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Original Article

Influence of hypoxia induced by minimally invasive prostatectomy on gene expression: implications for biomarker analysis

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Abstract: Handling and processing of clinical specimens during and after surgical resection may significantly skew the molecular data obtained from analysis of those samples. Minimally invasive prostatectomy was used as a model to specifically study effects of surgical ischemia on gene expression in human clinical samples. Normal prostatic urethra cup biopsies were procured from 12 patients at three time points during laparoscopic radical prostatectomy. Homogeneous cells (stroma and epithelium) were microdissected. Transcript analysis of 3 oxygen-dependent, 3 oxygen-independent, and 3 control class genes was performed using quantitative RT-PCR. Data were analyzed by relative quantitation and two-sided t-test. Patient demographic and time covariates were fit by a linear mixed model. VEGF, an oxygen-dependent gene, showed significant expression alterations across three time points in epithelium ($p=0.008$), but not in stroma ($p=0.66$). Expression levels of VHL, STAT5B, and CYPA showed significant changes at the $p<0.05$ level in the stroma only. Effects of age, PSA, prostate size, Gleason score, surgery type, total surgery time, total ischemia time, and estimated blood loss on VEGF expression over time were not significant at the $p<0.01$ level. Therefore, surgical manipulation and tissue processing methods need to be taken into account when assessing prostatic biomarkers; however, resection does not dramatically alter mRNA profiles in prostate specimens.

Keywords: Laparoscopic surgery, prostatectomy, warm ischemia, hypoxia, tissue microdissection, gene expression analysis

Introduction

Tissue based biomarkers have the potential to provide clinical and molecular information concerning tumor development and progression [1] and predict clinical endpoints [2]. However, variability in tissue handling and processing of resected samples may complicate the integrity, reproducibility, and interpretation of results from profiling assays (e.g. microarrays and protein arrays). Several surgical variables, including tissue manipulation and anoxia [3, 4], can potentially alter mRNA or protein expression levels and cast concern over the validity of studies

preformed with these clinical specimens [5].

The development of minimally invasive surgery has led to increasing use of laparoscopic and robotic-assisted laparoscopic surgical techniques in management of prostate cancer, significantly altering intraoperative handling of the specimen, including the early devascularization of the prostate. This results in significantly longer warm ischemia times before the organ is removed and available for procurement, potentially impacting the quality of expression profiles for prognosis. Using minimally invasive prostatectomy as a model, we investigated sequential effects of intra-operative ischemia on selected genes in microdissected cells from non-malignant clinical specimens to explore confounding effects on mRNA expression.

Methods

Tissue origin and study design

The study consisted of 12 patients with clinically localized prostate cancer undergoing minimally invasive prostatectomy as first-line therapy (Table 1). The protocol was approved by the Institutional Review Board of the National Cancer Institute. Patients received no prior therapy before surgery. An antegrade Montsouris approach to dissection of the prostate and seminal vesicles with early division of the vascular pedicles was performed. Upon complete dissection of the prostate from the patient, the prostate was isolated within a sealed specimen bag and left within the abdomen until completion of the procedure as is typically performed. A bilateral pelvic lymph node dissection and vesicourethral anastomosis were then completed followed by extraction of the intact specimen from the patient.

To minimize the potential confounding effects associated with random sampling of prostate adenocarcinoma, normal prostatic urethra was chosen as test tissue due to its uniform blood supply, accessibility, and relative histologic homogeneity. Non-malignant proximal prostatic urethra biopsies from the bladder neck were obtained from patients at defined time points during laparoscopic or robotic-assisted laparoscopic radical prostatectomy (**Table 1**). 36 snap-frozen biopsies from 12 cases were examined and 30 biopsies from 10 cases (**Table 2**) were included based upon epithelial and stromal cell content, histologic homogeneity, and quality of the mRNA.

Tissue sampling and processing

An identical transperitoneal approach and specimen handling procedure was used in all cases. Three urethral samples from the proximal prostatic urethra were obtained intraoperatively using 5 mm laparoscopic biopsy forceps during each case at defined time points of the procedure (**Figure 1**), immediately imbedded in OCT, frozen on dry ice, and stored at -80°C. First biopsy time = 0 (T0) was taken before the blood supply to the prostate was disrupted, when incision through the anterior bladder neck allowed access to the anterior portion of the prostatic urethra. The urethra had not yet been exposed to ischemia and basolateral vascular pedicles were still intact. Second biopsy time = 1 (T1) was collected after the prostate was dissected from the patient just prior to isolating the prostate in the laparoscopic bag (mean of 87 min after T0). Prostate and associated urethra was without blood perfusion representing the point at which tissue samples could be procured for research if immediate retrieval was performed. Third biopsy time = 2 (T2) was taken after extraction of the specimen from the patient (mean of 127 min after T1). The prostate had been exposed to a significant period of warm ischemia and T2 marks the time at which research tissue samples would normally be procured from most laparoscopic or robotic radical prostatectomy cases.

Microdissection and RNA isolation

Microdissection was employed to improve tissue sample homogeneity by isolating homogeneous cellular material (epithelium, stroma) for subsequent transcript analysis by quantitative RT-PCR (qRT-PCR). Each tissue sample was cut, stained, and dissected according to routine methods [6, 7]. Approximately 7,000 epithelial cells (~ 2,000 laser shots) and 10,000 stromal cells (~ 3,000 laser shots, superficial and deep stroma) were microdissected separately [8].

Total RNA of each individual sample was extracted, isolated, and assessed for quantity and quality [9] with NanoDrop (NanoDrop Technologies) and BioAnalyzer (Agilent Technologies Inc.), according to manufacturer protocols. Total RNA was also assessed by RNA Integrity

Table 1. Patient demographics and clinical information

Patient No.	Age (yrs)	Race / Ethnicity	PSA (ng/dL)	pStage	Prostate Size (g)	Gleason Score	Surgery Type	Total Surgery Time (min)	Total Ischemia Time (min)	Nerve Sparing	Estimated Blood Loss (mL)	T0 Biopsy Size (mm)	T1 Biopsy Size (mm)	T2 Biopsy Size (mm)
1	63	C	4.9	pT2b	47	3+4=7	RL	345	115	bilateral	250	2.0	2.2	3.6
2	64	C	3.9	pT2a	44.2	3+3=6	RL	335	190	bilateral	200	4.3	4.0	6.4
3	57	C	2.35	pT2c	21.4 *	3+4=7	L	653	219	bilateral	300	5.0	4.7	5.0
4	63	C	4.8	pT3a	32	3+4=7	L	462	230	bilateral	400	2.9	4.1	3.9
5	48	C	3.2	pT2c	35	3+3=6	L	352	168	bilateral	600	4.8	7.6	9.0
6	56	C	14.9	pT2b	32	3+4=7	RL	393	248	bilateral	300	3.2	2.7	4.5
7	61	C	2.3	pT2c	67	3+4=7	RL	401	260	bilateral	700	2.3	3.8	4.9
8	45	C	1.3	pT2a	33.5	3+3=6	RL	382	280	bilateral	700	3.1	2.0	4.0
9	65	C	6	pT3a	43.6	4+3=7	RL	371	240	bilateral	250	3.7	3.9	4.1
10	65	AA	4.3	pT3a	37	4+4=8	RL	360	234	bilateral	350	3.4	5.1	4.7
Mean	60		4.8		41.3	7		405.4	218.4		405	3.7	3.9	5.0

pStage = pathologic stage, according to criteria of the 2002 American Joint Committee on Cancer.
RL = Robot assisted laparoscopic prostatectomy
L = Laparoscopic prostatectomy
C = Caucasian
AA = African American
* Reported as cubic centimeters. Converted to gram weight assuming density equal to 1.071g/cc (according to http://www.rwc.uc.edu/koehler/biophys/1prob.html)

Table 2. Histological review of biopsy tissue specimens

Case	Inflammation (characteristics)	Edema
1A*	0	+
1B	+(S)	+
1C	++(IE)	0
2A	+(S)	0
2B	++(S)	+
2C	++(IE)	0
3A	++(IE)	0
3B	+(S)	+
3C	++(S)	++
4A	+(S, C)	0
4B	+++(S)	+
4C	++(S)	++
5A	+(S, C)	0
5B	++(S, IE)	+
5C	++(S, IE)	+
6A	0	+/-
6B	+/-(IE)	0
6C	+/-(IE)	+
7A	++(A, C)	+
7B	++(A, C)	++
7C	+++(A, C)	++
8A	+(M, SE, C)	0
8B	+(SS, C, A)	++
8C	+++(M, IE, C, A)	++
9A	+(C, M)	+
9B	++(C, M)	++
9C	++(C)	+
10A	++(C, S)	0
10B	++(C, S)	0
10C	++(A, C)	+/-

Case designations: A = Time 0 (T0), B = Time 1 (T1), C = Time 2 (T2); * Possible prostatic tissue – sample was not used in analysis; Inflammation characteristics: S = Stroma, IE = Intraepithelial, A = Acute, CH = Chronic, SE = Subepithelial, SS = Superficial stroma, M = Mucosa

Number (RIN) using 2100 Expert software (Agilent Technologies Inc.).

Gene selection

Because biopsies were obtained from the proximal prostatic urethra, which is intimately associated with the prostate during the entire surgical procedure, results seen in these tissue biopsies are extrapolated to be representative of surgical ischemia effects on gene expression in the prostatic epithelium and stroma. Ten genes constitutively expressed in various human tissues, including prostate, were chosen for gene expression analysis and segregated into three groups (**Table 3**). The oxygen-dependent group included genes involved in the cellular pathways responsive to hypoxia [10]. The oxygen-independent group included genes known to be unresponsive to hypoxia. These genes were identified from previous prostate gene expression array studies and found to be dysregulated in both moderate- and high-grade prostate cancer [11] and/or androgen-independent prostate cancer [9]. Control group included endogenous housekeeping genes routinely used as internal controls for qRT-PCR gene expression analysis

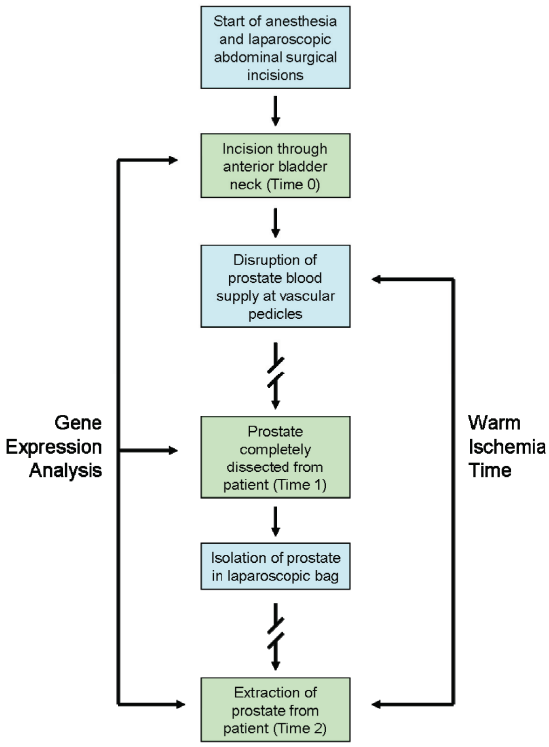


Figure 1. Warm ischemia tissue collection study design. Normal urethral biopsies taken at three time points: Time 0 = T0, Time 1 = T1, Time 2 = T2. T1 represents the time point at which samples would be collected during routine open prostatectomy. T2 represents the time point at which samples would be collected during routine laparoscopic or robotic prostatectomy.

Table 3. Genes evaluated as oxygen-dependent, oxygen-independent, and controls for analysis of warm ischemia effects on gene expression from tissue biopsies collected at three distinct time points during minimally-invasive prostatectomy

Symbol* (Entrez Gene Official Symbol)	Entrez Gene GeneID	Gene name	Gene Group**	Cellular Function	Map
HIF1a (HIF1A)	3091	Hypoxia-inducible factor 1, alpha subunit	Oxygen-dependent	Transcription factor activity	14q21-q24
VEGF (VEGFA)	7422	Vascular endothelial growth factor A	Oxygen-dependent	Vascular endothelial growth factor binding	6p12
VHL (VHL)	7428	von Hippel-Lindau tumor suppressor	Oxygen-dependent	Transcription factor binding	3p26-p25
LAMP2 (LAMP2)	3920	Lysosomal-associated membrane protein 2	Oxygen-independent	Lysosome transport	Xq24
MTR (MTR)	4548	5-methyltetrahydrofolate-homocysteine methyl-transferase	Oxygen-independent	Dihydropteroate syn-thase activity	1q43
STAT5B (STAT5B)	6777	Signal transducer and activator of transcription 5B	Oxygen-independent	Signal transduction activity	17q11.2
18s (LOC100008588)	100008588	18s ribosomal RNA	Control	Ribosome subunit	***
ACTB (ACTB)	60	β-actin	Control	Cytoskeletal structural protein	7p15-p12
CYPA (PPIA)	5478	Cyclophilin A	Control	Serine-threonine phosphatase inhibitor	7p13-p11.2
TFRC (TFRC)	7037	Transferrin receptor	Control	Cellular iron uptake	3q29

* Gene symbol used in this paper; ** Gene class assigned for the purposes of this paper; *** 18s rRNA is a ribosomal subunit and not a gene that is mapped to a specific chromosome; therefore, no map location is given.

Table 4. TaqMan assay primer probe set locations related to length of mRNA or rRNA

Gene	RefSeq	RNA Length (bp)	Exon Boundaries	Amplicon Length (bp)	Assay Center Location (bp)
HIF1a	NM_181054.1	3812	4 - 5	76	746
VEGF	NM_001025366.1	3665	1 - 2	77	1096
VHL	NM_000551.2	2968	2 - 3	72	679
LAMP2	NM_013995.1	4006	1 - 2	93	200
MTR	NM_000254.1	7122	1 - 2	57	319
STAT5B	NM_012448.3	5171	1 - 2	91	297
18s	X03205.1	1869	-	187	603
CYPA	NM_021130.3	2276	4	98	435
TFRC	NM_003234.1	5010	14	105	1765
ACTB	NM_001101.2	1793	1	171	40

[8, 12]. ACTB is one of the most stable genes in human prostate and bladder cancer and was used in this study as the endogenous control for normalization of gene expression [8, 13].

qRT-PCR gene expression

Approaches for qRT-PCR normalization of micro-dissected tissues employed were cell count during microdissection, total RNA measurement, and endogenous control genes [8]. qRT-PCR was performed in triplicate according to previously described methods [8]. Unique primer/probe sets were used to assess the 10 genes (**Table 4**). Observance of a minimum 10 cycle threshold (C_T) values difference between RT-

negative and RT-positive samples confirmed negligible (< 1 %) genomic DNA contamination [14, 15]. In all runs, controls consisting of sterile molecular grade water were negative and controls consisting of total human prostate RNA (~12ng/µl; Ambion, Austin, Texas) were positive. Relative quantitation of gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method [16, 17]. Gene expression fold change levels were reported according to suggested threshold levels [18].

Statistical Analysis

For each time point, target gene expression C_T values were normalized to ACTB expression C_T values, yielding the normalized ΔC_T value. For each gene, we fit a linear mixed model (with a random effect to account for correlation among observations taken on the same individual) with fixed effects corresponding to tissue type (epithelium versus stroma) and time (T0, T1, and T2). Analyses were done separately by tissue type. An F-test from the linear mixed model was conducted to determine whether there was any change over time from T0 to T1 to T2 (global test). A global test was conducted at the P-value = 0.01 significance level to informally correct for multiple comparisons. When the global test was significant for a particular gene, pairwise comparisons between expression levels at different time points were performed based on testing contrasts between time points using a linear mixed model. Pairwise tests were conducted at the 0.01 significance level.

A global test of whether there was a changing profile across the three time points (T0, T1, and T2) was done using a linear mixed model testing for time by covariate interaction [age, prostate specific antigen (PSA) concentration, pathologic stage (pStage), prostate size, surgery type, total surgery time, total ischemia time, and estimated blood loss]. Each of the continuous variables at the median value was dichotomized. Level of significance was considered at P-value = 0.01.

Results

Study participants, surgical data, and biopsy tissue specimens

In this study, minimally invasive prostatectomy specimens were used as a model of sequential effects of intra-operative ischemia on selected genes in microdissected cells from non-malignant clinical specimens to explore confounding effects on mRNA expression. **Table 1** provides details of patient demographics, clinical information, and biopsy sizes for 10 patients whose specimens were used in the study. Participant demographics were: mean age = 60 years, mean PSA = 4.8 ng/dL. Mean operative time was 405.4 minutes, with estimated blood loss of 405 mL. Mean prostate size was 41.3 g, with mean Gleason score of 7. Mean total surgical ischemia time was 218.4 minutes. Elapsed time between biopsy time points was measured (**Figure 1, Table 1**). Mean time between T0 and T1 was 87 min (SD = 0.02); T0 and T2 was 210 min (SD = 0.04); and T1 and T2 was 127 min (0.03). Median time between T0 and T1 was 74 min (range 54-133 min) and T2 and T1 was 158 min (range 40-190 min).

Each time point biopsy was measured at the widest diameter of the tissue sample (**Table 1**). Biopsy size means were: T0 = 3.5 mm, SD = 0.95; T1 = 4.0 mm, SD = 1.54; T3 = 5.0 mm, SD = 1.52; with an overall (T0, T1, and T2) biopsy size mean equal to 4.2 mm (SD = 1.4). Following tissue sectioning and hematoxylin and eosin (H&E) staining, each of the biopsies from the initial 12 cases was subjected to histological analysis to assess tissue quality. Overall, histological analysis revealed no significant morphologic changes between biopsies taken at the three time points (**Table 2**), and 10 cases were chosen for downstream analysis.

RNA quantity and quality

Following total RNA extraction and isolation, quantity and quality was assessed to determine whether the total RNA was acceptable for downstream qRT-PCR analysis. Total RNA quantities were within expected limits for dissected sample template concentrations (Epithelium: mean = 13.87 ng/ml, SD = 20.38, median = 5.50 ng/ml; Stroma: mean = 4.81 ng/ml, SD = 6.08, median = 3.45 ng/ml). RNA qualities were typical of that from dissected frozen prostate tissue (Epithelium: mean RIN = 6.11, SD = 0.93; Stroma: mean RIN = 4.77, SD = 1.98).

Due to the unknown effects of the minimally invasive surgery over time, each time point (T1, T2) total RNA sample was compared to T0. Total RNA quantity decreased slightly over time (T0: mean = 9.72 ng/ml, SD = 17.92, median = 4.10 ng/ml; T1: mean = 8.45 ng/ml, SD = 9.39,

median = 3.85 ng/ml; T2: mean = 7.21 ng/ml, SD = 14.84, median = 3.23 ng/ml). However, total RNA quality did not decrease over time (T0: mean = 4.56 ng/ml, SD = 2.17, median = 4.50 ng/ml; T1: mean = 5.16 ng/ml, SD = 1.66, median = 6.00 ng/ml; T2: mean = 5.57 ng/ml, SD = 1.67, median = 6.15 ng/ml).

Gene expression changes associated with laparoscopic surgery time points

The effects of minimally invasive surgery were explored by gene expression analysis. Gene expression differences between T0, T1 and T2, separated by tissue type (i.e. epithelium or stroma) were assessed. C_T values were within expected ranges for non-malignant dissected prostate tissue RNA of epithelium and stroma, respectively (Oxygen-dependent: HIF1a = 29.93, 30.45; VEGF = 31.01, 35.22; VHL = 33.66, 34.86. Oxygen- independent: LAMP2 = 30.70, 32.26; MTR = 33.02, 33.33; STAT5B = 32.50, 32.74. Controls: 18s = 19.91, 19.94; CYPA = 30.01, 30.42; TFRC = 33.54, 34.47).

Biologic fold change ($2^{-\Delta\Delta CT}$) between T0, T1, and T2 was analyzed by pairwise comparison. VEGF and VHL were the only genes presenting mean fold expression changes > 1.5 fold (-1.5 > x <1.5 fold). The other seven genes had no change in gene expression (-1.5 < x > 1.5 fold) in epithelium or stroma (**Table 5**). In epithelium, VEGF was up-regulated as surgical ischemia induced hypoxia progressed from T0 to T1 to T2 (T0 to T1 = 3.14 fold up-regulation; T1 to T2 = 1.60 fold up-regulation; T0 to T2 = 4.67 fold up-regulation). In stroma, VEGF was up-regulated from T0 to T1 (1.69 fold), down-regulated from T1 to T2 (-1.75 fold), and demonstrated no change in mean fold expression overall from T0 to T2. VHL was down-regulated in stroma from T0 to T2 (-2.0 fold), but presented no difference in expression at the other time intervals.
Comparison between T1 and T2 is of interest since it represents the additional ischemia time between an open prostatectomy and a laparoscopic procedure. Mean biologic fold change and mean $\Delta\Delta C_T$ analysis revealed no significant changes in gene expression, except VEGF in epithelium and VHL in stroma (**Table 5**, **Figure 2**).

Mean normalized ΔC_T values were compared across time using a global F-test. VEGF was the only gene to significantly change across the three time points (T0 to T1 to T2) over time ($P < 0.01$; **Figure 3**). VEGF was significant in epithelium ($P = 0.008$), not stroma ($P = 0.66$).

Individual pairwise comparisons of mean normalized ΔC_T values were conducted for T1 to T0, T2 to T0, and T2 to T1. VEGF (epithelium) was the only gene with individual pairwise comparisons significant at $P \leq 0.01$ and whose global P-value was < 0.01 (**Table 5**).

Without screening using the global F-test, changes in mean T2 to T1 ΔC_T values were analyzed and considered significantly different from zero at the 0.01 level. No genes showed a significant change.

Gene expression changes associated with other surgical and clinical characteristics

Effects of age, PSA, prostate size, Gleason score, surgery type, total surgery time, total ischemia time, and estimated blood loss on VEGF expression in epithelium were examined by linear mixed model testing for a time by covariate interaction. None of the covariates was significant at the $P = 0.01$ level.

Discussion

Effects of minimally invasive surgical induced hypoxia on qRT-PCR gene expression analysis of dissected prostate tissue were analyzed. Interestingly, the effects of surgical ischemia were small, with only one gene studied, VEGF, showing a greater than two-fold variation in expression. Though it is expected that the majority of effects would be in oxygen-dependent genes, other genes analyzed, including oxygen-independent and control gene classes, did not show significant changes These data are similar to findings from the initial report by Lin et al. on open surgical manipulation in which expression levels in only 1.5% of genes were altered [19].

Due to sample variability concerns, normal prostatic urethra was used in the present study instead of prostate tissue. This provided a more homogeneous tissue type from which to procure epithelium and stroma. Sample heterogeneity did not appear to be a problem given that normalized values of genes did not change significantly across time points. However, to ensure that our experimental design, normalization strategies, and assay methodology was capable

Table 5. Changes in expression of oxygen-dependent, oxygen-independent and control genes over time in epithelium and stroma during surgical warm ischemia induced hypoxia

Tissue	Gene	Mean Biologic Fold Change per Time Interval			P- value[a]
Epithelium		T1-T0	T2-T0	T2-T1	
	O_2-dependent				
	HIF1a	< 1.5↑	< 1.5↑	< 1.5↓	0.33
	VEGF*	3.14↑	4.67↑**	1.60↑	0.008***
	VHL	< 1.5↓	< 1.5↑	< 1.5↑	0.83
	O_2-independent				
	LAMP2	< 1.5↓	< 1.5↑	< 1.5↑	0.38
	MTR	< 1.5↓	< 1.5↑	< 1.5↑	0.31
	STAT5B	< 1.5↓	< 1.5↑	< 1.5↑	0.39
	Control				
	18S	< 1.5↓	< 1.5↑	< 1.5↑	0.24
	CYPA	< 1.5↓	< 1.5↑	< 1.5↑	0.66
	TFRC	< 1.5↑	< 1.5↑	< 1.5↑	0.64
Stroma					
	O_2-dependent				
	HIF1a	< 1.5↑	< 1.5↓	< 1.5↓	0.44
	VEGF*	1.69↑	< 1.5↓	-1.75↓	0.66
	VHL*	< 1.5↓	-2.00↓	< 1.5↓	0.03
	O_2-independent				
	LAMP2	< 1.5↓	< 1.5↓	< 1.5↓	0.23
	MTR	< 1.5↓	< 1.5↓	< 1.5↑	0.12
	STAT5B	< 1.5↓	< 1.5↓	< 1.5↓	0.02
	Control				
	18S	< 1.5↓	< 1.5↓	< 1.5↑	0.90
	CYPA	< 1.5↓	< 1.5↓	< 1.5↓	0.03
	TFRC	< 1.5↓	< 1.5↓	< 1.5↑	0.81

<1.5 = -1.5 > x < 1.5; suggested threshold of no change in gene expression; [a] P-values represent a global test of whether there is any change in mean ΔC_T values across the three time points (T0 to T1 to T2) over time. P-value < 0.01 is considered statistically significant; ↑ Fold change > 0.0; ↓ Fold change < 0.0; * Genes demonstrating a Grade 1 change in gene expression. Only two of nine genes (VEGF and VHL, both oxygen-dependent genes) showed a change in gene expression at any of the time point comparisons; ** Individual pairwise comparisons of mean ΔC_T values significant at P ≤ 0.01 and whose global P-value is < 0.01 (data not shown); *** Statistically significant global P-value (P < 0.01).

of detecting gene expression differences if they did exist, we compared epithelium to stroma samples. And, in fact expression level differences between the two tissue types were seen (e.g. VEGF ΔC_T: epithelium = -1.30, stroma = 0.15; LAMP2 ΔC_T: epithelium = -1.29, stroma = 0.21; TFRC ΔC_T: epithelium = 0.91, stroma = 2.27).

Total RNA quantity did decrease slightly over time (T0 to T1 to T2), but was unaffected by surgery type (laparoscopic or robot assisted laparoscopic). Total RNA quality of samples over time was relatively unaffected. There was adequate template to perform the qRT-PCR analysis; however, biopsy sample size and the inability to procure additional patient samples

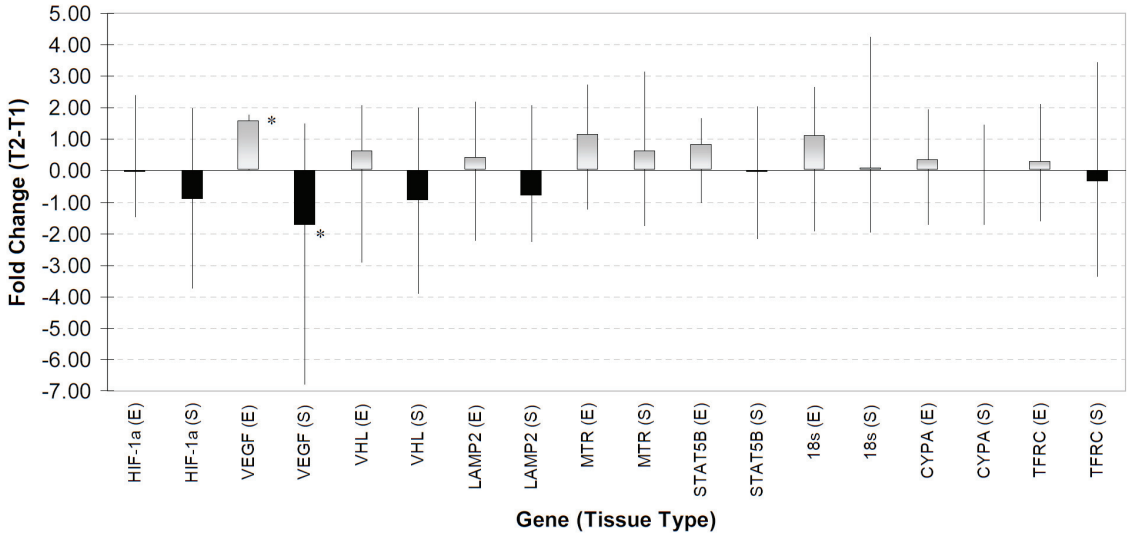


Figure 2. Mean biologic fold change in gene expression between biopsy time points (Time 2 and Time 1) per tissue type. VEGF was the only gene presenting > 1.5 fold change. Bar = mean fold change. Whiskers = fold change range. Tissue types: E = epithelium; S = stroma. * = >1.5 fold change.

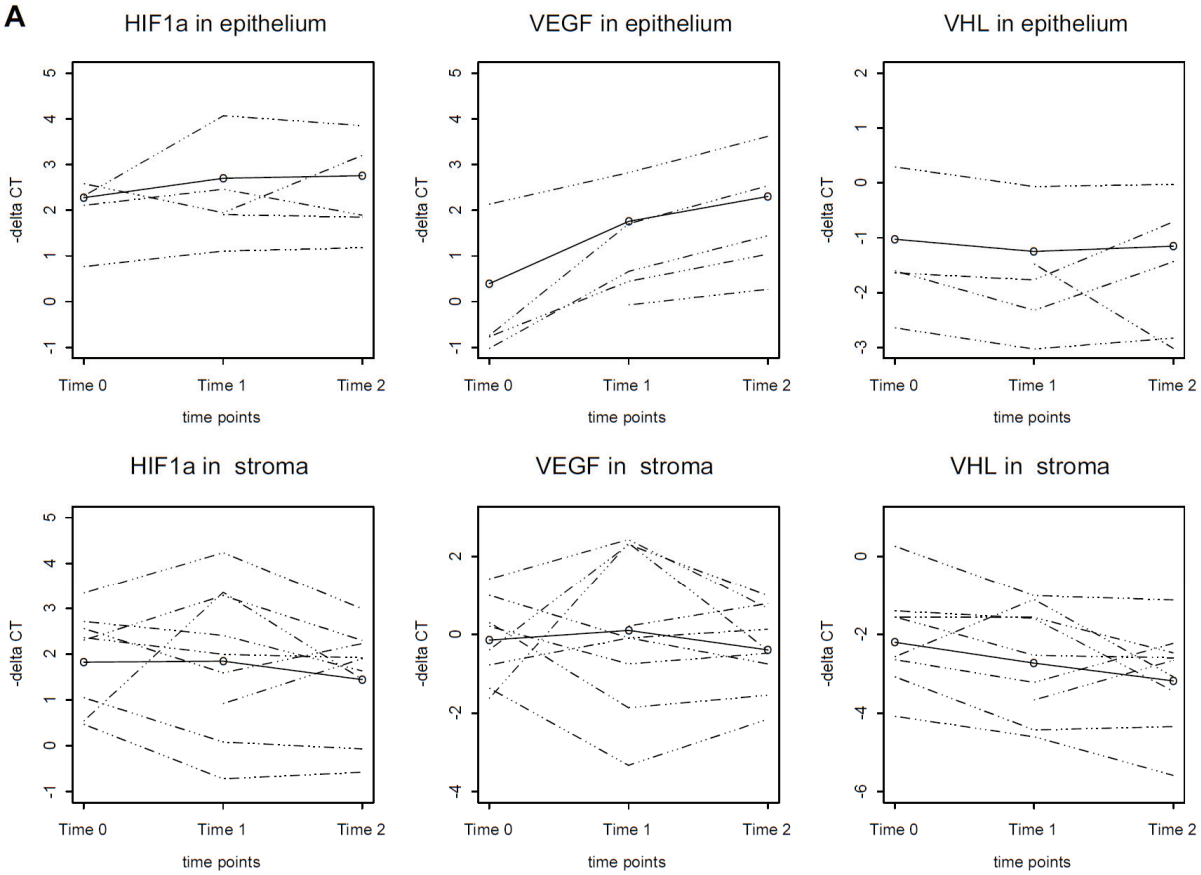
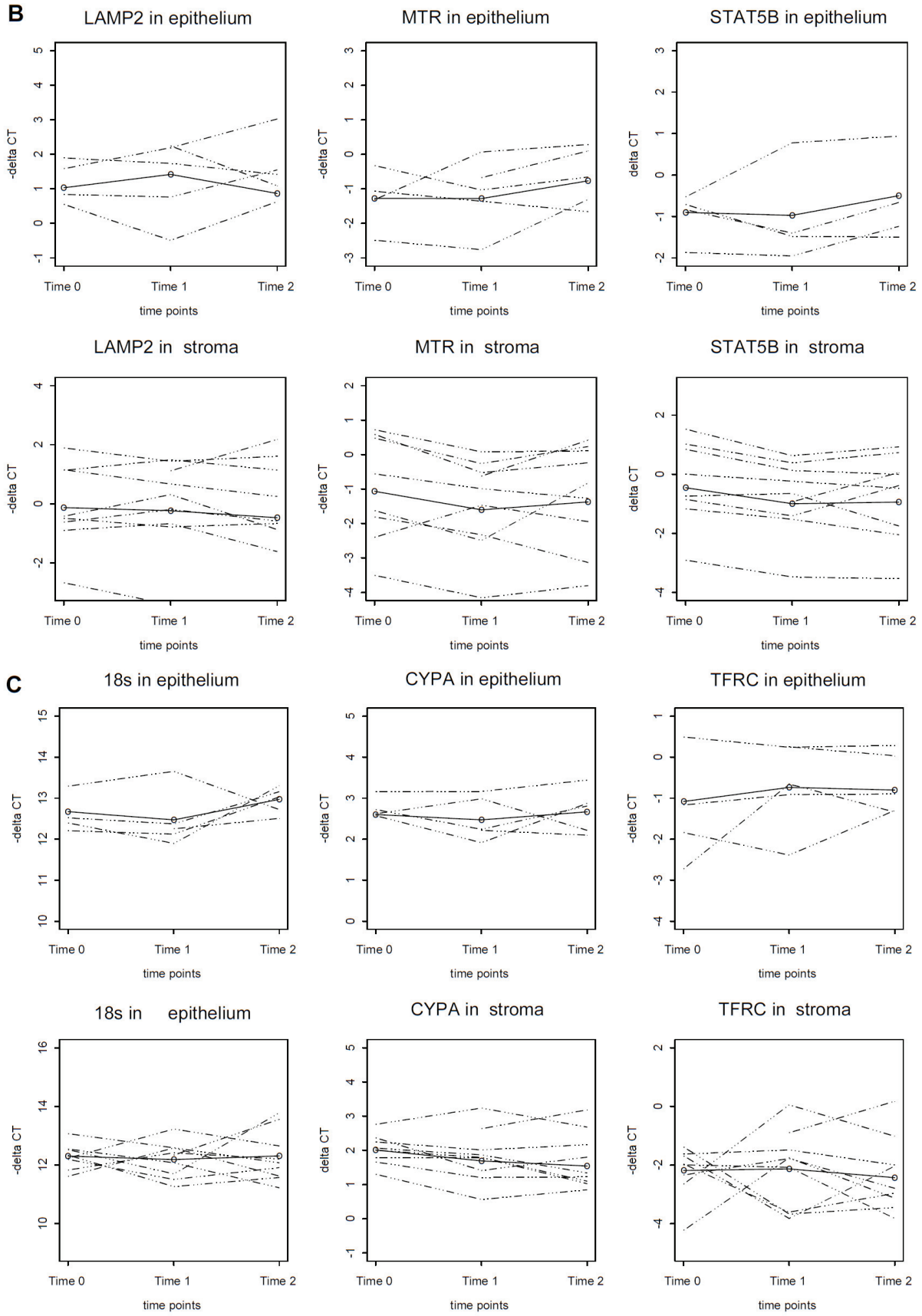


Figure 3. Gene expression changes over time. A. Oxygen-dependent gene group. B. Oxygen-independent gene group. C. Control gene group. Negative ΔC_T values are presented to reflect up regulation and down regulation of gene expression. Dark line with circles = mean of all ten cases $-\Delta C_T$s. Dotted lines = individual case $-\Delta C_T$ value profiles. (see B & C in next page)


B
LAMP2 in epithelium
MTR in epithelium
STAT5B in epithelium
LAMP2 in stroma
MTR in stroma
STAT5B in stroma
C
18s in epithelium
CYPA in epithelium
TFRC in epithelium
18s in epithelium
CYPA in stroma
TFRC in stroma
-delta CT
delta CT
Time 0
Time 1
Time 2
time points

precluded expression microarray or additional gene/pathway analysis.

Many studies have shown that hypoxia stresses cells and leads to multiple molecular changes. For example, HIF-1a protein is up-regulated at the protein level via decreased breakdown during periods of hypoxia while the RNA transcript was constitutive and unchanged [20]. Conversely, during times of exercise, skeletal muscle cells show up-regulation of HIF-1a at the mRNA level [21]. Hypoxia related genes assessed in the present work are regulated by the PI3-K pathway in which HIF-1a protein directly effects expression of other regulatory factors including VEGF and VHL [22-24]. VHL is known to regulate HIF-1a at the protein level by controlling the ubiquination of HIF-1a protein [25], but regulation at the transcript level is unknown. No significant variation in HIF-1a at any time point comparisons was observed due to surgery. Moreover, other than VEGF in epithelium and VHL in stroma, both oxygen-dependent genes, no other genes had significant fold change differences (>1.5 fold). Possibly, ischemia time from T0 to T2 (mean = 210 min) was not long enough to actively degrade the transcripts. Alternatively, prostate gland sizes may have been large enough to minimize gene expression changes due to an "adequate" blood supply, although no significant correlation has been established between gene expression stability or instability and prostate gland volume and vascularity [26].

The literature suggests that a 1.5 fold change in expression is a biologically meaningful threshold [18] and is the level at which researchers assign biologic significance. The comparison of T2 to T0 revealed a directional trend of up-regulation of gene expression in epithelium and down-regulation in stroma. The meaning of this phenomenon is unknown. The trend towards down-regulation of gene expression seen at T1 and T2 may be due to physical mRNA degradation and not hypoxia pathway related effects per se.

There are several limitations to the present study and additional work is necessary to further understand the effects of surgery on molecular profiles of tissues. The physical size of punch biopsy samples allowed for only one microdissection and RNA extraction from each specimen, including a lack of epithelium in a few of the cases. Thus, a high throughput expression array-based study could not be performed and only nine genes of interest and one endogenous control gene were analyzed. In addition, standardization of time intervals is not possible. It is this variability in time to processing that may alter gene expression. As such, our data potentially underestimate the transcription changes due to laparoscopic surgery. To account for these limitations, a more stringent P-value of <.01 was utilized and strict normalization strategies for qRT-PCR of MD tissues [8] were followed, using ACTB as a normalizer for all comparisons. ACTB may itself be affected by hypoxia although it was noted to be stable in previously published prostate and bladder studies [8, 13].

This study also has several strengths. First, it is the first to examine relatively non-invasive (laparoscopic) surgical manipulation induced hypoxia on gene expression. Second, the study design used optimum tissue handling and processing procedures [27]. Third, qRT-PCR was used as the analysis method, which is well-known to be more sensitive and specific than microarray analysis [28]. Fourth, patient demographics and time interval covariate interactions of gene expression were comprehensively analyzed.

In summary, our data suggest that although all steps should be taken to minimize warm ischemia time, only a limited amount of artifactual gene expression change is induced during surgical resection of the prostate gland. Surprisingly, even in those genes related to hypoxia, not much change occurs, while no variations were noted in genes not known to be involved in the cellular response to hypoxia. However, the full extent of expression changes and pathway correlations that happen during prostatectomy are not yet fully understood and further efforts are needed to extend our understanding of surgical ischemia as a confounder in studies involving human clinical samples.

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