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Integrity and Amplification of Nucleic Acids From Snap-Frozen Prostate Tissues From Robotic-Assisted Laparoscopic and Open Prostatectomies

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Abstract

Context—Recently, robotic-assisted laparoscopic prostatectomy has replaced open retropubic radical prostatectomy as the surgical procedure of choice. This less-invasive approach offers many advantages but exposes prostate tissue to longer periods of warm ischemia that may affect subsequent analysis of biomarkers.

Objective—To analyze the nucleic acid quality and quantity isolated from open versus laparoscopic prostatectomies.

Design—Nucleic acids were isolated from 10 open-obtained and 10 laparoscopic-obtained tissues stored in our prostate sample repository. Nucleic acid integrity was assessed via electrophoresis and polymerase chain reaction amplification of RNA and DNA targets ranging in size from 125 to 939 base pairs.

Results—The DNA yield, integrity, and polymerase chain reaction amplification were identical between samples obtained from both surgical approaches. The RNA integrity number and yield were similar, as was β -2 microglobulin mRNA amplification up to 652 base pairs. However, 2 of 10 samples (20%) collected robotically showed decreased real-time reverse transcriptase-polymerase chain reaction amplification of prostate-specific antigen messenger RNA, especially with targets larger than 300 base pairs.

Conclusions—Generally, the quality and quantity of nucleic acids isolated from prostate tissue obtained via open or laparoscopic approaches are equivalent, suggesting that procurement of tissues is appropriate from either procedure. However, some loss of reverse transcriptase-polymerase chain reaction amplification of larger RNA targets was noted in the laparoscopic samples; appropriate design of assays to keep amplicon sizes small and the use of internal controls to assess RNA integrity is recommended.

Open retropubic radical prostatectomy (RRP) has been the standard surgical treatment for localized prostate cancer for decades. In recent years, however, robotic-assisted laparoscopic prostatectomy (RALP) has rapidly become the surgical procedure of choice.¹ This less-invasive approach offers patients the potential advantages of a smaller abdominal incision, reduced blood loss, and morerapid postoperative recovery. During RALP, the blood supply to prostate tissue is interrupted long before the specimen is removed from the body,

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exposing the tissue to longer periods of warm ischemia.² This may affect the quality of the sample for subsequent research purposes, including analysis of nucleic acid biomarkers. Studies have demonstrated that ischemic tissues exposed to higher temperatures are subject to greater and more-rapid RNA degradation. Factors such as warm ischemia and time at room temperature before tissue treatment affect downstream results of messenger RNA (mRNA) expression analysis of tissue specimens obtained during surgery.³

As part of routine quality control assessment of tissues procured into our specimen bank, we assess the quantity and quality of nucleic acids isolated from representative tissues. We have expanded this evaluation to examine in detail the quality of nucleic acids obtained from RALP and RRP. To more fully assess the effect of these approaches on downstream biomarker studies, we have compared the quality of DNA and RNA resulting from each surgical procedure using real-time polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of various sized targets.

MATERIALS AND METHODS

Sample Selection

Twenty frozen tissue samples were randomly selected from our prostate biospecimen repository of tissues obtained from patients who had prostatectomies, with the consent of an institutional review board–approved protocol, including 10 samples of RRP and 10 samples of RALP. Six procedures (30%) occurred in 2009 (3 RRP and 3 RALP) and 14 (70%) in 2006. Frozen aliquots of LNCaP, DU145, and PC3 prostate cancer cell lines ($1-3 \times 10^6$) were used as positive controls for amplification of various DNA and RNA targets, and the T47D breast cancer cell line was used as a negative control for the prostate-specific antigen (PSA) mRNA assays.

Tissue Sampling

Immediately after surgical removal, the prostate was transported to surgical pathology, and the exterior surface inked per routine procedure. The gland was cut at 0.5-cm intervals from apex to base, and each level arrayed and cut into quadrants. Depending on the size of the gland, 1 or 2 full slices were selected for research procurement; a 0.3-mm margin was removed for histologic examination, and the internal portions, constituting most of the slice, were harvested. These tissue slices were placed in plastic molds with embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek USA, Torrance, California) and snap-frozen in liquid nitrogen. The samples were then transferred to freezers at -80°C for long-term storage. The time between removal from the patient to snap-freezing averaged just more than 40 min for both RALP and RRP samples.

Sample Preparation

Using a cryostat at -20°C , 10 to 15 slices, at 10 μm thick, were cut from frozen tissue blocks. All surfaces and equipment used for tissue preparation were cleaned with 10% bleach, followed by 70% alcohol, to minimize contamination. Additionally, in conditions involving RNA extraction, equipment and surfaces were wiped with RNaseOut (G-Biosciences, Maryland Heights, Missouri) to minimize RNase exposure. Excess embedding medium was cut away to ensure maximal nucleic acid extraction quality. Tissue for DNA extraction was placed into Puregene Cell Lysis Solution (formerly, Gentra Systems, Minneapolis, Minnesota, now Qiagen, Valencia, California). Tissue for RNA extraction was placed into a Buffer RLT (RNeasy Mini kit, Qiagen). Samples were kept on wet ice during processing.

Nucleic Acid Extraction and Yield

DNA and RNA were extracted from each tissue sample (5 sections each of 10 μm) and cell line according to kit directions. Manual extraction of DNA was performed using Puregene DNA Isolation Kit (formerly Gentra Systems, Qiagen), according to the manufacturer's protocol, with a final sample elution volume of 50 μL . All DNA samples were stored at 4°C. Manual extraction of total messenger RNA was performed using RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol, with a final sample elution volume of 50 μL . All RNA samples were chilled on ice during use or stored at -80°C. DNA and RNA concentration and purity were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). All DNA measurements showed A_{260}/A_{280} readings of at least 1.8. All RNA measurements showed A_{260}/A_{280} readings of at least 2.0. All DNA and RNA sample concentrations were adjusted to 20 ng/ μL and 50 ng/ μL , respectively, for later experiments.

DNA Analysis

The integrity of the DNA was assessed qualitatively by electrophoresis on a 0.8% agarose gel and quantitatively by real-time PCR of genomic targets within the *TP53* gene of either 125 bp or 493 bp in length. Reactions were performed on a Light-Cycler 1.5 Instrument (Roche Diagnostics, Indianapolis, Indiana) using Light-Cycler DNA FastStart Master HybProbe (Roche Diagnostics) and *TP53* gene primer pairs (Table 1). An equal amount (40 ng) of DNA was added to each reaction to permit differences in C_t to reflect differences in DNA quality. The PCR mixture (20 μL in volume) consisted of LightCycler DNA FastStart Hybridization Probe reaction mix and enzyme, 0.5 μM each of forward and reverse primer, and donor and acceptor fluorescence resonance energy transfer probes (0.2 μM concentration each), 3 mM or 5 mM MgCl_2 for p53fwd125 or p53fwd493 respectively, and 2 μL of template DNA. After 10 minutes of enzyme activation and DNA denaturation at 95°C, 50 cycles of PCR were performed: 95°C for 10 seconds, 55°C for 5 seconds, and 72°C for 10 seconds. The reaction was then cooled to 40°C. Relative quantitative results were assessed by comparative cycle threshold (C_t) values.

The DNA was also assessed using the hemoglobin- β chain gene target of 262 bp in length. The PCR mixture (20 μL in volume) consisted of LightCycler FastStart DNA SYBR green reaction mix and enzyme, 0.07 μM each of forward and reverse primer (Table 1), 4mM MgCl_2 , and 2 μL of template DNA. After 10 minutes of enzyme activation and DNA denaturation at 95°C, 45 cycles of PCR were performed: 95°C for 15 seconds, 62°C for 10 seconds, 72°C for 24 seconds. The reaction was then cooled to 40°C. Relative quantitative results were assessed by comparative C_t values.

RNA Analysis

The RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) with the Nano 6000 LabChip kit (Agilent). This chip-based nucleic acid separation system determines RNA integrity using an algorithm that compares the intensity of the ribosomal RNA peaks to the low molecular weight (degraded) RNA ratio, generating a score of 1 through 10 (a RIN score or RNA integrity number). A RIN score of 10 represents a perfectly intact RNA sample.⁴ The RIN numbers between the RALP and RRP samples were compared using the Student *t* test.

Quantitative RT-PCR on a LightCycler 1.5 (Roche Diagnostics) was carried out targeting differently sized amplicons for β -2 microglobulin (*B2M*) mRNA (either 157 bp or 652 bp long), CK19 mRNA (either 148 bp or 743 bp long), and PSA mRNA (sizes ranged from 229 bp to 939 bp); primers and probes are listed in Table 1. As with the quantitative PCR for DNA, an equal amount of RNA (100 ng) was added to each reaction to permit differences in

C_t to reflect differences in RNA quality. The RT-PCR assay (20 μ L in volume) for *B2M* consisted of RNA SYBR Green buffer (Roche Diagnostics), RT-PCR enzyme mix, 4mM $MgCl_2$, forward and reverse primer (each at 0.5 μ M concentration), and 1 μ L of RNA template. Reverse transcription was carried out at 50°C for 10 minutes. After 10 min at 95°C for enzyme activation, 50 cycles of PCR were performed: 95°C for 10 seconds, 52°C for 10 seconds, 72°C for 30 seconds.

Quantitative RT-PCR assay for CK19 consisted of RNA Hybridization Probes 5 $\times$ buffer (Roche Diagnostics), RT-PCR enzyme mix, 1.28mM $MgCl_2$, forward and reverse primer (each at 0.5 μ M concentration), CK149-Prb TaqMan probe at 0.3mM concentration, and 2 μ L of RNA template. Reverse transcription was carried out at 50°C for 10 minutes. After 3 minutes at 95°C for enzyme activation, 50 cycles of PCR were performed: 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 15 seconds.

The RT-PCR assay for PSA consisted of RNA Hybridization Probe buffer, RT-PCR enzyme mix, 4mM $MgCl_2$, forward and reverse primer (each at 0.5 μ M concentration), PSATaq1 probe (a 6-FAM dye 5'-exonuclease probe) at 0.2 μ M concentration, and 1 μ L of RNA template. Reverse transcription was carried out at 55°C for 10 minutes. After 2 minutes at 95°C, 50 cycles of PCR were performed: 95°C for 10 seconds, 52°C for 10 seconds, and 72°C for 20 seconds.

RESULTS

Characteristics of the Patients

The patients selected for study showed virtually identical demographic and disease profiles, as summarized in Table 2. Although the total operative time was longer in cases of RALP than it was in cases of RRP, and blood loss was higher for patients with RRP than it was for patients with RALP, as expected, differences in results were not statistically significant. Warm ischemia time was not specifically available in this retrospective study; removal of the prostate from the body cavity occurs late in the course of RALP, whereas tissue removal is done shortly following devascularization in RRP. Our cases showed an average total operative time that was 40 minutes longer for the robotic prostatectomy cases, with the warm ischemia times being much longer.

Nucleic Acid Yield

The DNA extracts yielded 20 to 380 ng/ μ L per sample, with A_{260}/A_{280} values within the 1.8 to 2.0 range. The RNA extractions measured 50 to 100 ng/ μ L per sample, with A_{260}/A_{280} values consistently around 2.0. The amount of nucleic acid recovered varied because of differences in the sample processed. Histologic evaluation of each sample showed cellularity varying from 5% or 10% to 90% of each section, with the surface area of the frozen samples varying from approximately 1 to 4 cm²; thus, nucleic acid yields would be expected to vary considerably.

DNA Integrity

The DNA degradation was evaluated by 0.8% agarose gel electrophoresis, which showed a high-molecular-weight band for each sample, as shown in Figure 1. No differences between DNA isolated from RALP and RRP was visible. The DNA integrity was further assessed via a comparison of quantitative real-time PCR performed on various-sized amplification targets. No significant difference in positivity, as measured by C_t value, of either the shorter (125 bp), intermediate (262 bp), or longer (493 bp) amplified products was observed between DNA samples isolated from RALP and RRP specimens (Figure 2). The C_t values of these samples are equivalent to those of DNA prepared from LNCaP cells.

RNA Integrity

The RIN scores of 7 to 9, as measured by capillary electrophoresis, were obtained for most RNA samples as shown in Figure 3. The RIN scores of RALP samples were slightly lower on average (mean [SD], 7.8 [1.8]) than were RRP samples (8.3 [1.6]), although the difference was not statistically significant. One RALP and one RRP sample showed evidence of degradation with lower RIN scores of 3.6 and 4.0, respectively. Interestingly, these lower RIN scores did not correlate with operative time or time for sample procurement and freezing nor did they correlate with poor RT-PCR results.

The RNA integrity was further evaluated using quantitative RT-PCR to amplify mRNA targets of various lengths. Three mRNA targets were chosen for study: CK19 and *B2M* are housekeeping genes expressed in all cells, whereas PSA expression is restricted primarily to prostate epithelial cells. Degradation of PSA mRNA could reflect the epithelial cell integrity in these samples. No difference in quantitative RTPCR C_t values was noted for the larger- and smaller-sized CK19 and *B2M* amplicons as summarized in Figure 2 between RALP and RRP. However, the C_t values of some samples did vary for the PSA mRNA amplification. Two of the 10 RALP samples failed to show amplification of the largest (939 bp) amplicon length as shown in Figure 2. These same samples also showed suboptimal amplification of the smallest (229 bp) amplicon. When studied in more detail across a range of amplicon sizes, one of these samples showed no amplification of PSA mRNA beyond 368 bp, and the other failed amplification beyond 654 bp (data not shown). Of note, these RALP samples with decreased PSA mRNA were the 2 cases with the longest RALP operative times (290 and 366 minutes); however, both had adequate RIN scores of 8.3 and 6.6, respectively. The 2 samples (one RALP lane 2 and one RRP lane 7 in Figure 3) that showed degradation as evidenced by RIN numbers < 4.0 showed excellent amplification of all sizes of PSA transcript targets across all sizes in this study.

COMMENT

Prostate tissues procured into tumor repositories are increasingly likely to be obtained by RALP than the previously standard RRP. However, tissues harvested via RALP are removed from the circulating blood supply long before removal from the abdominal cavity resulting in longer periods of warm ischemia, which may in turn lead to nucleic acid degradation, compromising the quality of studies of these samples. In a study⁵ specifically examining RALP and RRP resections of the prostate gland, immunohistochemical staining, DNA gel analysis, and RNA integrity calculated by microfluidic capillary electrophoresis have been compared, suggesting that little degradation occurred in samples collected following RALP. Another study examined tissue microarray results for immunohistochemical analysis, and quantitative PCR gene expression, again suggesting that mRNA profiles from RALP and RRP were adequate for expression analysis.⁶ However, RT-PCR targets in that study⁶ were all smaller than 150 bp, and that study did not examine PCR amplification results in a quantitative manner.

Studies have shown that quantitative RT-PCR performance is affected by RNA integrity, with more intact RNA yielding better amplification, as evidenced by lower cycle numbers by real-time PCR.⁷ The importance of RNA quality rises with increasing length of the amplified product, with amplification products larger than 400 bp being strongly dependent on good RNA quality (RIN score of at least 5).⁷ Interestingly, our series included 2 samples showing no reduction in amplification of mRNA targets, despite RIN scores of 4 or below.

In contrast, this study expands the investigation to include more RRP and RALP samples and analyzes the RNA both quantitatively and qualitatively by targeting increasing amplicon sizes up to nearly 1 kb in length. An equivalent amount of nucleic acid was added to each

quantitative reaction so that a decrease in nucleic acid quality would be directly reflected in increased C_t values. Similar to previous studies, the amplification of small targets is equivalent in RALP- and RRP-derived samples. However, one-fifth of the RALP samples failed amplification of the largest PSA target (936 bp); this correlated with the length of operative time in RALP procedures. Possibly, the extended period of warm ischemia could have led to some deterioration of markers, specifically the PSA mRNA. Unfortunately, warm ischemia time was not recorded for each of the procedures and, therefore, can not be analyzed to confirm this hypothesis. The observation of failed RT-PCR for PSA, but not CK19 or *B2M*, could reflect differences in degradation rates of various mRNA species or, perhaps, a different response to hypoxia of the epithelial compartment within the prostate tissue. CK19 and *B2M* were chosen as overall reference genes for mRNA quality because of broad patterns of expression in different cell types, whereas PSA expression is restricted primarily to prostate epithelial cells.

We have demonstrated that there is no difference between RALP and RRP in C_t values in DNA targets (493 bp), but there is some difference in RNA targets regarding the RALP surgical method, which was evident in PSA gene expression analysis. Results showed that, although the PSA gene was expressed for all samples tested (this is shown by the amplification of the smallest target in all samples), the RNA for 2 of the 10 laparoscopic samples did not amplify at longer base pair lengths.

CONCLUSION

Past studies have suggested equivalency in sample quality between prostate tissue obtained from RALP and RRP; these studies examined immunohistochemical results, viability in tissue culture, nucleic acid integrity by gel electrophoresis, and mRNA expression profiles.^{3,5,8} We demonstrated similar results between RALP and RRP in C_t values for DNA targets (493 bp), but amplification failures were observed in some larger RNA targets from RALP specimens. We have performed more detailed studies to demonstrate some reduction of RT-PCR quality for large amplicons from some mRNA transcripts, suggesting that the longer period of warm ischemia may affect downstream studies. However, the quality of DNA amplification and most RNA amplification studies was uncompromised. Thus, prostate tissues obtained from RALP are suitable for nucleic acid evaluation, provided that appropriate controls are assessed, and amplicon sizes are kept small.

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Figure 1. Agarose gel analysis of DNA with DNA/HindIII markers (L). Lanes 1 through 5 are samples obtained from laparoscopic prostatectomies. Lanes 6 through 10 are samples obtained from robotic prostatectomies.

Figure 2.

Reverse transcriptase-polymerase chain amplification of various DNA and RNA targets reflects the quality of nucleic acid in each sample. Increasing cycle threshold (C_t) values reflect poor amplification. The C_t values for each target (target and size noted at the top of each column) are shown for the robotic-assisted laparoscopic prostatectomy (RALP), retropubic radical prostatectomy (RRP), and cell line samples. The open circles among the RALP samples signify the results of the 2 cases with the longest operative time.

Figure 3. Microcapillary gel analysis of RNA. Lanes 1 through 5 are robotic-assisted laparoscopic prostatectomy (RALP) samples; lanes 6 through 10 are radical retropubic prostatectomy (RRP) samples. Lanes 2 and 7 (one RALP sample and one RRP sample) show degradation, with RNA integrity scores of 3.6 and 4.0. However, there are no significant signs of difference between the surgical methods.

Table 1

Primer and Probe Sequences

Primer	Sequence	Length, bp
p53		
p53rev	GAC TGG AAA CTT TCC ACT TG	
p53fwd125	TTC CTA GCA CTG CCC AAC A	125
p53fwd493	CTT CTC CTC CAC CTA CCT G	493
p53DonorProbe	CCC CAG CCA AAG AAG AAA CCA CTG GAT GGA GAA T-FITC	
p53AcceptorProbe	Cy5.5-TTT CAC CCT TCA GGT ACT AAG TCT TGG GAC CTC TT-phos	
Hemoglobin β chain		
BG07	GGT TGG CCA ATC TAC TCC CAG G	
BG08	TGG TCT CCT TAA ACC TGT CTT G	262
β -2 microglobulin		
β 2-UP2	CTT GTC TTT CAG CAA GGA CTG	
β 2-DN2	CCT CCA TGA TGC TGC TTA CAT	157
β 2-DN7	AGA TTA ACC ACA ACC ATG CCT	652
Cytokeratin-19		
CK148FWD	CAG AGC CTG TTC CGT CTC AAA	
CK148REV	CAG AGC CTG TTC CGT CTC AAA	148
CK149-PRB	6FAM-CAC CAT TGA GAA CTC CAG GAT TGT CCT GC-BBQ	
CK743FWD	GAC TAC AGC CAC TAC TAC ACG ACC	
CK743REV	AGC CGC GAC TTG ATG TCC ATG AGC C	743
Prostate-specific antigen		
PSA231F18	CTG CCC ACT GCA TCA GGA	
PSA440R20	ATG ACC TTC ACA GCA TCC GT	229
PSA580R19	CGC ACA CAC GTC ATT GGA A	368
PSA619R22	AGC ACA CAG CAT GAA CTT GGT C	410
PSA703R20	TGA TAC CTT GAA GCA CAC CA	492
PSA782R19	TCC ACT TCC GGT AAT GCA C	570
PSA864R21	GCC TGG TCA TTT CCA AGG TTC	654
PSA1086R22	CCC CAC AAA TAA CAC AGA CAC C	877
PSA1153R17	TGT CCA GCA CAT GTC AC	939
PSATaq 1Probe	6FAM-CAG CTT CCC ACA CCC GCT CT-TAMRA	

Table 2

Patient Demographics

Characteristic	RALP, n = 10, mean (SD)	RRP, n = 10, mean (SD)
Age, y	61.1 (5.8)	61.9 (4.1)
Gleason score	6.5 (0.5)	7.2 (0.6)
Blood loss, mL	312.5 (224)	640 (324)
Total operative time, min	201.1 (84)	159.3 (42)
Time until tissue received into freezer, min	45.5 (20)	42.8 (8.5)

Abbreviations: RALP, robotic-assisted laparoscopic prostatectomy; RRP, retropubic radical prostatectomy.