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

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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 indicate that samples obtained from RALP specimens may be suitable for prostate cancer biomarker studies—an important finding given the current shift in surgical procedures for prostate cancer treatment. *Prostate 74: 61–69, 2014.*

KEY WORDS: **biobanking; DNA; RNA; biomarker; biorepository; prostate cancer**

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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 of 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 min 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 man-

agement, by following the basic principles outlined recently by Samaratunga et al. [19]. From each case, the prostate capsule was inked black then tattooed in blue and green to orient left and right sides, respectively. The seminal vesicles and vas deferens were removed and the prostate was weighed. A section was taken for routine processing (formalin fixation and paraffin embedding) where the prostate meets the seminal vesicles to determine if there was seminal vesicle invasion. The proximal margin was taken as a thin shave in the region where the prostate was separated from the bladder neck and submitted for routine processing. The distal aspect of the specimen was truncated and perpendicular margins were taken for this region. Next, serial transverse sections of the prostate, perpendicular to the posterior surface, were made from apex to base to expose any visible lesions. Punch biopsies of any visible tumors as well as macroscopically normal appearing areas were then taken (7–12 mm punches are used depending on the prostate size). If no visible lesions are present, then 2–6 punch biopsies ranging from 7 to 12 mm in diameter (depending on the prostate size) were taken from various aspects of the peripheral zone. These harvested tissues were coated in Optimal Cutting Temperature (OCT) media (Sakura Finetek), frozen, and then cryosectioned for Hematoxylin & Eosin (H&E) staining for histological characterization prior to immersion in liquid nitrogen and eventually storage in cryovials at −80°C in our tissue bank. The remaining prostate tissue is formalin-fixed and processed into quadrants in a manner that preserves the three dimensional orientation of all tissues [11] for further processing and placement into individual cassettes for histopathological diagnosis.

TABLE I. Clinical and Pathologic Parameters of Patient Samples

Age of specimen (weeks)	Gleason score; tertiary Gleason grade	Pathologic stage	Patient age
10 RRP cases			
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

Histological Characterization and Sample Selection

The H&E stained microscopic slide obtained prior to liquid nitrogen freezing of the prostate tissue was reviewed by a pathologist to identify regions of tumor and benign tissues. For regions containing significant fractions of tumor and benign tissues, the pathologist helped to determine benign areas that could be removed from the block to help enrich for tumor tissue. Each tumor-enriched block (either with or without trimming) was then evaluated by the pathologist to estimate the percentage of tumor epithelial cells present within the block. In each case, fifty 10 μm frozen sections were collected into two cryovials (100 sections total) for extraction of DNA and RNA from each sample. H&E stained microscopic slides were obtained immediately before and after the 50 frozen sections collected for sample preparation. These H&E slides were further examined by a pathologist as an extra step to control for the quality and percentage of tumor or benign tissue within the collection tubes. By histopathological estimates, tumor samples typically contained between 70% and 90% tumor. 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 I).

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 presence of the TMPRSS2-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 TMPRSS2-ERG primer/probe (IDT) sequences are shown in 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 fold differences were determined using the $\Delta\Delta C_t$ method normalized to U6 expression and the matched benign tissues.

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 C_t values for housekeeping genes in real-time PCR assays for DNA or RNA samples collected from RRP versus RALP (data not shown).

Figure 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 ± 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 (Fig. S1).

We next analyzed the RIN distribution for samples collected from RRP versus RALP. As shown in Figure 2,

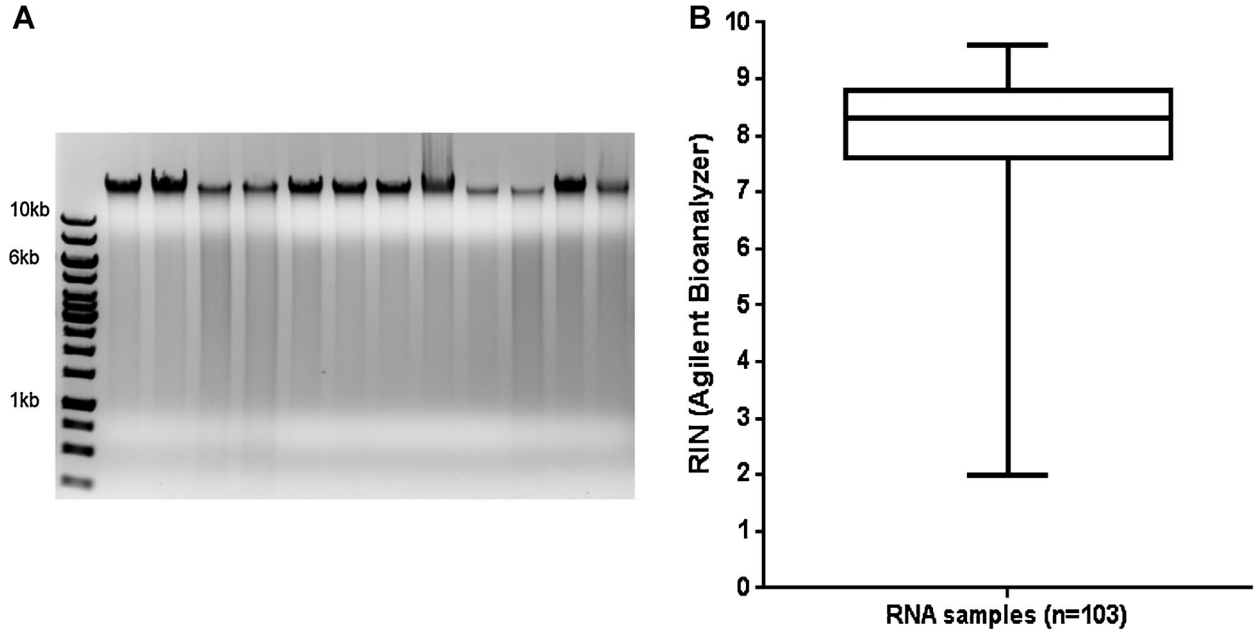

Fig. 1. Assessment of nucleic acid quality as part of SOP development. **A**: Agarose gel of DNA samples extracted from frozen prostate tissue specimens. **B**: RIN of RNA samples extracted from frozen prostate tissues (Agilent Bioanalyzer). Mean RIN = 7.7 ± 1.8, median RIN = 8.3.

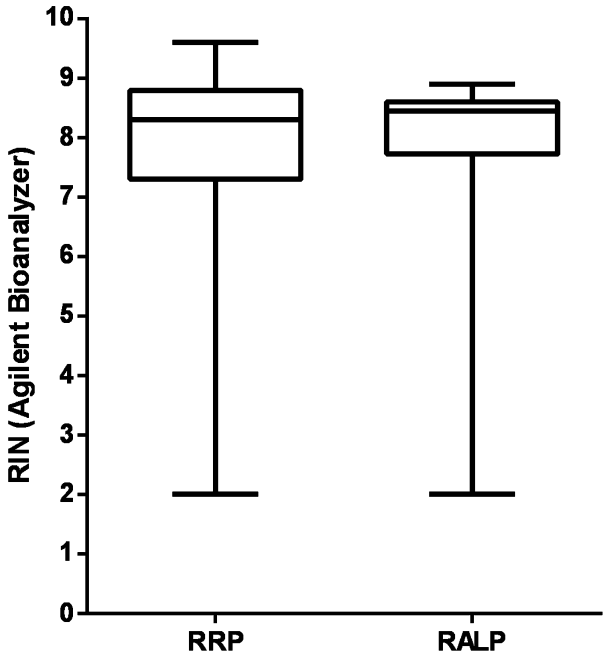

Fig. 2. Distribution of RIN numbers obtained for RNA extracted from radical retropubic prostatectomy (RRP, n = 83) versus robot-assisted laparoscopic prostatectomy (RALP, n = 20) specimens. Median RIN for RRP versus RALP specimens was 8.3 (interquartile range 7.3–8.8) and 8.5 (interquartile range 7.7–8.6),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

the median RIN did not differ significantly for RRP versus 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 I. Patient's samples were varied by Gleason score (ranging from 6 to 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 I). 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 I). 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 $P = 0.932$, respectively, Fig. 3).

The proto-oncogene ERG (ETS related gene) is known to be over-expressed in a subset of prostate

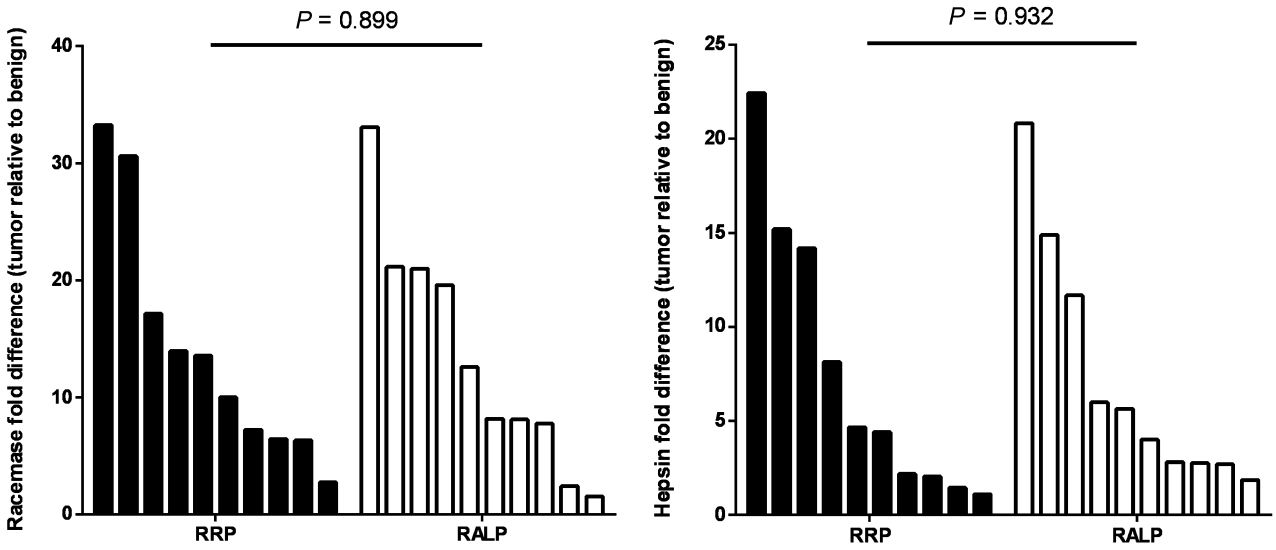

Fig. 3. Fold difference in racemase and hepsin expression in tumor as compared to benign among tumor/benign RNA pairs from RRP and RALP.

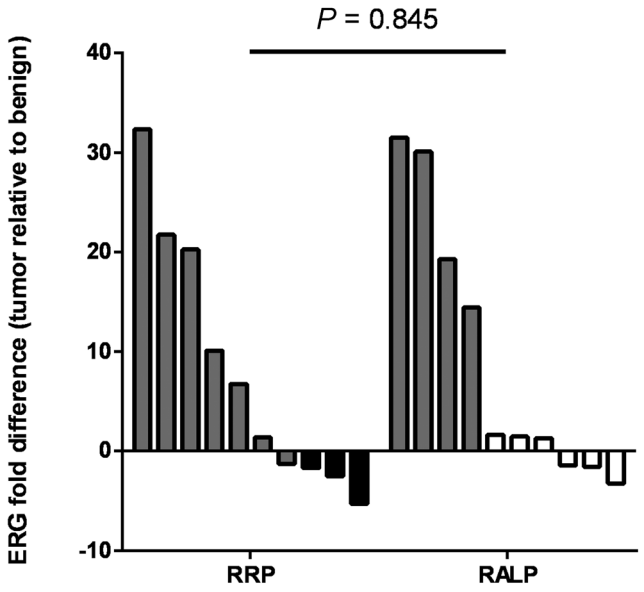

Fig. 4. Fold difference in ERG expression in tumor samples as compared to benign among RNA pairs from RRP and RALP. Samples shown in grey represent cases where the tumor sample was found to be positive for TMPRSS2-ERG gene fusions via qPCR.

cancers due to the presence of TMPRSS2-ERG gene fusions [18]. As shown in Figure 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 four 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 Figure 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* in RRP and upregulation of *miR-141* in RRP and RALP in tumor samples relative to matched benign 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 10 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) ($P = 0.010$, 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 (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 over-expression 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 appar-

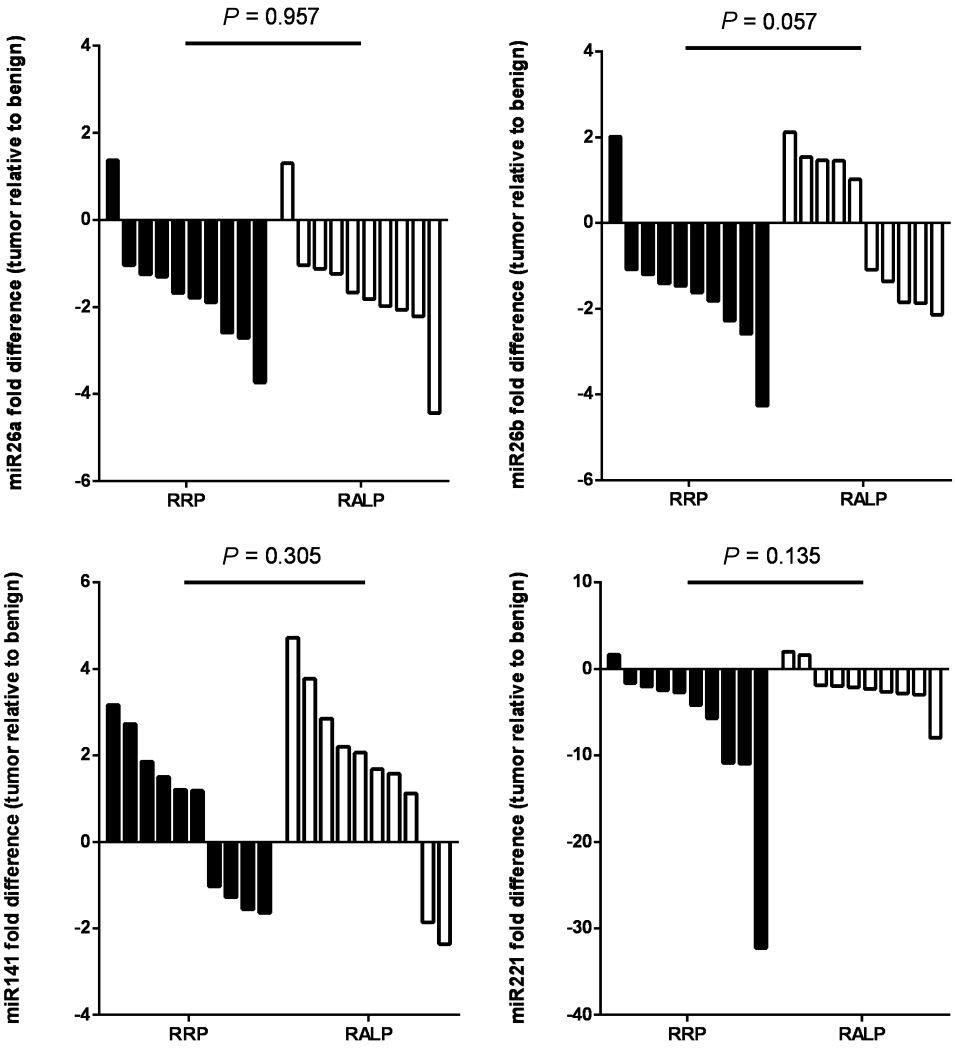

Fig. 5. Fold difference in *miR-26a*, *miR-26b*, *miR-141*, and *miR-221* expression in tumor samples relative to benign among RNA pairs from RRP and RALP as assessed by TaqMan® small RNA assay.

ent in RRP and RALP specimens, half of the RALP showed greater *miR-26b* expression in tumor relative to benign, and 4 of 10 of the RRP specimens showed downregulation of *miR-141* in tumor relative to benign (Fig. 5). Importantly, there was no significant difference in expression levels of any of the microRNAs examined in RRP versus RALP. It is possible that since we observed upregulation of *miR-26b* in half 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 intratumoral expression of *miR-141* is predictive of a

reduced relapse-free interval [21]. In the present study we found that *miR-141* is upregulated in tumor versus benign tissues in a subset of prostate cancer patients (Fig. 5), and that upregulation of *miR-141* was significantly correlated with higher Gleason score in RALP specimens and showed a similar trend in RRP specimens (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.

CONCLUSIONS

In all, the biomarkers that were tested did not demonstrate a difference between RRP and RALP specimens, however, we note the possibility that there may be other biomarkers that are more affected by warm ischemia, and therefore our results may not be completely generalizable. 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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