Testosterone and Dihydrotestosterone Tissue Levels in Recurrent Prostate Cancer

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Abstract

Purpose: Prostate cancer eventually recurs during androgen deprivation therapy despite castrate levels of serum androgens. Expression of androgen receptor and androgen receptor–regulated proteins suggests androgen receptor activation in recurrent prostate cancer. Many groups have pursued mechanisms of ligand-independent androgen receptor activation but we found high levels of testicular androgens in recurrent prostate cancer tissue using RIA.

Experimental Designs: Prostate specimens from 36 men were procured preserving blood flow to prevent ischemia and cyropreserved immediately. Recurrent prostate cancer specimens from 18 men whose cancer recurred locally during androgen deprivation therapy and androgen-stimulated benign prostate specimens from 18 men receiving no hormonal treatments were studied. Tissue levels of testosterone and dihydrotestosterone were measured in each specimen using liquid chromatography/electrospray tandem mass spectrometry. Testosterone and dihydrotestosterone levels were compared with clinical variables and treatment received.

Results: Testosterone levels were similar in recurrent prostate cancer (3.75 pmol/g tissue) and androgen-stimulated benign prostate (2.75 pmol/g tissue, Wilcoxon two-sided, $P = 0.30$). Dihydrotestosterone levels decreased 91% in recurrent prostate cancer (1.25 pmol/g tissue) compared with androgen-stimulated benign prostate (13.7 pmol/g tissue; Wilcoxon two-sided, $P < 0.0001$) although dihydrotestosterone levels in most specimens of recurrent prostate cancer were sufficient for androgen receptor activation. Testosterone or dihydrotestosterone levels were not related to metastatic status, antiandrogen treatment, or survival (Wilcoxon rank sum, all $P > 0.2$).

Conclusions: Recurrent prostate cancer may develop the capacity to biosynthesize testicular androgens from adrenal androgens or cholesterol. This surprising finding suggests intracrine production of dihydrotestosterone and should be exploited for novel treatment of recurrent prostate cancer.

Prostate cancer recurs in almost all men receiving androgen deprivation therapy (ADT) and is the main cause of death in prostate cancer. Recurrent prostate cancer retains androgen receptor protein expression, with androgen receptor remaining active in growth signaling despite castrate levels of circulating androgens (1). Androgen receptor protein and androgen receptor–regulated proteins are expressed in prostate cancer that recurs during ADT in both the primary (2–5) and bone metastases (6, 7). Androgen receptor activation in recurrent prostate cancer may occur by a variety of mechanisms that alter the sensitivity (8–10) or specificity (11) of androgen receptor (reviewed in refs. 12–14). Recent studies using androgen-independent prostate cancer cell lines (8, 9) and xenografts (9) showed that androgen receptor overexpression allowed recurrent prostate cancer growth in the presence of castrate levels of circulating androgens. However, androgen receptor mutations that prevented ligand-binding prevented recurrent growth; overexpressed androgen receptor required ligand to confer recurrent growth (8).

Androgens may be synthesized from adrenal androgens in prostate tissues using steroidogenic enzymes expressed in prostate cells (15). ADT decreases dihydrotestosterone formation from adrenal androgens (16) but dihydrotestosterone levels remained measurable (17) in metastatic prostate cancer. Furthermore, clinical specimens of recurrent prostate cancer analyzed using RIA contained levels of testicular androgens, testosterone, and dihydrotestosterone sufficient for androgen receptor activation (2).

These reports suggest that prostate cancer which recurs after medical or surgical castration may retain androgen dependence. Herein, we report the quantitation of testosterone and dihydrotestosterone levels using liquid chromatography tandem

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Table 1. Tissue testosterone (T) and dihydrotestosterone (DHT) levels in androgen-stimulated benign prostate (AS-BP) and recurrent prostate cancer (RCaP) specimens using LC/MS/MS and RIA

Recurrent prostate cancer patient	Age (y)	Race	Serum prostate–specific antigen* (ng/mL)	Gleason sum	Bone metastases	Androgen deprivation therapy	Interval (mo) from androgen deprivation therapy to tissue acquisition
1	69	CA	7.1	8	$T_{3c}N_0M_0$	LHRH + flu	30
2	67	AA	534.6	8	$T_{2c}N_xM_x$	orch	92
3	81	AA	9.3	8	$T_{3c}N_xM_{1b}$	orch + flu	13
4	75	CA	28.3	9	$T_4N_xM_0$	LHRH	43
5	69	CA	79.0	10	$T_4N_xM_{1b}$	LHRH + flu	19
6	57	AA	5.8	9	$T_{3c}N_xM_0$	orch	19
7	65	CA	1.1	9	$T_4N_xM_{1b}$	LHRH	37
8	61	AA	199.0	9	$T_4N_2M_{1b}$	orch	49
9	78	AA	24.0	9	$T_{2c}N_xM_0$	1° hypogonad	N/A
10	62	AA	32.3	9	$T_{3c}N_1M_0$	flu	11
11	86	AA	28.0	9	$T_{1b}N_xM_{1b}$	orch	48
12	82	CA	53.0	10	$T_{3c}N_xM_{1b}$	orch	77
13	60	CA	0.0	10	$T_{2b}N_xM_{1b}$	LHRH → DES	36
14	86	CA	10.3	10	$T_xN_1M_0$	Lupron	72
15	89	CA	36.5	9	$T_{2a}N_xM_0$	orch	76
16	86	AA	5.6	10	$T_{2b}N_xM_0$	orch	84
17	69	CA	3.0	10	$T_{3c}N_1M_0$	DES → orch	27
18	69	CA	120.5	10	$T_{3c}N_2M_{1b}$	flu → DES	27
Maximum	89		534.6				92
Upper quartile	82		53				72
Median	69		26				37
Lower quartile	65		5.8				27
Minimum	57		0				11

Abbreviations: CA, Caucasian; AA, African-American.

mass spectrometry (LC/MS/MS) in specimens of androgen-stimulated benign prostate (AS-BP) and recurrent prostate cancer.

Materials and Methods

Patient and tissue samples. Eighteen men who presented with recurrent prostate cancer had a median age of 69 years and median ADT duration of 37 months. Eighteen men with AS-BP had a median age of 66 years and received no hormonal treatments. Prostate specimens were frozen in liquid nitrogen within 5 minutes of procurement to prevent ischemic damage to prostate tissue. Recurrent prostate cancer cells were obtained from men who underwent transurethral resection to relieve urinary retention from local recurrence during androgen deprivation therapy and frozen immediately (Table 1). AS-BP specimens were obtained from radical prostatectomy done for clinically localized prostate cancer. The superior vascular pedicles were left intact until all other portions of the procedure were completed. Upon removal of the operative specimen, the surgeon took the prostate to a side table, the specimen was inked and samples were obtained and frozen immediately. Histologic diagnoses were confirmed by examination of frozen and corresponding formalin-fixed, paraffin-embedded tissue specimens. Research data was merged with clinical data from prospectively maintained databases. All prostate specimens were acquired in compliance with the guidelines of the University of North Carolina Lineberger Comprehensive Cancer Center Clinical Protocol Review Committee and Institutional Review Board and the Federal Health Insurance Portability Accountability Act and protected health information regulations.

Specimen preparation. Cryopreserved prostate specimens were homogenized at 50 mg/mL in 4°C purified distilled water. One milliliter aliquots were removed and 1 ng each of deuterated testosterone and dihydrotestosterone internal standards were added and samples extracted thrice with 3 mL of 60:40 hexane/ethyl acetate. Collected organics were evaporated under vacuum. Analyte purity was improved using solid phase extraction cartridges (SPEC-C18AR, Varian, Palo Alto, CA) and high-performance liquid chromatography to control matrix effects and separate androgens. Solid phase extraction cartridges were conditioned with methanol and water. Samples were applied in 1:6 methanol/water. Samples were eluted in methanol, dried under vacuum, and reconstituted in 50% methanol for analysis. An Agilent 1100 capillary LC system consisting of G1376A CapPump equipped with vacuum solvent degasser, G1377A autosampler and Therm column compartment containing Zorbax SB-C18 (5 μm, 150 × 0.5 mm, Agilent) column was used to separate testosterone and dihydrotestosterone. The column was maintained at 40°C. A gradient profile using mobile phase A (0.1% acetic acid in water) and B (0.1% acetic acid in methanol) at a flow rate of 20 μL/minute was used as follows: 60% B from 0.0 to 1.0 minutes, 70% to 100% B from 1.1 to 9.0 minutes, 100% B from 9.0 to 14.0 minutes, and 100% to 60% B from 14.0 to 14.5 minutes. The column was equilibrated at 60% B for 12 minutes prior to sample injection.

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Table 1. Tissue testosterone (T) and dihydrotestosterone (DHT) levels in androgen-stimulated benign prostate (AS-BP) and recurrent prostate cancer (RCaP) specimens using LC/MS/MS and RIA (Cont'd)

Survival (mo) after tissue acquisition	Tissue androgen assays (pmol/g tissue)				AS-BP Patient	Age (y)	Race	Tissue androgen assays (pmol/g tissue)			
	LC/MS/MS		RIA					LC/MS/MS		RIA	
	Testosterone	Dihydrotestosterone	Testosterone	Dihydrotestosterone				Testosterone	Dihydrotestosterone	Testosterone	Dihydrotestosterone
12	1.6	0.0	2.1	5.3	1	59	CA	3.4	23.6	6.59	6.54
17	3.7	0.0	3.0	0.9	2	50	AA	0	14.5	4.16	9.79
15	13.6	4.9	10.2	2.3	3	77	CA	1.2	16.8	4.51	6.73
46	1.2	4.6	1.6	0.4	4	66	AA	1.8	11.3	5.03	2.52
6	1.7	0.0	2.1	0.3	5	73	AA	2.5	12	2.95	5.66
7	3.8	7.8	<0.87	0.5	6	67	CA	2.9	20.5	9.37	23.34
4	5.4	3.9	0.9	0.4	7	65	CA	13	17.1	2.78	5.85
19	8.6	6.7	2.1	0.5	8	59	CA	1.2	13.2	2.43	5.11
11	9.8	2.8	2.1	0.7	9	71	CA	2.9	9.8	5.21	7.09
13	11.4	1.2	19.78	13.2	10	65	CA	1.4	14.3		
48	1.1	0.0	2.6	<0.26	11	66	AA	1.6	11.2		
36	2.5	0.4	3.1	0.7	12	64	CA	2	6.5		
4	7.2	1.3	<0.87	<0.26	13	44	CA	2.7	10.7		
7	0.0	0.0			14	71	CA	2.8	13.7		
6	1.6	0.7			15	62	n/a	2.8	13.7		
6	6.7	5.2			16	67	CA	3.2	20.3		
23	9.1	1.5			17	69	CA	3.3	38.3		
3	1.1	0.0			18	57	CA	3.9	12.4		
48	13.6	7.8	19.78	13.2		77		13	38.3	9.37	23.34
19	8.6	4.6	2.95	0.9		69		3.2	17.1	5.21	7.09
11.5	3.75	1.25	2.08	0.46		65.5		2.75	13.7	4.51	6.54
6	1.6	0	1.56	0.42		59		1.6	11.3	2.95	5.66
3	0	0	0.87	0.26		44		0	6.5	2.43	2.52

Optimized electrospray ionization conditions resulted in detection limit for underivatized testosterone and dihydrotestosterone of 0.7 fmol or 203 fg (signal to noise = 3). Validation data confirmed calibration of testosterone and dihydrotestosterone within the linear dynamic range 0.7 fmol to 3.5 pmol. Limit of quantitation was 3.5 fmol.

Analysis of androgens. Testosterone and dihydrotestosterone were ionized using an elecrospray ionization source in positive ion mode and measured using an MDS Sciex API3000 triple quadrupole mass spectrometer. Stable isotope labeled androgen internal standards and quantitation of unique testosterone and dihydrotestosterone product ions allowed accurate LC/MS/MS measurements. The parent/product ion pairs (Fig. 1) of *m/z* 289.2 to 97.0 for testosterone, *m/z* 292.2 to 96.8 for internal standard testosterone-d_3, *m/z* 291.2 to 255.2 ($-2H_2O$) for dihydrotestosterone, and *m/z* 295.2 to 259.2 ($-2H_2O$) for internal standard dihydrotestosterone-d_4. Mass spectrometer parameters were optimized for strongest product ion signal intensities. Optimized declustering potential, focusing potential, and collision energy settings for testosterone and dihydrotestosterone were 40, 180, and 25.8 V, and 50, 240, and 23.5 V, respectively. Other mass spectrometry parameters were turbo gas 6 L/minute (300°C), nebulizer gas 6, curtain gas 12, collision activated dissociation gas 4, ionspray 4,400 V, entrance potential 10 V, and collision exit potential 17.7 V. Nitrogen was used for all gas inputs.

Statistical analysis. The Wilcoxon rank sum test was used for statistical comparison of testosterone and dihydrotestosterone levels in patients with AS-BP and recurrent prostate cancer. Testosterone and dihydrotestosterone levels in the presence or absence of metastases or antiandrogen treatment were compared using the Wilcoxon rank sum test. The correlation between testosterone or dihydrotestosterone levels and clinical descriptors (age, serum prostate–specific antigen, interval from ADT to tissue procurement, and survival) were analyzed using Spearman rank correlation coefficients. Wilcoxon tests were two-sided and P values < 0.05 were considered statistically significant.

Results

Tissue androgen levels. LC/MS/MS analysis of 18 AS-BP and 18 recurrent prostate cancer specimens showed similar testosterone but different dihydrotestosterone levels (Table 1). Testosterone levels were 2.75 pmol/g in AS-BP and 3.75 pmol/g tissue in recurrent prostate cancer (Wilcoxon two-sided, P = 0.30) (Table 1). Median tissue levels of dihydrotestosterone were 91% lower in recurrent prostate cancer (1.25 pmol/g tissue) than AS-BP (13.70 pmol/g tissue; Wilcoxon two-sided, $P < 0.0001$). Six patients had undetectable levels of dihydrotestosterone and one of those patients also had an undetectable level of testosterone. Recurrent prostate cancer patient 14 suggests that prostate cancer can recur completely independent of testicular androgens. Thirteen of these 18 recurrent prostate cancer specimens and 9 of these 18 AS-BP

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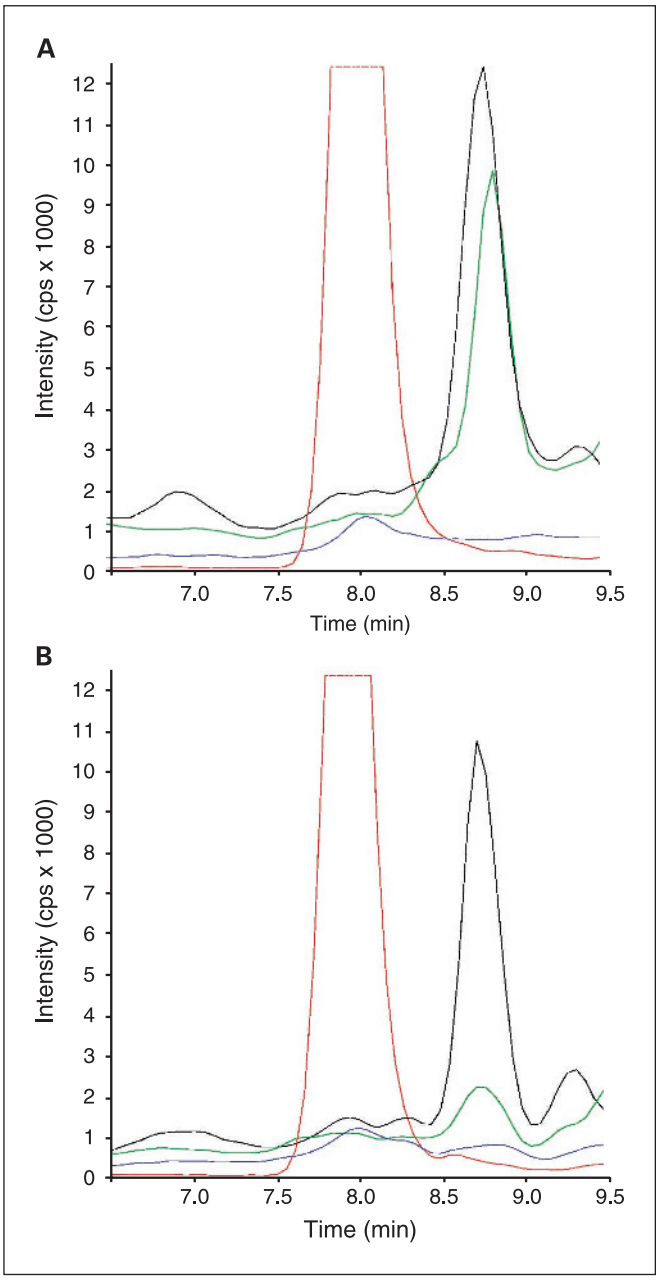

Fig. 1. LC/MS/MS multiple reaction monitoring profile of representative samples of AS-BP (*A*) and recurrent prostate cancer (*B*); cps, counts per second. The parent/product ion pairs for testosterone (blue), internal standard testosterone-d_3 (red), dihydrotestosterone (green), and internal standard dihydrotestosterone-d_4 (black) were monitored and product ion used for androgen quantitation.

specimens had been analyzed previously using RIA (2), which permitted comparison of results obtained using the two methods. Median dihydrotestosterone levels were similar in recurrent prostate cancer specimens measured using LC/MS/MS (1.25 pmol/g tissue) and RIA (0.46 pmol/g tissue). Both methods showed that median dihydrotestosterone levels decreased by ~90% in recurrent prostate cancer compared with AS-BP.

Correlation between androgen levels and clinical variables. Recurrent prostate cancer tissue levels of testosterone and dihydrotestosterone were unrelated to age, prostate-specific antigen, and time from ADT to tissue procurement, and survival (Spearman rank correlation r values not significantly different than 0 and Wilcoxon two-sided, P values > 0.11). Median tissue testosterone levels were similar for men with bone metastasis (n = 8; 4.0 pmol/g tissue) and men without bone metastasis (n = 10; 3.79 pmol/g tissue; Wilcoxon two-sided, P = 1.0). Median dihydrotestosterone levels were similar in the presence (0.89 pmol/g tissue) or absence (1.4 pmol/g tissue) of bone metastasis (Wilcoxon two-sided, P = 0.79). Median testosterone levels were similar between 5 men who received flutamide (1.68 pmol/g tissue) and 13 men who were not treated with antiandrogens (3.8 pmol/g tissue; Wilcoxon two-sided, P = 0.79). Dihydrotestosterone levels did not differ between 5 men who received flutamide and 13 men who did not (Wilcoxon two-sided, P = 0.21).

Discussion

These measurements using LC/MS/MS in 18 specimens each of AS-BP and recurrent prostate cancer confirm our previous report using RIA in 30 specimens of AS-BP and 15 specimens of recurrent prostate cancer (2). In that report, testosterone levels were similar in AS-BP (3.26 pmol/g tissue) and recurrent prostate cancer (2.78 pmol/g tissue) and dihydrotestosterone levels were decreased in recurrent prostate cancer (1.45 pmol/g tissue) compared with AS-BP (8.16 pmol/g tissue; Wilcoxon two-sided, P < 0.0001). Dihydrotestosterone levels in recurrent prostate cancer compared with AS-BP decreased 91% measured using LC/MS/MS in this report compared with 82% measured using RIA in the previous report (2). Geller et al. reported that dihydrotestosterone levels measured using RIA decreased 75% from an average of 17.6 pmol/g tissue in 17 AS-BP to an average of 4.47 pmol/g tissue in prostate cancer recurrent after castration (five men) or castration and diethylstilbesterol (four men; ref. 18). Two recent reports using LC/MS/MS (19, 20) support these findings. Mizokami et al. showed that average tissue dihydrotestosterone levels decreased 75% in 15 AS-BP specimens (2.48 ng/g tissue, equivalent to 8.53 pmol/g tissue) compared with prostate cancer harvested from 12 radical prostatectomy specimens obtained after 3 to 6 months of ADT (0.619 ng/g tissue or 2.13 pmol/g tissue). Nishiyama et al. found that tissue dihydrotestosterone concentrations decreased 75% from 5.44 ng/g tissue (18.73 pmol/g tissue) to 1.35 ng/g tissue (4.65 pmol/g tissue) in 30 men undergoing ADT for 6 months. Tissue testosterone concentrations were not measured in either study.

In this and our prior report (2), 22 specimens (13 recurrent prostate cancer and 9 AS-BP) were analyzed using both methods (Table 1). LC/MS/MS yielded higher measurements than RIA for dihydrotestosterone (Wilcoxon two-sided signed-rank test, P = 0.01) but testosterone measurements were similar. Others reported that RIA and LC/MS/MS gave different measures for serum testosterone and these differences depended upon testosterone levels and the type of RIA assay used (21).

Data obtained using LC/MS/MS indicate that medical or surgical castration median testosterone tissue levels are similar in AS-BP and recurrent prostate cancer. Median dihydrotestosterone levels in recurrent prostate cancer specimens decreased 91% after a median of 37 months of ADT but remained

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sufficient in most men to transactivate androgen receptor (1.25 nmol/L) based on studies of prostate cancer cell lines (22, 23). Dihydrotestosterone tissue levels showed in a total of four referenced studies (this report and refs. 2, 19, 20) support ligand activation of androgen receptor from 3 to 92 months after institution of ADT. However, tissue testosterone and dihydrotestosterone levels did not correlate with clinically relevant variables such as duration or type of ADT and survival.

Decreased dihydrotestosterone levels in recurrent prostate cancer compared with AS-BP contrast to comparable testosterone levels in the two tissue types; these data suggest an altered 5α-reducing capability in recurrent prostate cancer. Kliman et al. reported a significant impairment in dihydrotestosterone formation in six specimens of metastatic prostate cancer (17). Dihydrotestosterone is synthesized from intracellular testosterone by steroid 5α-reductase isozymes I and II in prostate. Thigpen et al. (24) showed that steroid 5α-reductase isozyme II is the predominant isoform in prostate tissue. Recent evidence supports increased isozyme I expression in prostate cancer (25) and recurrent prostate cancer (26). Catalytic activity at 50% maximal rate requires higher substrate concentration (testosterone) for steroid 5α-reductase isozyme I than isozyme II (27). Increased steroid 5α-reductase isozyme I expression relative to isozyme II in recurrent prostate cancer tissue may decrease dihydrotestosterone formation.

The tissue levels of testosterone and dihydrotestosterone (Fig. 1; Table 1) measured in recurrent prostate cancer tissue supports a new paradigm. Prostate cancer that recurs after medical or surgical castration is not "androgen-independent" because recurrent prostate cancer usually has androgen levels sufficient to activate androgen receptor. Testosterone and dihydrotestosterone in recurrent prostate cancer tissue may result from intracrine metabolism of circulating adrenal androgens (28, 29) or plasma membrane cholesterol (30) located in lipid rafts (31). The high tissue levels of testosterone and dihydrotestosterone in recurrent prostate cancer is unexpected and suggests that these testicular androgens present a target for novel therapies.

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