Comparative Biomarker Expression and RNA Integrity in Biospecimens Derived from Radical Retropubic and Robot-Assisted Laparoscopic Prostatectomies

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Abstract

Background: Knowledge of preanalytic conditions that biospecimens are subjected to is critically important because novel surgical procedures, tissue sampling, handling, and storage might affect biomarker expression or invalidate tissue samples as analytes for some technologies.

Methods: We investigated differences in RNA quality, gene expression by quantitative real-time PCR, and immunoreactive protein expression of selected prostate cancer biomarkers between tissues from retropubic radical prostatectomy (RRP) and robot-assisted laparoscopic prostatectomy (RALP). Sections of tissue microarray of 23 RALP and 22 RRP samples were stained with antibodies to androgen receptor (AR) and prostate-specific antigen (PSA) as intersite controls, and 14 other candidate biomarkers of research interest to three laboratories within the Australian Prostate Cancer BioResource tissue banking network. Quantitative real-time PCR was done for *AR, PSA* (*KLK3*), *KLK2, KLK4*, and *HIF1A* on RNA extracted from five RALP and five RRP frozen tissue cores.

Results: No histologic differences were observed between RALP and RRP tissue. Biomarker staining grouped these samples into those with increased (PSA, CK8/18, CKHMW, KLK4), decreased (KLK2, KLK14), or no change in expression (AR, ghrelin, Ki67, PCNA, VEGF-C, PAR2, YB1, p63, versican, and chondroitin 0-sulfate) in RALP compared with RRP tissue. No difference in RNA quality or gene expression was detected between RALP and RRP tissue.

Conclusions: Changes in biomarker expression between RALP and RRP tissue exist at the immunoreactive protein level, but the etiology is unclear.

Impact: Future studies should account for changes in biomarker expression when using RALP tissues, and mixed cohorts of RALP and RRP tissue should be avoided. *Cancer Epidemiol Biomarkers Prev; 19(7); 1755–65. ©2010 AACR.*

Introduction

The da Vinci robot-assisted laparoscopic prostatectomy (RALP) procedure has provided a less debilitating alternative to performing radical retropubic prostatectomy (RRP) in men with early-stage, presumed organ-confined prostate cancer. The robotic procedure has been of immense benefit to patients in terms of shorter hospital stay and reduced blood loss, and has become the surgical procedure of choice for many men (1). The proportion of prostatectomy procedures performed using RALP continues to increase annually.

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New genomic, proteomic, and metabolomic technological approaches to translational research rely on high-quality biospecimens with accurate, comprehensive, clinical, and pathologic data annotation. However, there are potential drawbacks to the RALP procedure for translational medicine relating to increased warm ischemia with a potential for increased hypoxia, and consequently, affecting the integrity of RNA and expression of cancer biomarkers. With conventional RRP, the blood supply to the prostate is severed late in the procedure such that the period of warm ischemia, prior to removal of the gland and placement on ice, is restricted to a short period of ~30 minutes (2, 3). In the RALP procedure, the blood supply to the prostate is severed earlier in the procedure leading to longer period of warm ischemia within the peritoneal cavity before externalization of the prostate, during the learning phase up to 1 to 2 hours, but with more experience, this period is decreasing.

Currently, there is a worldwide focus on producing evidence-based standard operating procedures, which minimize preanalytic variation to achieve the highest quality samples possible as tissue analytes (4). Variation in protocols for collection, sampling, and storage of biospecimens could alter inherent biological characteristics and the molecular profile of the tissue, leading to anomalies in analysis that could be misinterpreted as disease-related or disease-specific traits. Prostate cancer staging tools such as nomogram-derived predictions of risk of extraprostatic extension of disease and outcome measures of disease-free survival following putative curative surgery frequently depend on the addition of immunoquantitation of cancer biomarkers to clinical staging and Gleason score. A recent study indicated that an exclusively clinical and pathologic nomogram developed for RRP patients was equally accurate for their RALP counterparts (5). Although a number of studies have investigated the effect of the ischemic time during RRP on gene expression (2, 3), or have used RRP tissues to study postsurgical delays in processing of up to 5 hours at ambient temperature (6), the effect of robotic surgery on prostate cancer biomarker expression has not been investigated to the same degree (7). In this study, we compare prostate cancer tissue samples from conventional RRP with samples from RALP for changes in histologic morphology, RNA integrity, gene expression by quantitative real-time PCR (qPCR), and immunostaining for 16 biomarkers of particular research interest to our laboratories, including markers of hypoxia.

Materials and Methods

Tissues

Histologically representative paraffin-blocked tissues from 22 RRP and 23 RALP procedures were provided by pathologists for tissue microarray (TMA), with ethical consent from the Royal Adelaide Hospital Research Ethics Committee (approval no. 041010), and were from procedures done by surgeons associated with the Royal Adelaide Hospital Urology Unit. Blocks were randomly provided from RRP procedures between October 2000 and April 2004, and from RALP procedures between November 2004 and May 2005. The TMA was constructed at and distributed in blinded fashion by the Sydney node (Garvan Institute) of the Australian Prostate Cancer BioResource (APCB) tissue banking network, which acted as an independent site in this study. Two or three 1-mm cores of prostate cancer tissue representative of the Gleason grades present were entered into the TMA for each participant. Quality assurance on the final TMA was done by a pathologist (J. Kench). TMA sections were received at the APCB nodes at Dame Roma Mitchell Cancer Research Laboratory (University of Adelaide), Australian Prostate Cancer Research Centre-Queensland (Queensland University of Technology), and the Prostate and Breast Cancer Research Group (Monash University), which were randomly assigned a nomenclature of site 1, 2, or 3.

Fresh prostate cancer samples (punch biopsy, 8 mm for RALP, 6 mm for RRP) were provided at diagnostic pathology work-up with informed patient consent, snap-frozen using Optical Cutting Compound as a cryoprotectant in liquid nitrogen, and banked at −80°C for future research at the Adelaide, Brisbane, and Melbourne nodes of the APCB during 2004 to 2008. The difference in gauge of punch biopsy reflects the personal preference of the pathologists concerned, with the aim to provide as large and representative sample of tissue as possible, while minimizing compromise of the surgical margins, and taking into account the overall size of the prostate. All tissues were derived with approval of the appropriate institutional ethics committees under consensus standard operating procedures across the APCB nodes. For this study, RALP was derived from one site, and RRP from the other two sites. Frozen prostate tissue cores from five RALP and five RRP randomly selected patients were retrieved from storage for RNA extraction. The cores were confirmed to contain >25% cancer by frozen section by a pathologist (S. Jindal).

Warm ischemia times for RRP and RALP were determined intraoperatively by notation of the times of clamping of the prostate blood supply, and exteriorization of the prostate and placement on ice. Warm ischemia time for RRP was ~30 to 40 minutes' duration, confirming previous studies (2, 3). The cohort of RALP tissues used for TMA construction were archived between November 2004 and May 2005, soon after surgeons started to use robotic surgery at our institution, when the median operative duration time for RALP was ~4.25 hours (range, 3.5-5.25 h). Median warm ischemia time was ~1.5 hours (range, 1.25-1.75 h). The fresh-frozen RALP tissues were banked between February 2006 and March 2008, when the median operative duration and warm ischemia time had reduced to ~3.25 hours (range, 2.74-4 h) and 1 hour (range, 0.5-1.75 h), respectively.

Immunohistochemical staining and measurement

Selection of the 16 biomarkers for immunohistochemical analysis was based on long-term expertise and research utilization by the three individual groups undertaking this study, to determine whether any differences in expression might be expected when using RALP tissue. Specific details of the antibodies, dilutions, and immunoreactions are detailed in Supplementary Table S1. In brief, sections (4 μm) of paraffin-embedded prostate tissue from the TMA were immunostained with the following specific antibodies: intersite controls, androgen receptor (AR; ref. 8) distributed by one site and used at two sites, and prostate-specific antigen (PSA; ref. 9) distributed by one site and used at all sites; at site 1, nuclear proliferation antigen (Ki67; ref. 10), chondroitin 0-sulfate (C-0-S; ref. 11) and versican (12); at site 2, cytokeratins (CK8/18 and high molecular weight, CKHMW), proliferating cell nuclear antigen (PCNA), tumor suppressor transcription factor p63, and vascular endothelial growth factor (VEGF-C; refs. 13-15); at site 3, kallikreins (KLK-2, -4, and -14; ref. 16), proteinase activator receptor (PAR2; refs. 16, 17), ghrelin (18), and Y-box binding protein (YB1; ref. 19). Immunostaining details in brief are endogenous peroxidase was blocked at all sites, and all sections were subjected to microwave antigen retrieval, except for PSA. Sections to be stained with CKHMW were additionally treated with trypsin. Blocking agent was used either prior to or as the diluent within the immune reaction. Sites 1 and 3 used a manual staining method with overnight primary antibody incubations at 4°C, whereas site 2 used an autostainer (Dako Autostainer Universal Staining System, Dako, Denmark) at room temperature for 30 to 120 minutes. Visualization of immunoreactivity was by commercial kit or individual immunoperoxidase and diaminobenzidine tetrahydrochloride reagents (Dako) to yield an insoluble brown deposit. A whole paraffin tissue section from a prostate cancer block known to be positive for the specified antigen was used as a positive control, and negative control was replacement of the primary antibody with appropriate diluent. Immunostaining was visually assessed by two pathologists (S. Jindal and H. Samaratunga) and an experienced scientist (H. Wang). Nuclear immunostaining was assessed by the percentage of nuclei positive. Cytoplasmic and extracellular matrix antigens were graded on an intensity scale of 0 to 3+. Nuclear and cytoplasmic staining was assessed in malignant cells only for AR and PSA, respectively. For markers Ki67, CK8/18, VEGF-C, and PCNA, normal and malignant epithelial cells and stromal cells were assessed, and for basal cell markers, CKHMW and p63, benign elements and Prostatic Intraepithelial Neoplasia were evaluated. Versican and C-0-S were assessed in the cancer-associated stroma. All other markers were assessed in cancer cells only.

RNA integrity and cDNA synthesis

Assessment of RNA quality was done on an Agilent 2100 bioanalyzer using LabChip kit (Eukaryote Total RNA 6000 Nano assay; Agilent Technologies) to yield an RNA integrity number on a scale of 1 to 10. cDNA was synthesized from 600 ng of RNA that had been Turbo DNAfree-treated and isopropanol-precipitated, using the iScript cDNA synthesis kit (Bio-Rad) according to the instructions of the manufacturer. The controls for the reverse transcription reaction included: a "no RNA" control containing only the reverse transcriptase reaction mix, water, and enzyme, and an "RNA only" control that contained RNA template, water, and reverse transcriptase reaction mix, but no reverse transcriptase enzyme. cDNA samples were diluted 1:10 and 2 μL was used in subsequent qPCR.

qPCR

qPCR reactions were done in triplicate using iQ SYBR Green Supermix (Bio-Rad) as outlined by the manufacturer, at one APCB site only. Primers (Geneworks) were included at a concentration of 400 nmol/L each per reaction (see Supplementary Table S1 for primer sequences). Target cDNA was amplified in a 25 μL reaction on an iQ5 Cycler (Bio-Rad) using the following PCR conditions: 1 cycle at 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds, 95°C for 1 minute, 65°C for 1 minute followed by melt curve analysis for 60 cycles from 65°C to 95°C (0.5°C per cycle for 30 seconds). Reaction efficiency was determined using a standard curve of 1 μg of Universal Human Prostate RNA (Ambion) that had been reverse-transcribed and cDNA diluted serially to 1:2, 1:10, 1:50, 1:250, and 1:1,250. Each standard was amplified in duplicate with 2 μL of template per reaction. Gene expression data for *AR, PSA, KLK2, KLK4*, and *HIF1A* was normalized to both reference genes *GAPDH* and *MRPL19*. The primers used are listed in Supplementary Table S2.

Statistical analysis

All analyses were done using SPSS 16.0 for Windows software (SPSS, Inc.). Spearman's correlation was used to determine the correlation of AR and PSA immunostaining between the three sites. The Mann-Whitney U (continuous variables) and χ^2 (dichotomized variables) tests were used to determine statistical significance of expression between RALP and RRP tissue. Statistical significance was accepted at $P < 0.05$.

Results

Characterization of the RALP and RRP TMA cohorts

The clinical and pathologic characteristics of the RALP and RRP cohorts used in the TMA are shown in Table 1. Preoperative serum PSA was significantly lower in the 23 RALP patients (median, 5.7 ng/mL) than in the 22 RRP patients (median, 8.5 ng/mL; $P = 0.004$, Mann-Whitney U test), agreeing with published data that illustrates the conservative nature of the selection of patients for RALP (1). There was no significant difference in age range, pathologic stage, or Gleason score between the RALP and RRP cohorts. Comparative histologic examination

by a pathologist (S. Jindal) on H&E-stained sections of the TMA cohort indicated that there was no difference in prostate tissue morphology in tissues derived from RALP and RRP operative procedures. Representative images of RALP and RRP Gleason grade 3 tissues are shown in Fig. 1A.

Comparative immunostaining of biomarkers in RALP and RRP tissues

Sections of the RALP and RRP TMA were immunostained for intersite control antigens AR (two sites) and PSA (three sites). Although there was an overall lower level of AR staining at site 2 compared with site 1 (Fig. 1B), there was no difference in staining for AR between RALP and RRP operative procedures at either site (Mann-Whitney U test). The lower level of AR staining at site 2 might be due to methodologic differences in antigen retrieval, staining conditions, or detection reagents dictated by use of an autostainer. A small but statistically significant increase in PSA expression was observed for the RALP cohort at site 2 ($P = 0.002$, Mann-Whitney U test), with a slight but nonsignificant increase or decrease at the other two sites (Fig. 1C). Significant correlations by Spearman test were observed in the PSA ($r = 0.216$, $P = 0.026$) and AR ($r = 0.361$, $P < 0.0001$) measurements between sites 1 and 2. PSA measurements between sites 1 and 3 were also significantly correlated ($r = 0.210$, $P = 0.029$, Spearman correlation test). All other biomarker protein stainings were conducted at individual sites, using markers of particular research interest for which considerable staining experience existed at each site. Markers that showed a significant change in expression between the RALP and RRP cohorts are shown in Fig. 2. The cytokeratins CK8/18 and CKHMW were increased in the RALP cohort (Mann-Whitney U test, $P < 0.0001$ and <0.0001, respectively). The three kallikreins studied also

showed significant changes in immunostaining with KLK4 being increased in RALP ($P < 0.0001$), and KLK2 and KLK14 both significantly reduced in the RALP cohort ($P = 0.017$ and <0.0001, respectively). Markers demonstrating no significant differences in protein expression between the two tissue sources were C-0-S, ghrelin, Ki67, PAR2, PCNA, p63, VEGF-C, versican, and YB1 (Fig. 3).

Characterization of frozen RALP and RRP tissues and RNA extracts

The clinical and pathologic characteristics of the cohort of five RALP and five RRP tissues used for RNA extraction are recorded in Table 2. There were no significant differences in age range, preoperative serum PSA, Gleason score, or pathologic stage between the RALP and RRP tissues. Duration of storage of the tissues at –80°C prior to extraction was not significantly different. Investigation of RNA integrity using the Agilent bioanalyzer determined that high-quality RNA could be obtained from both RALP and RRP tissues, with no significant difference in RNA integrity number values (Table 2). Quantitative PCR was used to evaluate gene expression for *AR* and *PSA*, two additional prostatic androgen-regulated genes, *KLK2* and *KLK4*, and hypoxia-induced factor *HIF1A* (20), which were normalized to the reference genes *GAPDH* and *MRPL19*. No differences in expression of the five genes tested were observed between RALP and RRP RNA preparations (Mann-Whitney U test; Fig. 4).

Discussion

Whenever new surgical procedures or different tissue sampling, storage, or analytic techniques arise, it is important to understand precisely what effect these might have on molecular and protein analyses used in discovery and
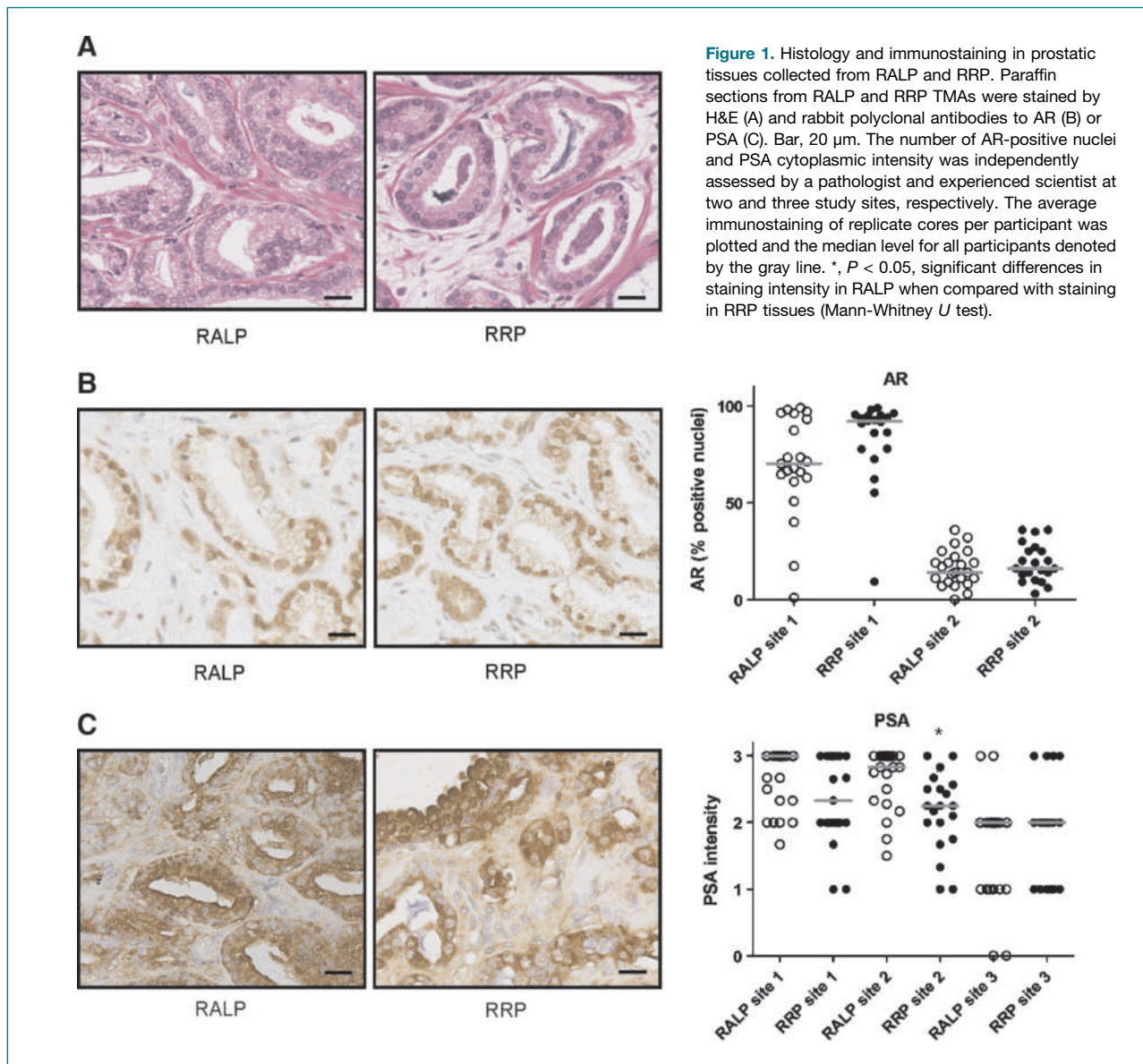

Table 1. Clinical and pathologic characteristics for RALP and RRP TMA cohorts

	RALP cohort	RRP cohort	*P*
Patients (*n*)	23	22	
Median age at diagnosis (y)	64.6 (50.5-73.4)	62.1 (51.9-72.3)	0.196*
Median preoperative serum PSA (ng/mL)	5.7 (1.2-16.0)	8.5 (5.1-19.0)	0.004*
Unknown	2	2	
Pathologic stage (*n*)			
pT2	12	11	1.00[†]
pT3	11	11	
Gleason grade (*n*)			
3 + 3	11	13	0.697[‡]
3 + 4	7	6	
4 + 3	5	3	

*Mann-Whitney *U* test.
[†]Fisher's exact test.
[‡]Pearson χ^2 test.

Figure 1. Histology and immunostaining in prostatic tissues collected from RALP and RRP. Paraffin sections from RALP and RRP TMAs were stained by H&E (A) and rabbit polyclonal antibodies to AR (B) or PSA (C). Bar, 20 μm. The number of AR-positive nuclei and PSA cytoplasmic intensity was independently assessed by a pathologist and experienced scientist at two and three study sites, respectively. The average immunostaining of replicate cores per participant was plotted and the median level for all participants denoted by the gray line. *, $P < 0.05$, significant differences in staining intensity in RALP when compared with staining in RRP tissues (Mann-Whitney U test).

biomarker studies. Currently, there is a focus on producing evidence-based standard operating procedures for biobanking, which minimizes preanalytic variation to achieve the highest quality samples possible as tissue analytes (4), and to bring into universal practice a preanalytic code[11], outlining the treatment or production history of all tissue samples. These initiatives are being driven by international organizations such as the International Society for Biological and Environmental Repositories through its Working Group on Biospecimen Science, as well as the U.S. NIH via its Office of Biorepositories and Biospecimen Research.

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[11] Betsou F, Lehmann S, Ashton G, et al. Standard Preanalytical coding for biospecimens: Defining the sample PREanalytical code. Cancer Epidemiol Biomarkers Prev 2010;19:1004-11.

The potential for hypoxic conditions to be generated during the period of intraoperative warm ischemia with resultant change in gene expression is a key issue for studies using RRP and RALP tissues. Two recent publications report ischemia-induced changes in gene expression profiling during RRP (2, 3), whereas a single publication has compared tissue integrity in postoperative biopsies derived immediately after RRP and RALP (7). Each study provides valuable insights into the effect of prostatectomy-induced ischemia. Lin et al. (2) compared gene expression profiling of *in situ* biopsies of the prostate taken immediately after surgical exposure of the prostate with *ex vivo* biopsies taken after RRP. A later study by Schlomm et al. (3) compared biopsies taken after prostate exposure, and immediately after RRP, but also intraoperative biopsies just before and just after cutting the
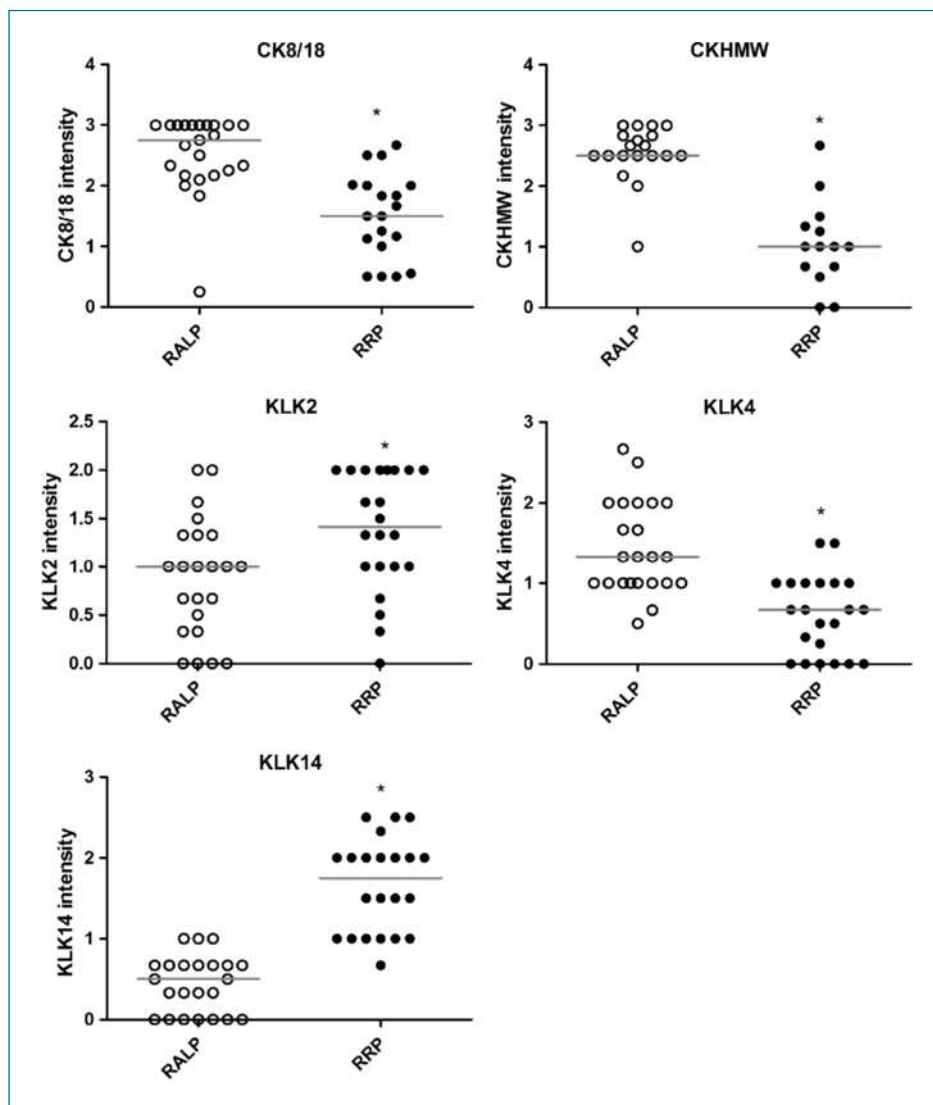
Figure 2. Immunoreactive biomarker expression significantly affected by surgical procedure. Paraffin sections from TMAs of RALP and RRP were stained with specific antibodies to CK8/18, CKHMW, KLK2, KLK4, and KLK14, and intensity of staining assessed by a pathologist and experienced scientist at each site. The average immunostaining of replicate cores per participant was plotted and the median level for all participants denoted by the gray line.
*, $P < 0.05$, significant differences in staining intensity in RALP when compared with staining in RRP tissues (Mann-Whitney U test).

prostate blood supply. An important conclusion from both of these studies was that surgical manipulation alone induced changes in gene expression profiling, suggesting that preoperative biopsies were optimal for studying molecular changes in prostate tissue. Unfortunately, neither preoperative diagnostic biopsy nor *in situ* biopsy prior to prostate removal are sustainable sources of tissue for research study. Best et al. (7) studied integrity differences between RRP and RALP at the tissue, DNA, RNA, immunoreactive protein, and viable cell culture levels. Their samples were all derived after surgical removal of the prostate, as was the situation in our study. A case could be mounted for taking serial intraoperative biopsies during RALP for both of these studies, as it is technically possible to exteriorize biopsies through a port. However, modification of the robotic procedure is considered not in the best interest of patients and is difficult to defend ethically. Intraoperative sampling in RALP

would lead to delays in operative progress, extra bleeding, and from a pathology viewpoint, any alteration to the integrity of the prostate capsule might compromise the pathologic assessment of surgical margins and/or the assessment of small foci of extracapsular extension, which reflect staging of the tumor. Consequently, we adopted a more conservative approach, similar to that of Best et al. (7), sampling tissue immediately postsurgery for snap-freezing. We also used replicate sampling of archived tissue blocks from a larger number of patients and construction of a TMA for our immunoreactive protein analysis. We examined 16 putative prostate cancer biomarkers under intensive investigation in our laboratories and for which we had considerable immunostaining experience, with the question being: would there be any major consequences for continuing research programs due to the surgical move from RRP to RALP tissues? To maximize any difference between RRP and RALP tissue,

we constructed our TMA from RALP tissues collected early in our experience with the robotic procedure, in which the warm ischemia time was up to 60 minutes longer than for RRP. Another prime consideration was that our postoperative biopsies should be snap-frozen within 10 to 15 minutes of surgical removal of the prostate, to obviate any delay in tissue processing, which has been shown to cause additional preanalytic changes in gene expression profiling (3, 6).

Studies in numerous tissues have reported hypoxia-induced changes in HIF1A and VEGF associated with malignancy (20, 21). In this study, the focus has been on observing the effect of extended warm ischemia times during RALP compared with RRP and the potential for the generation of hypoxia on RNA integrity and candi-date prostate cancer cell biomarkers, including HIF1A and VEGF-C. Contrary to some expectations, there were no effects from the extended warm ischemia in RALP on either tissue morphology or RNA integrity. This agrees with the study of Lin et al. (2), in which change in gene expression was not related with the duration of ischemia, and with Best et al. (7), in which there was no difference in RNA quality. It also seems that little additional hypoxia may be generated during the extended period of warm ischemia in RALP (average 60 minutes longer than RRP for the TMA tissues) as no change in immunostaining was observed for VEGF-C. This view was reinforced by the observed unchanged expression of the *HIF1A* gene in RNA from RALP tissues (average 30 minutes longer warm ischemia period than RRP for the frozen tissues).
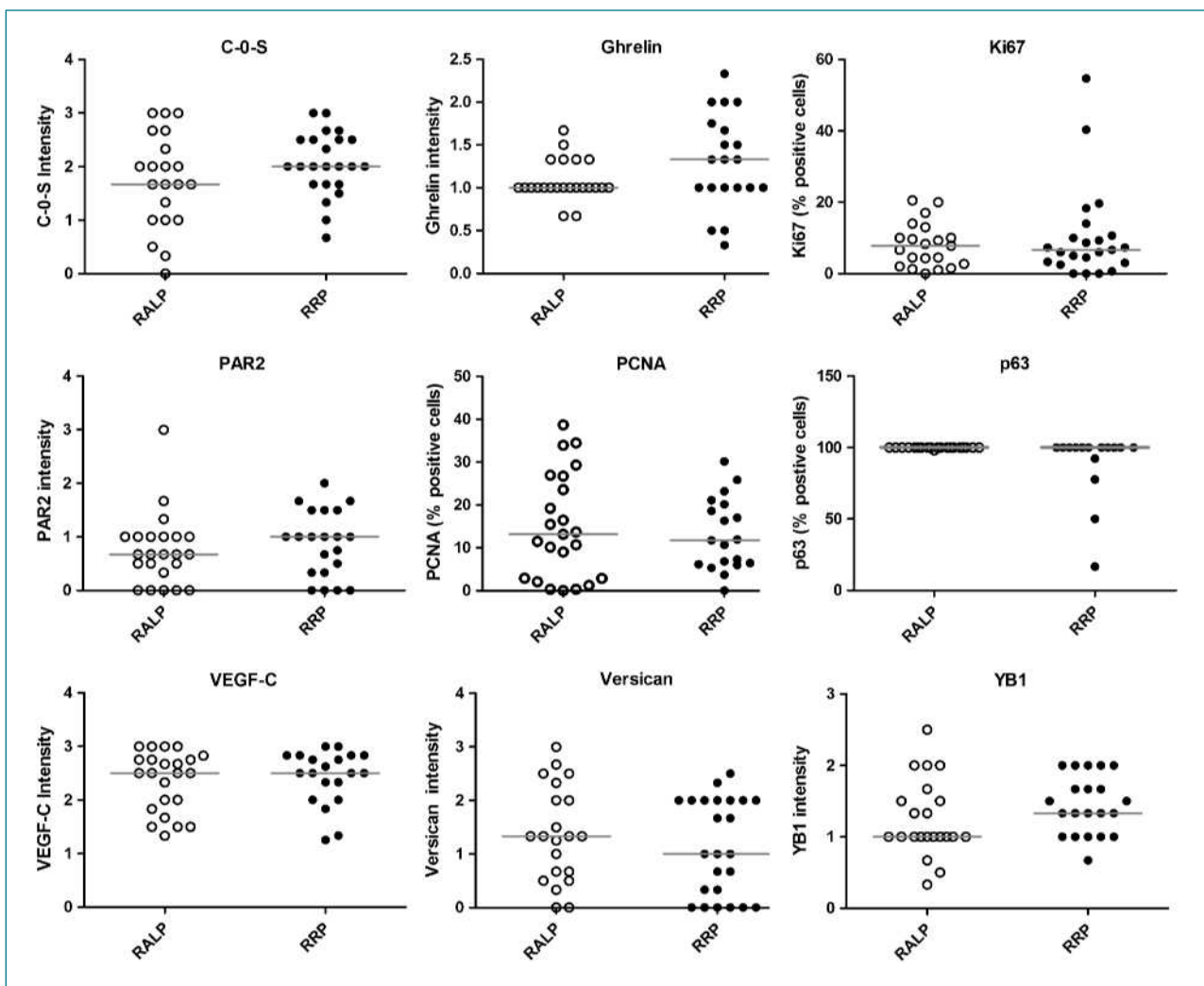

Figure 3. Immunoreactive biomarker expression unchanged by surgical procedure. Paraffin sections from TMAs of RALP and RRP were stained with specific antibodies to C-0-S, ghrelin, Ki67, PAR2, PCNA, p63, VEGF-C, versican, and YB1. Staining intensity was scored for C-0-S, ghrelin, PAR2, VEGF3, versican, and YB1, and the percentage of positive nuclei were scored for Ki67, PCNA, and p63 by a pathologist and experienced scientist at each site. The average immunostaining of replicate cores per participant was plotted and the median level for all participants denoted by the gray line. No significant differences were determined (Mann-Whitney *U* test).

Table 2. Clinical characteristics, RNA integrity number, and tissue storage time at −80°C for RALP and RRP tissues used for qPCR

	RALP cohort	RRP cohort	P
Patients (n)	5	5	
Median age at diagnosis (y)	64.3 (54.1-69.2)	63.2 (60.3-67.4)	0.421*
Median preoperative serum PSA (ng/mL)	7.9 (7.0-12.4)	7.6 (2.4-15.1)	0.690*
Pathologic stage (n)			0.524†
pT2	3	1	
pT3	2	4	
Gleason grade (n)			0.198†
3 + 3	1	0	
3 + 4	4	2	
4 + 3	0	2	
4 + 5	0	1	
RNA integrity number	9.0 (8.0-10.0)	9.6 (8.7-10.0)	0.310*
Storage time (mo)	13.8 (4.0-27.0)	17.4 (3-29.0)	0.754*

*Mann-Whitney U test.
†Pearson χ^2 test.

Although the induction of hypoxia seems unchanged between the two surgical procedures, changes in the protein expression of certain candidate prostate cancer biomarkers were observed in this study.

Markers with significant increases in protein expression between RALP and RRP tissues were PSA (KLK3), KLK4, CK8/18, and CKHMW. The markers demonstrating a significant reduction in protein expression in RALP tissues were KLK2 and KLK14. Two recent studies using LNCaP prostate cancer cells reported increased expression of PSA under hypoxic conditions (22). Hypoxia was reported to increase the recruitment of nuclear AR to the *PSA* gene promoter with resultant increases in ARE-reporter gene activation and accumulation of PSA (23), and in a similar experimental model, the use of chromatin immunoprecipitation showed that HIF1 interacts directly with AR on the *PSA* gene promoter and activates PSA expression (22). A recent study (24) identified decreased PSA associated with higher Gleason grade, but this is unlikely to contribute to the change in PSA immunoreactive protein seen in this study as there was no significant difference in Gleason scores between the RRP and RALP cohorts used in the TMA.

Hypoxia has also been shown to increase the level of microRNAs (miRNA), small noncoding RNAs that regulate gene expression in cancer cells (25). miRNAs predominantly function by imperfect complementarity to the 3′-untranslated region of the target gene mRNA, causing suppression of protein translation, in the absence of mRNA degradation, through an RNA-induced silencing complex pathway. Recent evidence suggests that kallikreins are a potential target for miRNAs, using multiple target sites in kallikrein mRNA to exert quantitative control of kallikrein expression at the posttranscriptional level (26). Because the increased expression of miRNAs

induced by HIF1 leads in general to reduced protein levels, this might explain the reduction in KLK2 and KLK14 observed in this study. Although the role for miRNA in regulating kallikrein expression is speculative due to the lack of evidence for increased hypoxia in RALP compared with RRP tissues, the fact that the kallikreins as a group showed differential changes in expression under the same conditions is particularly interesting, but the mechanism for increased production of KLK4 is unclear. Although changes in the kallikrein profile may relate to their enzymatic role within cancer progression, the relevance of increased expression of normal epithelial and basal cell cytokeratins as observed in this study is unclear. Although biological explanations might account for the observed changes in immunoreactive protein expression, we cannot rule out that some changes are artifacts. Most of the biomarkers (14 of 16) were examined at an individual site only, using long-term expertise with established staining protocols at the respective sites. The other biomarkers were examined at two (AR) or three (PSA) sites. No differences in the number of AR-stained nuclei were detected between the operative procedures at either site, but the overall level of staining between the sites was significantly different and may have been related to methodologic differences, including the use of an autostainer at one site. For PSA, only site 2 recorded a small but significant difference between the surgical procedures. The disparity between this and the lack of detection at the other sites is obviously intersite variation.

Examination of gene expression by qPCR indicated no significant differences between RALP and RRP RNA steady state levels for any of the biomarkers examined (*AR, PSA/KLK3, KLK2, KLK4*, and *HIF1A* genes) suggesting, at least for the markers examined, that differences in the duration of warm ischemia did not alter gene

transcription or mRNA stability. This lack of difference in gene expression, although protein levels for PSA, KLK2, KLK4, and KLK14 changed at the immunoreactive protein level, is interesting. Whether this difference is due

to the shorter ischemic time for the frozen RALP biopsies compared with the earlier TMA cohort is unknown. Increases in protein expression through changes in protein synthesis are unlikely to manifest themselves within such
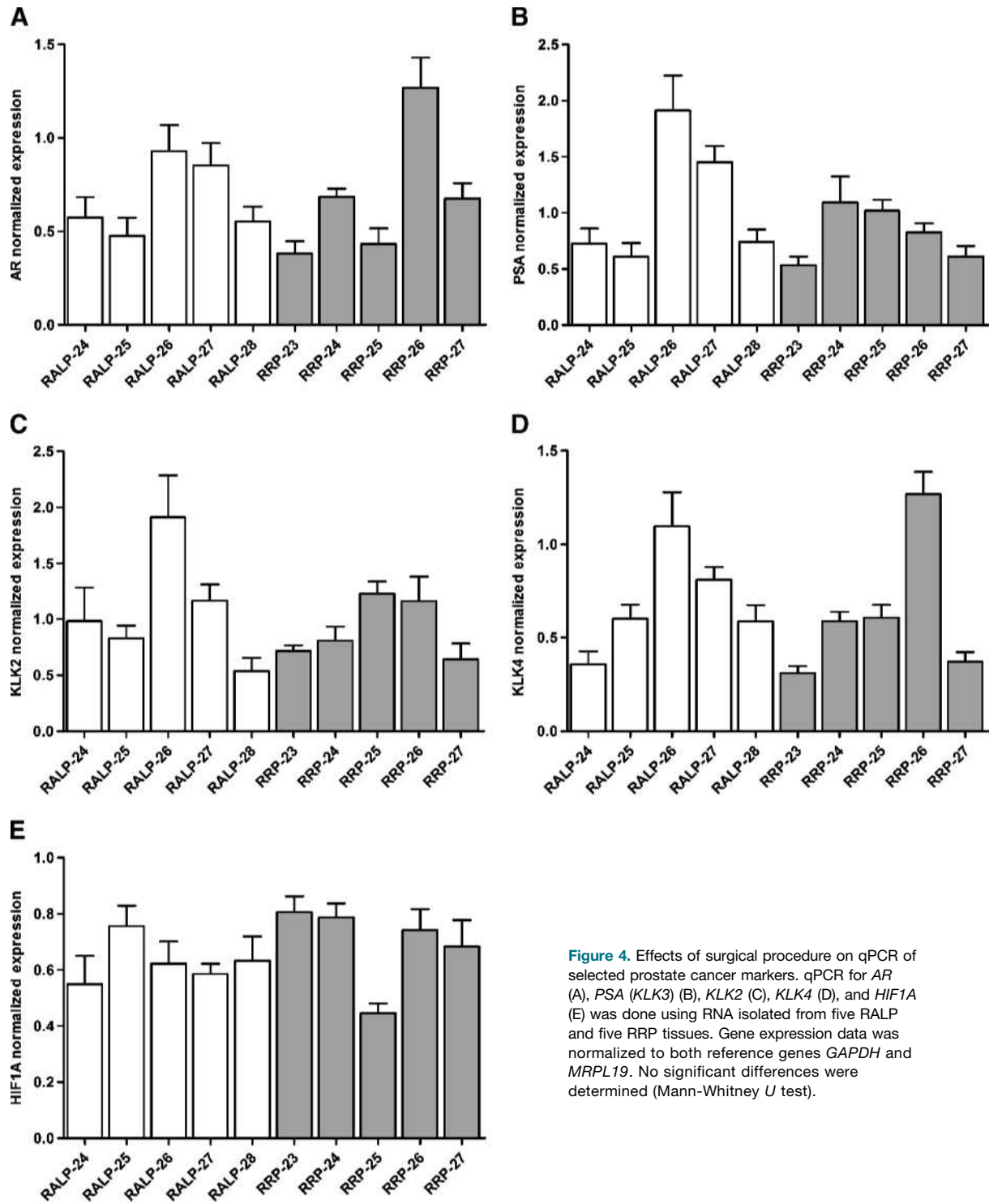

Figure 4. Effects of surgical procedure on qPCR of selected prostate cancer markers. qPCR for *AR* (A), *PSA* (*KLK3*) (B), *KLK2* (C), *KLK4* (D), and *HIF1A* (E) was done using RNA isolated from five RALP and five RRP tissues. Gene expression data was normalized to both reference genes *GAPDH* and *MRPL19*. No significant differences were determined (Mann-Whitney *U* test).

a short time during a surgical procedure, even in the presence of changes in gene expression at the RNA level which did not occur in this study. Decreases in protein expression could, however, potentially occur via increased protein turnover and protein degradation. Differences in grade and stage between surgical cohorts are unlikely to explain these observed changes as there were no significant differences in the variables between the groups. The statistically significant reduction in serum PSA level in the RALP cohort at diagnosis is, we believe, due to conservative cohort selection for RALP, rather than any real difference in cellular PSA production as the observed postsurgical increase in this cohort is counterintuitive. One possible explanation is that the assessment of immunoreactive protein expression was confined to the cancer cell compartment of tissue sections, whereas RNA was produced from whole tissue cores that contained at least 25% cancer by volume, but would also have contained variable amounts of nonmalignant cell elements. This variable stromal and benign epithelial component in the tissues used to extract RNA might have affected the estimate of gene expression in the tumor cell component. We also recognize that this is a preliminary study in which most of the candidate biomarkers were examined at one or two sites only according to the research interest and expertise at that site, and that further studies are required to confirm our observations.

In conclusion, this study has shown that RALP tissue is morphologically indistinguishable from RRP tissue, and that the RALP procedure imposes no detrimental effect on the quality of RNA extracted. In addition, there is no apparent change in steady state gene expression levels for those genes examined due to the adoption of a new operative procedure. Changes in the expression of immunoreactive protein of undefined origin, both increased and decreased, were observed for a range of biomarkers, leading to the conclusion that cohorts of tissue assembled for research should be comprised of tissues from one procedure or the other, but not mixed, and that the effect of RALP and RRP surgical procedures should be investigated for all new biomarker studies. Where this is not possible due to the unavailability of diagnostic core biopsies for research, tissue samples should be processed as soon as possible after surgery. Because our RALP study cohorts were drawn from patients operated on between 2004 and 2008, and show reductions in the duration of the operative procedure and the duration of warm ischemia, it is likely that these times will be further reduced. This means that our study could be considered as a worse case scenario.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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