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Integrity of Prostate Tissue for Molecular Analysis After Robotic-Assisted Laparoscopic and Open Prostatectomy

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Abstract

Objectives—Tissue warm-ischemia time prior to fixation for pathological analysis has been linked to changes in cell morphology, as well as nucleic acid and protein integrity. Robotic-assisted laparoscopic prostatectomy(RALP) results in longer warm-ischemia times compared to open radical retropubic prostatectomy(RRP). To assess the effect of longer ischemia times on biomolecular integrity we analyzed DNA, RNA and protein collected from robotic and open prostatectomies.

Methods—Specimens obtained from 22 consecutive RALP (11) or RRP (11) operations were examined after H&E staining by light microscopy. To assess protein integrity, immunohistochemical staining for p63, E-cadherin, and AE1/AE3 was performed. DNA was assessed by gel analysis. An RNA integrity score was determined by microfluidic capillary electrophoresis and calculated based on the electropherogram and simulated gel view. Finally, epithelial cells were cultured on collagen-coated plates.

Results—No differences in clinicopathologic characteristics existed between groups with the exception of a significantly longer warm-ischemia time during RALP (82+/-23min) versus RRP (23 +/-2min) (<0.001). Tissue integrity was suitable for the assessment of pathologic grade and stage for all samples. Protein and DNA analyses demonstrated no evidence of degradation in any samples. No significant differences in the RI scores were demonstrated between surgical approaches. Prostate epithelial cells were cultured successfully in 66% of RALP specimens.

Conclusion—Robotic prostatectomy, though it involves additional exposure to warm-ischemia, does not significantly affect histopathological characteristics or the biomolecular integrity of the specimen. Provided a rapid response occurs for tissue banking after specimen removal, molecular research studies utilizing prostate tissue harvested via RALP appear feasible.

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Keywords

robotic-assisted laparoscopic prostatectomy; tissue banking; RNA

Introduction

Over the last five years, the use of robot-assisted laparoscopic prostatectomy (RALP) has increased dramatically. It is expected that 16,000 RALPs, or roughly 20% of the total number of radical prostatectomies, will be performed in 2006. As the use of RALP becomes more widespread, prostate tissue available for research use will have to increasingly be obtained via this method. During robotic prostatectomy, the prostate is devascularized and remains intracorporeal at body temperature (37°C) until the laparoscopic ports are removed¹. This results in the prostate tissue being subject to a greater period of warm ischemia than that seen with standard open prostatectomy. Studies have demonstrated more rapid RNA degradation in ischemic tissues exposed to higher temperatures². To date, studies have not been published examining whether tissues obtained by RALP are suitable for molecular studies and cell culture, and furthermore whether they are comparable to samples obtained by open radical robotic prostatectomy (RRP).

Materials and Methods

Specimen Collection and Processing

Tissues were obtained for histological and molecular analysis from consecutive robot-assisted laparoscopic prostatectomies (RALP) or radical retropubic prostatectomy (RRP) under an IRB approved protocol between March and June 2006. All surgeries were performed for removal of biopsy-proven prostate adenocarcinoma and patient characteristics are displayed in Table 1. A total of 11 consecutive specimens were analyzed from each group (22 patients). After removal of the specimen, the tissue was rapidly transported to Pathology and a segment flash-frozen in liquid nitrogen and stored at -80°C. The remainder of the specimen was then processed in a standard fashion using 10% formalin overnight, paraffin-embedding and sectioning on a microtome³. Times to specimen fixation and freezing were recorded. Hematoxylin and Eosin (H&E) staining was performed in a standard fashion and evaluated by a genitourinary pathologist (WH).

Immunohistochemistry

Immunohistochemistry was performed as previously described⁴. Four µm formalin-fixed, paraffin-embedded sections were subject to a heat induced epitope retrieval (HIER) procedure and loaded onto an automated immunohistochemistry stainer (gen^{ITM}, Ventana Medical Systems, Inc., Tucson, AZ). Antibodies used included an epithelial cell membrane protein E-cadherin (1:100 dilution (Clone ECH-6) Cell Marque, Hot Springs, AZ), a nuclear basal cell protein p63 (1:100 dilution (Clone BC4A4) BioCare Medical, Concord, CA), and a cytokeratin stain for epithelial cells AE1/AE3 (1:200 dilution; DakoCytomation, Carpinteria, CA). Negative control slides were incubated with Mouse or Rabbit Isotype Controls (Zymed Laboratories, Inc., South San Francisco, CA). Staining percentage was measured as negative (<5%), 1+(6–25%), 2+(26–50%), 3+(51–75%) and 4+(76–100%) as previously described⁴.

DNA isolation and analysis

DNA was generated from 6 open and 6 robotic tissue specimens using the QIAamp DNA mini kit (QIAGEN, Valencia, CA) per the manufacturer's protocol. Briefly, tissue was homogenized and lysed with Proteinase K in buffer for 2 hours. The samples were treated with RNase A followed by 100% ethanol and placed on a QIAamp spin column for the isolation of DNA.

Once DNA was isolated and quantitated, one microgram was placed on a 1% agarose gel using GenePure LE agarose (GeneMate, Kaysville, UT) and subject to electrophoresis and imaging. All DNA samples were stored at 4°C.

RNA isolation and analysis

RNA was generated from the samples after tissue homogenization using a using RNeasy Mini Kit (QIAGEN, Valencia, CA) per protocol. DNase treatment was used on the column to eliminate DNA from the final product. RNA integrity and quantitation was analyzed using the Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA)⁵. In brief, 1ul of RNA was loaded onto the microfluidic chips in the priming station. Samples were then automatically subject to gel-based electrophoresis, staining, destaining, band detection, and imaging. RNA integrity was evaluated by examination of the electropherogram and simulated gel view⁶. Criteria included baseline flatness, existence of additional or noise peaks between ribosomal peaks, low molecular weight species contamination, and the presence of genomic DNA among others. An integrity number from 1–5 (1 being greatest integrity) was given to each sample based on these criteria by two blinded analyzers (V.X.F., N.A.) using previously published criteria⁶. Ribosomal peak heights are automatically generated and the ratios determined. RNA was stored at –80°C.

Epithelial Cell Culture

Epithelial cell cultures were then established from 3 consecutive fresh tissue samples as we have described⁷. Briefly, fresh prostate tissues were minced and plated on 100-mm collagen dishes in F-12+ medium with penicillin/streptomycin. F-12+ is a supplemented Ham's medium (Life Technologies, Inc., Gaithersburg, MD; intermediate Ca²⁺, 0.3 mM) also containing 1% FBS, bovine pituitary extract (25 µg/ml; Life Technologies, Inc.) and cholera toxin (100 ng/ml; Life Technologies, Inc.). Cultures were passaged routinely when confluent after dispersion with trypsin-EDTA (Life Technologies, Inc.).

Results

Patient age, pathology and other clinicopathological factors were similar between the open and robotic groups (Table 1). To determine ischemia time for the robotic and open cases, the minutes from the incision of the bladder neck and posterior pedicles until specimen removal from the body were documented. This is the point where the first arterial inflow, the urethral branches of the prostatic artery (in turn arising from the inferior vesical artery), is disrupted. The mean for robotic surgery (82 min) was significantly longer than open surgery (23 min). We initially reviewed the histopathology, using light microscopy, from all patients in both groups to determine if there were any alterations in cellular morphology. No significant ischemia-related differences including eosinophilia, cellular swelling, karyolysis or karyorrhexis were noted. Tissue integrity was suitable for assessment of pathologic grade and stage for all samples. There were rare focal areas of ischemia, recognized by cellular eosinophilia and epithelial breakdown, in sections of both robotic and open radical prostatectomy specimens that were attributed to the standard fixation process.

Protein integrity was then assessed in 6 RALP and 6 RRP by immunostaining using antibodies against a variety of proteins in both the nuclear and cytoplasmic compartments of epithelial and stromal cells. (Figure 1) Quantitation of staining revealed no statistical differences in staining scores between open and robotic specimens for E-cadherin (SI(mean) = 4 open; 4 robotic), p63 (SI(mean) = 4 open; 4 robotic), and AE1/AE3 (SI(mean) = 3.6 open; 3.8 robotic). Thus, there was no degradation of these immunohistochemically detectable antigens within these specimens.

To assess DNA integrity, gel electrophoresis was performed and the lanes assessed for low molecular degradation products and laddering (Figure 2). No differences were noted and no significant degradation of DNA was found when the two approaches were compared.

RNA was then analyzed for 6 samples from each group and compared using both microfluidic capillary electrophoresis tracings and simulated gel views (Figure 3). Determination of RNA integrity (RI) value using gel analysis was based on the combination of electropherogram (peak), gel views, and ratio of 28S/18S as described⁶. Assignment of an RNA integrity number, as well as comparison of the 28S/18S ratio, demonstrated no significant differences between RALP and RRP samples (Table 2). We found significant RNA degradation in 2 RRP and 1 RALP specimens. Thus, variation in RNA integrity appears not to be associated with the surgical approach, but rather with other aspects of tissue handling.

Our final approach to assess tissue integrity was to mince the tissue and attempt to generate pure epithelial cultures as we have described with RRP specimens⁷. This technique is routinely performed at our institution and roughly 75% of RRP samples generate pure epithelial cultures (data not shown). A total of three prostate samples from the RALP group were cultured which resulted in the growth of pure epithelial cells within 7–10 days in two of the three samples. No fibroblast or stromal growth was observed on the plates. This finding indicates that, in the majority of cases, the epithelial cells obtained by RALP are functionally intact and able to replicate.

Discussion

Current research to clarify the molecular basis of prostate cancer relies on the integrity of DNA, RNA, and protein. Tissue is usually collected from a specimen obtained during RRP and rapidly processed. As RALP becomes more common, the tissue available to scientists will be increasingly harvested via this method. However, differences in the techniques result in the prostate, after devascularization, being left intracorporeal at a higher temperature (37°C) for a longer period of time with RALP. In this first study to compare open and robotic tissues, we find tissues obtained via both techniques to be acceptable for tissue banking and molecular studies.

We specifically examined RALP tissue early in our experience when operative times were longer to present a more extreme situation. During the time period examined, this additional time was roughly 60 minutes greater in the RALP specimens than RRP specimens. Warm ischemia has been associated with morphological changes in cells, including cellular swelling and autolysis⁸. These changes, if sizeable enough, could theoretically impact the pathological examination of tissues. In our series, we did not find that this increased exposure to warm ischemia time significantly impacted the microscopic appearance of our RALP specimens. Gleason grading and margin analyses were able to be performed.

Our work specifically compared prostate tissues collected via both RRP and RALP techniques and found that DNA, RNA, and protein integrity was equivalent. RNA is a transient molecule and breaks down rapidly during cellular damage. Previous studies using real-time reverse transcription polymerase chain reaction have found the incubation of mouse tissues at 37°C profoundly decrease mRNA gene levels to 10% after 4 hours, however little change occurs before 80 minutes⁹. We utilized newer, comprehensive analytical methods, based on a microfluidic chip analysis, to examine both the spectrum of RNA fragments and generate an integrity score. Microfluidic chip analysis is a highly sensitive method for examining RNA integrity and confirming purity and is increasingly being used in gene expression profiling platforms¹⁰. Degradation in both RALP and RRP samples we attributed to factors other than surgical approach. These include delayed pathologic processing or unequal storage. Other

studies have looked at the impact of warm ischemia on RNA integrity. Ohashi *et al.* processed benign human breast tissue left at room temperature at intervals between 10–180 minutes and found no loss of RNA integrity during this time period¹¹. In spontaneous canine tumors, significant variation in RNA degradation rates occurred with degradation occurring as early as 15 minutes¹². However, this study failed to use RNase-free buffers during processing. Our approach utilizing microcapillary electrophoresis on human prostate tissues provides reassurance that RNA from these RALP specimens is largely functional.

We utilized immunohistochemistry to assess antigen preservation of three different markers (E-cadherin, AE1/AE3, and p63) within different cellular compartments, and find no differences in integrity between surgical approaches. E-cadherin immunostaining, in particular, has been documented to be sensitive to ischemia and degradation¹³. Antigen stability detected by immunohistochemistry is clearly affected by tissue handling between excision and freezing¹⁴. Given our RNA data and success in initiating successful cell cultures, further protein testing with additional markers or other methodological approaches (ELISA, western blot) were deemed unnecessary. Epithelial cells are more sensitive to ischemic damage than fibroblasts lending a rationale for the culture of this cellular subtype¹⁵.

Conclusions

Given these findings of biomolecule integrity, we can recommend proceeding with molecular research studies utilizing prostate tissue harvested via RALP. It is important however to emphasize that a rapid and standardized tissue processing approach, involving multiple services, will reduce changes in molecular profiles¹⁶. In a large study of a cooperative tissue bank, DNA was useable in over 80% of samples while RNA was functional in only 60%¹⁷. It is recommended that an analysis of individual samples be performed prior to their use in molecular studies given the variation in RNA integrity found between samples.

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Abbreviations

RALP	Robotic-assisted laparoscopic prostatectomy
RRP	Radical retropubic prostatectomy
SI	staining intensity

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Figure 1. Analysis of prostate tissue by immunohistochemistry for E-cadherin protein expression. (A) Specimen surgically removed utilizing a robotic-assisted laparoscopic approach and (B) Open radical retropubic approach. No alterations in the expression of these or other proteins (see text) were detected between surgical approaches. Tissue architecture is maintained in both specimens.

Figure 2.

Analysis of DNA by gel electrophoresis. Lanes 2–7 represent specimens from conventional open radical retropubic prostatectomies. Lanes 8–13 represent specimens from robotic prostatectomies. No smaller DNA fragments (or laddering) representing breakdown are seen with either surgical approach. A *Bam-HindI* ladder is displayed in lane 1.

Figure 3.

Analysis of RNA by microcapillary, automated gel analysis. Lanes 1–2 and 6–9 are specimens obtained from open radical retropubic prostatectomies. Lanes 3–5 and 10–12 were obtained from robotic assisted laparoscopic prostatectomies. The two distinct bands at 2000 and 4000 are 18S and 28S ribosomal RNA respectively. Significant RNA degradation (multiple lower bands) is seen in lane 6, with minor degradation in lanes 5, 9 and 12. Integrity of the RNA is assessed by visualization of the 28S and 18S ribosomal RNA bands and by the generated electropherogram (not shown). Microfluidic chip analysis is a highly sensitive method for examining RNA integrity and purity prior to gene expression analysis.

Table 1

Patient Characteristics and Sample Ischemia Time.

	RRP (n=11)	RALP (n=11)	p-value
PSA (ng/ml)	12.47(+/-12.2)	7.94(+/-3.2)	<0.001
Age (yrs)	58.3(+/-8)	61.9(+/-6)	0.39
Gleason Score	6.3(+/-0.5)	6.6(+/-0.5)	0.8
Size (grams)	83.5(+/-61.8)	46(+/-21.6)	0.003
Ischemia time * (min)	23(+/-2)	82(+/-23)	<0.001

* Ischemia time is defined for RRP and RALP is defined as the point at which the bladder neck/posterior pedicles are transected until the specimen is flash frozen.

Table 2

RNA concentration and 28S/18S ratio calculated for open and robotic prostatectomy specimens using the Experion™ electrophoresis system. The mean 28S/18S ratio was calculated for both groups (n=6).

	Sample Number	RNA Concentration (ng/μL)	28S/18S Ratio	RNA Integrity Value [*]
RRP	1	9.16	1.46	1
	2	27.81	1.17	1
	6	5.27	1.40	5
	7	24.96	1.27	1
	8	13.53	1.33	1
	9	18.05	2.42	4
			Mean = 1.508	Mean = 2.17
RALP	3	7.80	1.58	1
	4	17.94	1.31	1
	5	16.73	0.91	3
	10	21.12	1.19	1
	11	15.22	1.02	1
	12	15.52	1.50	2
			Mean = 1.252	Mean = 1.5

* No significant differences in RNA integrity between groups.