum test).

Figure 3

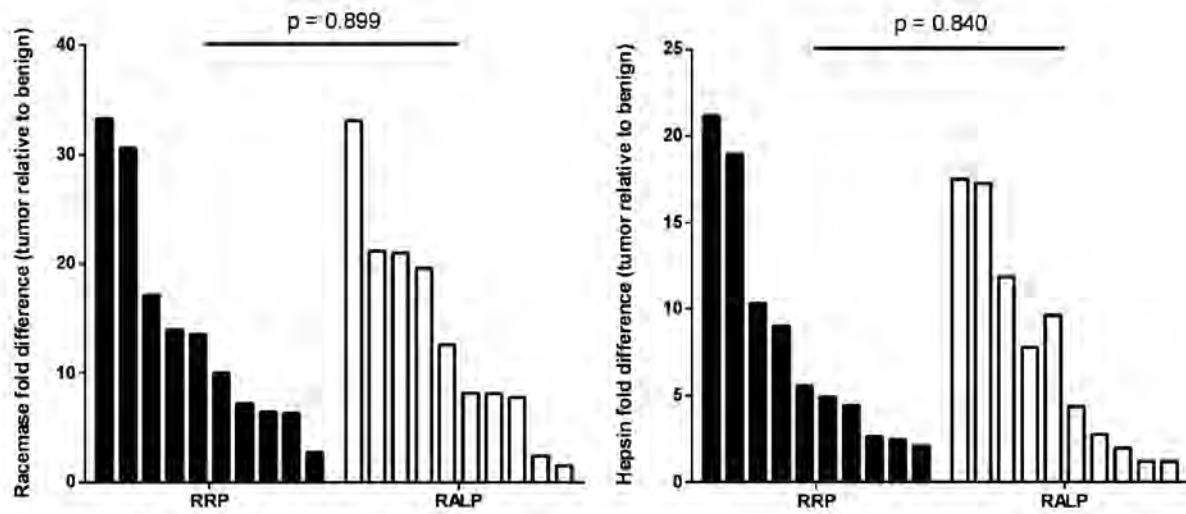


Figure 3. Fold difference in racemase and hepsin expression in tumor as compared to benign among tumor/benign RNA pairs from RRP and RALP.

Figure 4

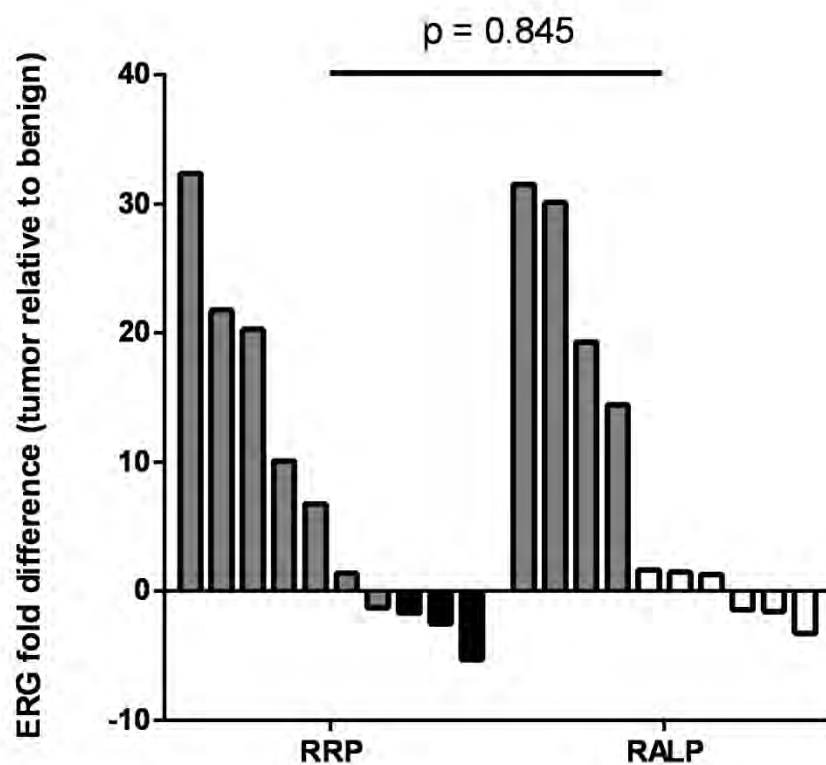


Figure 4. Fold difference in ERG expression in tumor samples as compared to benign among RNA pairs from RRP and RALP. Samples shown in grey represent cases where the tumor sample was found to be positive for TMPRSS-ERG gene fusions via qPCR.

Figure 5

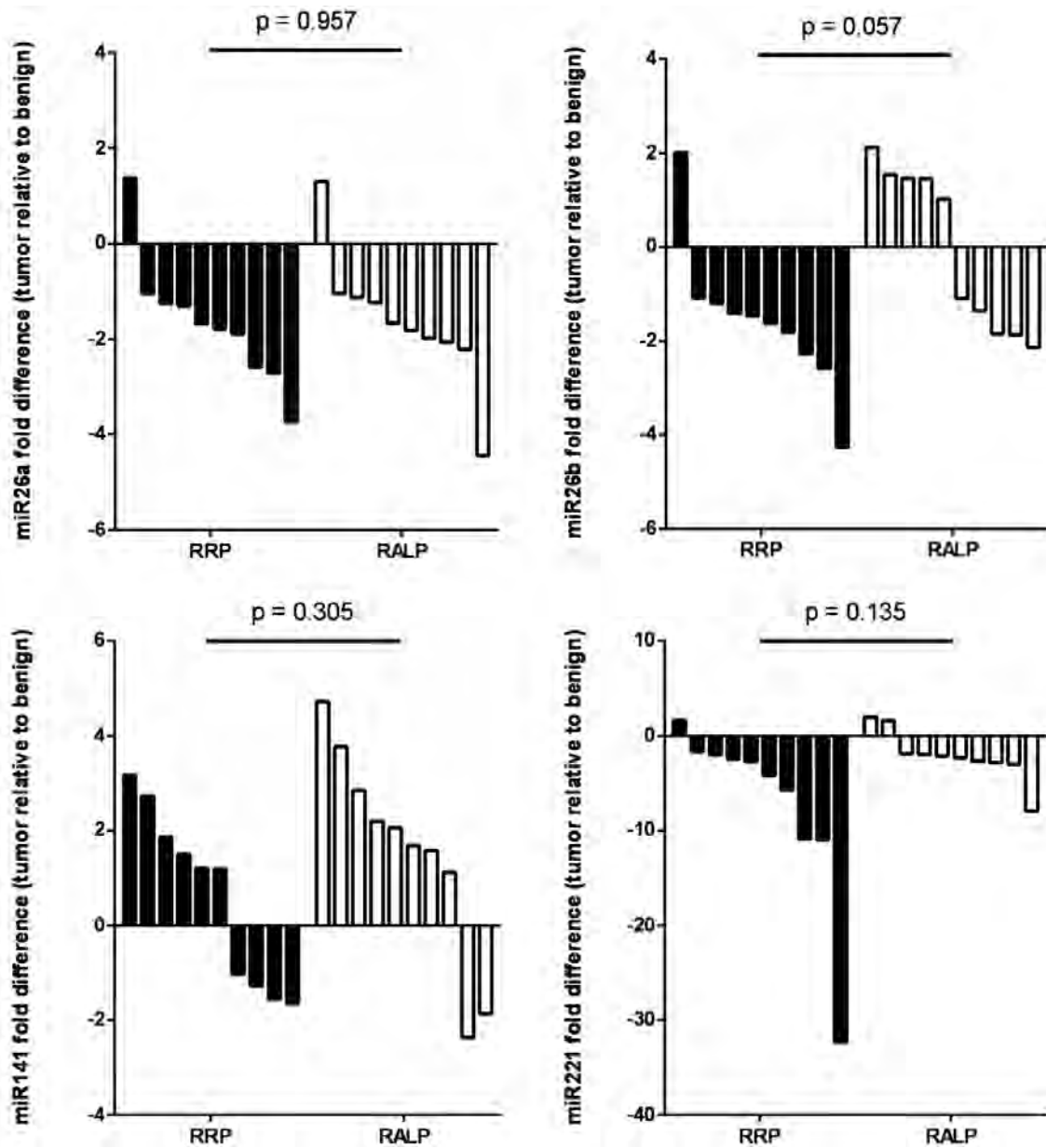
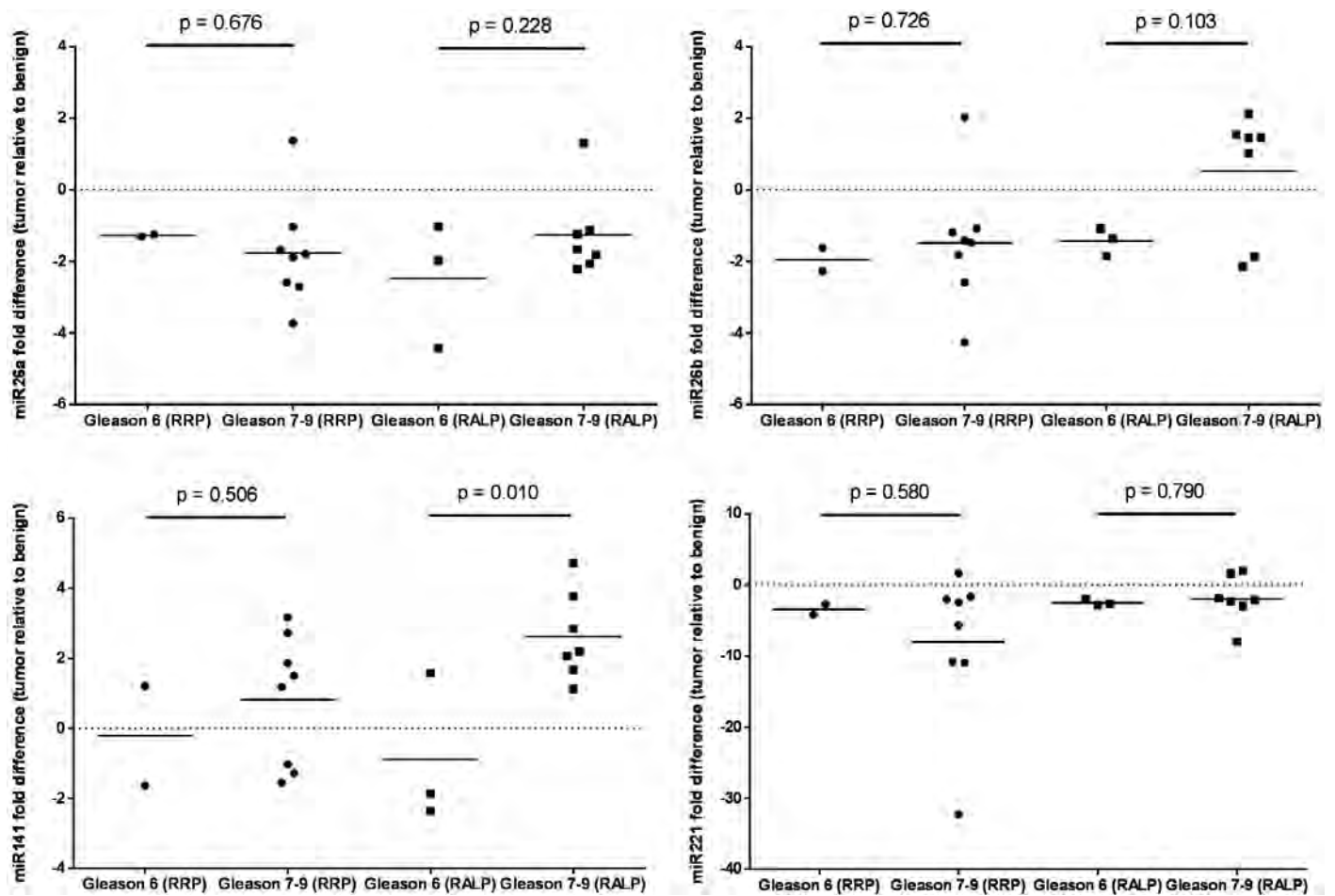


Figure 5. Fold difference in miR26a, miR26b, miR141, and miR221 expression in tumor samples relative to benign among RNA pairs from RRP and RALP as assessed by TaqMan® Small RNA Assay.

Supplementary Figure S2



Supplementary Figure S2. Assessment of fold difference in miR26a, miR26b, miR141, and miR221 expression levels (tumor/benign) and Gleason score of the primary tumor.