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Detection of Increased Choline Compounds with Proton Nuclear Magnetic Resonance Spectroscopy Subsequent to Malignant Transformation of Human Prostatic Epithelial Cells[1]

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ABSTRACT

In this study, a panel of normal human prostate cells (HPCs) and tumor cells derived from metastases were studied by ^{1}H NMR spectroscopy to determine whether the malignant transformation of HPCs results in the elevation of choline compounds. Although an elevated choline signal has been observed previously in clinical studies, the contribution of the different Cho compounds to this elevation, as well as their quantification, has not been established until now. Here we have shown that HPCs derived from metastases exhibit significantly higher phosphocholine as well as glycerophosphocholine levels compared with normal prostate epithelial and stromal cells. Thus the elevation of the choline peak observed clinically in prostate cancer is attributable to an alteration of phospholipid metabolism and not simply to increased cell density, doubling time, or other nonspecific effects. Androgen deprivation of the androgen receptor-positive cell lines resulted in a significant increase of choline compounds after chronic androgen deprivation of the LNCaP cell line and in a decrease of choline compounds after a more acute androgen deprivation of the LAPC-4 cell line. These data strongly support the use of proton magnetic resonance spectroscopic imaging to detect the presence of prostate cancer for diagnosis, to detect response subsequent to androgen ablation therapy, and to detect recurrence.

INTRODUCTION

Prostate cancer is the second leading cause of cancer related death among United States men[3] (1). Early detection and diagnosis of prostate cancer are critically important factors in the prognosis of this disease. Currently, serum analysis of prostate-specific antigen levels, digital rectal examination, transrectal ultrasound, and biopsy are the standard methods for detecting and diagnosing prostate cancer (1). Several noninvasive imaging technologies are currently being evaluated with the purpose of obtaining a reliable *in vivo* marker to differentiate between benign and malignant tissue (2–4). These noninvasive techniques are also being explored for a "watchful waiting" approach to prostate cancer once diagnosed, to avoid unnecessarily aggressive treatments when not required. Of these technologies, localized ^{1}H MRSI[4] shows the most promise in detecting prostate cancer (2, 3). ^{1}H NMR spectra localized in malignant prostatic tissue typically exhibit low levels of citrate and increased levels of tCho, which results in a high tCho to citrate ratio (2, 5, 6).

The tCho peak consists of PC, GPC, and free Cho, but the low resolution of *in vivo* spectroscopy does not allow identification of individual peaks from these compounds. To better understand the pathways underlying the changes in spectra observed *in vivo*, it is necessary to identify and quantify the changes in these Cho compounds after malignant transformation. We recently established, for a panel of human mammary epithelial cells, that both tCho and PC levels increased significantly with malignant progression (7). To establish further this pattern of Cho compounds after malignant transformation for other epithelial cells, in this study we determined the phospholipid profiles of normal HPCs and epithelial tumor cells. Primary human stromal cells originating in the prostate were also included in this panel to determine whether there were significant differences between the phospholipid profiles of stromal and epithelial prostatic cells.

Androgen ablation remains one of the standard therapies for metastatic prostate cancer, but these cancers can recur subsequent to anti-androgen therapy. Both AR-positive and -negative prostate tumor cells as well as androgen-deprived subclones of AR-positive cell lines were therefore included in the panel of HPCs studied. The ability to detect significant differences in phospholipids between androgen-dependent and androgen-independent cells would be useful not only for predicting which cancers would respond to androgen deprivation, but also in understanding the metabolic outcome of prostate cancer transition from androgen dependence to androgen independence.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The phenotype and origin of the HPCs used in this study are described in Table 1. The PC-3, LNCaP, and DU-145 cells (purchased from American Type Culture Collection), TSU (8) and PPC-1 (9, 10) were grown in RPMI 1640 (Life Technologies, Inc.) containing 300 mg/liter L-glutamine supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml). The androgen-deprived cell lines LN95, LN96, LN97, and LN98 were derived from the AR-positive cell line LNCaP by growing them in the medium described but without phenol red and with charcoal-stripped FBS (Hyclone). The year of initiating androgen deprivation is denoted by the number with the cell line.

The LAPC-4 cell line, originally explanted by Klein *et al.* (11) and kindly provided by Robert E. Reiter (UCLA, Los Angeles, CA), was cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) containing 868 mg/liter L-alanyl-L-glutamine and 5.985 g/liter HEPES. Iscove's modified Dulbecco's medium was supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml). The LAPC-AD cell line was derived by growing LAPC-4 in Iscove's modified Dulbecco's medium without phenol red and supplemented with 10% charcoal-stripped FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml). The primary epithelial cells PrEC and PrSC were purchased from Clonetics (Walkersville, MD). The PrEC cells were cultured in Opti Mem I (Life Technologies, Inc.) containing 292 mg/liter L-glutamine and 2.4 mg/liter HEPES with penicillin (100 units/ml)/streptomycin (100 μg/ml) supplemented with the following reagents from BioWhittaker: (*a*) bovine pituitary extract (52 μg/ml); (*b*) human epidermal growth factor (10 ng/ml); (*c*) retinoic acid (0.1 ng/ml); (*d*) transferrin (50 μg/ml); (*e*) insulin (5 μg/ml); (*f*) hydrocortisone (1 μg/ml); (*g*) epinephrine (0.5 μg/ml); and (*h*)

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[4] The abbreviations used are: MRSI, magnetic resonance spectroscopic imaging; ^{1}H, proton; NMR, nuclear magnetic resonance; HPC, human prostate cell; AR, androgen receptor; FBS, fetal bovine serum; PCA, perchloric acid; Cho, choline; DT, doubling time; GPC, glycerophosphocholine; PC, phosphocholine; tCho, total choline; TSP, 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt; V_{cell}, cell volume.

Table 1 *Phenotype and origin of the panel of investigated HPCs*

Cell line	Phenotype	Origin
PrSC	Senescent	Prostate stromal cells (fibroblastic)
PrEC	Senescent	Prostate epithelial cells
LNCaP (12–14)	Malignant	Lymph node metastasis
LN-98	Malignant	Androgen deprivation of LNCaP since January 1998
LN-97	Malignant	Androgen deprivation of LNCaP since January 1997
LN-96	Malignant	Androgen deprivation of LNCaP since January 1996
LN-95	Malignant	Androgen deprivation of LNCaP since January 1995
LAPC-4 (11)	Malignant	Lymph node metastasis
LAPC-AD	Malignant	Androgen deprivation of LAPC-4 since October 1998
DU-145 (15–17)	Malignant	Brain metastasis
TSU (8)	Malignant	Lymph node metastasis
PC-3 (18, 19)	Malignant	Bone metastasis
PPC-1 (9)	Malignant	Stage D2 prostatic carcinoma[a]

[a] Karyotype analyses revealed chromosome identity between PPC-1 and PC-3 (20) indicating PPC-1 is a clonal derivative of PC-3.

Table 2 *Androgen receptor status (AR), doubling time (DT), and cell volume (V_{cell}) of the investigated HPCs*

HPCs	AR	DT	V_{cell} (mean ± SE) [10^3 μm^3]
PrSC	Positive	16.0 h	4.01 ± 0.45
PrEC	Positive	16.5 h	15.7 ± 1.9
LNCaP (12–14)	Positive	25.0 h	6.49 ± 0.81
LN-98	Positive, AD[a]	25.0 h	2.40 ± 0.15
LN-97	Positive, AD	22.0 h	2.37 ± 0.16
LN-96	Positive, AD	26.0 h	2.78 ± 0.19
LN-95	Positive, AD	30.0 h	2.31 ± 0.20
LAPC-4 (11)	Positive	19.0 h	1.78 ± 0.28
LAPC-AD	Positive, AD	22.0 h	2.52 ± 0.28
DU-145 (15–17)	Negative	25.0 h	3.63 ± 0.33[b]
TSU (8)	Negative	17.0 h	2.45 ± 0.19
PC-3 (18, 19)	Negative	27.0 h	3.12 ± 0.30
PPC-1 (9)	Negative	15.5 h	3.43 ± 0.30

[a] AD, androgen-deprived.
[b] $n = 250$; for the other cell lines $n = 100$ cells.

triiodothyronine (6.5 ng/ml). The PrSC cells were maintained in RPMI 1640 supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml). All HPCs were maintained at 5% CO_2 in air, 90% humidity, and 37°C in a CO_2 incubator. Cell-volume measurements, which are presented in Table 2, were calculated from the cell diameter assuming that a single cell in suspension has the shape of a sphere. The diameter was measured for single cells in suspension using a light microscope with ×1000 magnification. Diameter measurements were averaged over 100 cells for each cell line.

PCA Extraction and Preparation for NMR Spectroscopy. PCA extraction was performed as described before (7). Briefly, cells were grown to 80% confluency. Culture medium was changed 24 h and an additional 3 h before harvesting. After trypsinization, cells were washed in PBS [137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.47 mM KH_2PO4 (pH7.4)]. An equal volume of 0.8 M PCA was added, and the cells were homogenized, with subsequent centrifugation for 30 min. at 14,000 × *g*. Starting with trypsinization, all of the procedures including centrifugation were carried out on ice or at 4°C. After centrifugation, the lysates were transferred to Eppendorf tubes, the pH was adjusted to 7.0 with 1 N NaOH, and the lysates were lyophilized. For the ^{1}H NMR measurements the lyophilized samples were dissolved in 600 μl D_2O, traces of salt removed by centrifugation, and the supernatant transferred into a 5-mm NMR tube with 5 μl 0.75% TSP in D_2O.

Acquisition of NMR Spectra and Data Analysis. ^{1}H NMR spectroscopy measurements were performed on an 11.7 T Bruker NMR spectrometer. Fully relaxed, one-dimensional ^{1}H NMR spectra were acquired using a 30-degree flip angle, a 6024 Hz sweep width, 4.7-s repetition time, 32,000 block size, presaturated water suppression, and 256 scans. The acquired spectra were fourier-transformed. The chemical shift ∂ was assigned with reference to the internal standard TSP ($\partial = 0$ ppm).

To determine concentrations, peak amplitudes (*I*) for Cho, PC, GPC, and tCho-containing metabolites (PC + GPC + Cho) were compared with that of the internal standard TSP according to the equation:

$$[\text{metabolite}] = A_{TSP} \cdot \frac{I_{metabolite}}{I_{TSP} \cdot N_{cell} \cdot V_{cell}} \quad \text{(A)}$$

where [metabolite] is the molar concentration of the metabolite, A_{TSP} is the number of moles of TSP in the sample, N_{cell} is the cell number, and V_{cell} the cell volume calculated from the radius (*r*) of the cell (see Table 2) according to the equation, $V_{cell} = 4/3 \times \pi \times r^3$. Because the number of protons contributing to the signal of all of the Cho metabolites at 3.21–3.23 ppm and to the TSP peak at 0 ppm is the same, no correction for differences in the number of protons was required. For equation A to be valid, it is necessary that spectra are fully relaxed, as in this study, or to correct for saturation. The resulting metabolite concentrations were averaged for three separate cell culture experiments with separate batches of cells (five for TSU and four for LAPC-AD) for each cell line.

Statistical Analysis. Statistical analyses were performed using Statview II version 1.04, 1991 (Abacus Concepts, Inc., Berkeley, CA). The differences in metabolite levels between cell lines were considered significant for $P \leq 0.05$, using the unpaired student *t* test (two-tailed).

RESULTS

The AR status, the measured DT and V_{cell} of the HPCs used in this study are presented in Table 2. The DT of the cells ranged from 15 to 30 h. From this table, it is apparent that the DT was not related to androgen dependence or independence or to the malignant status of the cells.

^{1}H NMR spectra of the mortal prostate epithelial cells PrEC and the androgen independent PC-3 tumor cells presented in Fig. 1, revealed three water-soluble, Cho metabolites: free intracellular Cho at 3.207 ppm, PC at 3.224 ppm, and GPC at 3.233 ppm. The spectrum from PC-3 cells showed elevated PC and GPC signals compared with PrEC cells. The intracellular PC, GPC, and tCho concentrations for epithelial and stromal cells derived from healthy prostatic tissue compared with androgen-dependent and androgen-independent prostate cancer cell lines are summarized in Fig. 2. As evident in this figure, a significant ($P \leq 0.050$) elevation of PC, GPC, and tCho, compared with epithelial normal HPCs (PrEC), was consistently observed for all of the tumor cell lines irrespective of their androgen status. Compared with stromal PrSC cells, again all of the HPCs derived from metastases exhibited significantly higher PC levels, with the exception of LNCaP and DU-145, significantly higher GPC levels, with the exception of LNCaP, and significantly higher tCho levels, with the exception of LNCaP and DU-145.

The influence of androgen deprivation on PC, GPC, and tCho levels for the androgen-dependent tumor cell lines LNCaP and LAPC-4 is shown in Fig. 3. The response to androgen deprivation for LNCaP cells appears to be in marked contrast to that for LAPC-4 cells.

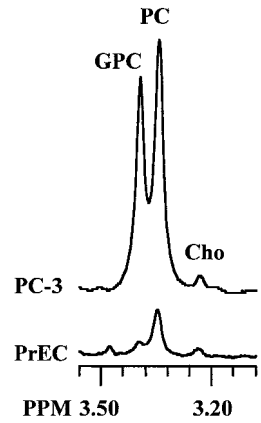

Fig. 1. Expanded one-dimensional ^{1}H NMR spectra of the Cho-containing region obtained from PCA extracts of PrEC and PC-3 cells. These data demonstrate the qualitative differences between the normal phenotype, characterized by low levels of Cho-containing compounds, *versus* the malignant phenotype, showing elevated concentrations of PC and GPC. Spectral assignments include free Cho at 3.207 ppm, PC at 3.224 ppm, and GPC at 3.233 ppm.

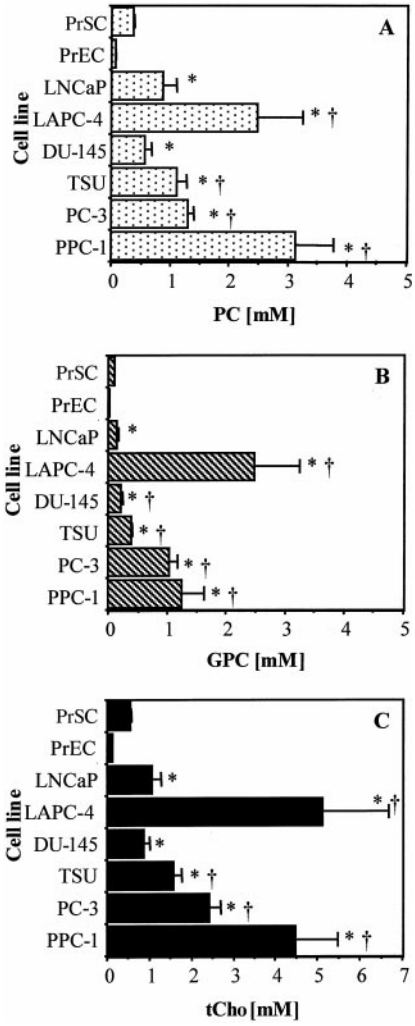

Fig. 2. PC (*A*), GPC (*B*), and tCho (*C*) concentrations (mean ± SE; three experiments) for a panel of HPCs comparing normal prostate cells of stromal and epithelial origin with androgen-dependent and -independent prostate tumor cells. Significant differences between tumor cell lines and the normal PrEC cells are marked by ∗ ($P < 0.050$), and between tumor and PrSC cells are marked by † ($P < 0.050$).

Long-term androgen deprivation (5–14 months) of the LNCaP cell line resulted in a significant increase in PC, GPC, and tCho concentrations in all subclones. Although the parent line, LNCaP did not exhibit significantly higher Cho metabolite levels compared with PrSC cells, and all of the androgen deprived subclones showed significantly increased Cho metabolites compared with PrSC cells. In contrast, androgen deprivation of >13 months in the LAPC-4 cell line, which exhibited much higher PC, GPC, and tCho levels to start with, produced a trend of decreased Cho compounds that were significant for GPC ($P \leq 0.05$) and tCho ($P \leq 0.07$), although all of the Cho metabolite levels were still significantly higher than in PrEC and PrSC cells.

DISCUSSION

The healthy adult prostate is enclosed in a capsule of fibrous connective tissue and smooth muscle fibers that extend into the gland and divide it into indistinct lobes. The stroma of the healthy prostate is comprised predominantly of smooth muscle corresponding with a high expression of smooth muscle α-actin and low expression of vimentin (21). Because clinical MRSI may provide localized voxels with stromal as well as epithelial cell components, we included normal prostate stromal cells in our panel of HPCs. The concentrations of Cho-containing compounds were elevated in epithelial tumor cells compared with normal epithelial and stromal prostate cells. This elevation was independent of the androgen sensitivity of the tumor cells. These data are consistent with the elevation of the tCho peak, detected as an increase in Cho to citrate, in clinical ^{1}H MRSI studies of prostate cancer (2), providing additional support for the use of the Cho signal as an *in vivo* marker of the presence of malignant prostate cells for diagnosis and treatment. Cho concentrations detected in the PCA extracts represent the water-soluble free Cho compounds. Studies have shown that the tCho peak detected *in vivo* is in excellent agreement with tCho measured in PCA extracts of the same tissue after excision (22, 23), and therefore the signal *in vivo* is also mainly from water-soluble free Cho compounds and not from membrane bound compounds. For purposes of *in vivo* detection, it is reasonable to assume that differences in tCho between normal and malignant prostate cells detected in PCA extracts will appear as differences in tCho *in vivo* with MRSI. Our observations are also consistent with recent PET studies that demonstrate increased uptake of ^{11}C-Cho in prostate cancers and other malignancies (4, 24).

Our previous study on human mammary epithelial cells (7) dem-

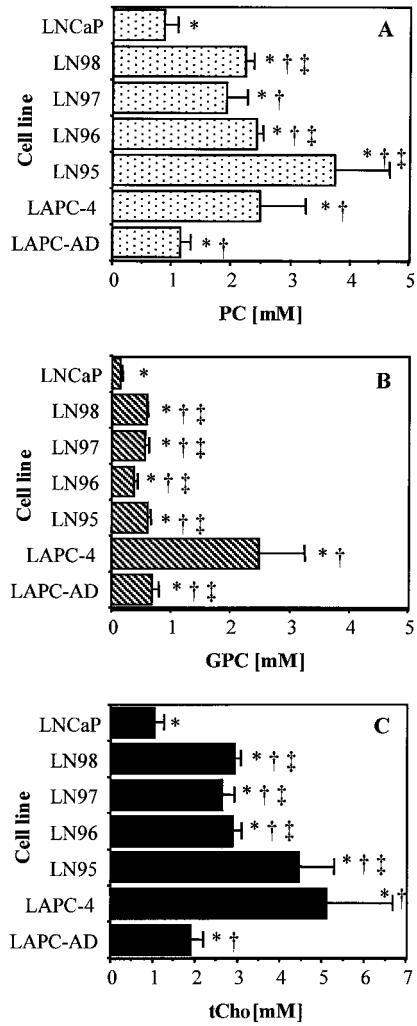

Fig. 3. Effect of androgen deprivation on PC (*A*), GPC (*B*), and tCho (*C*) levels for the androgen-dependent cancer cell lines LNCaP and LAPC-4. Concentrations are in mM (mean ± SE) and averaged over three experiments (four experiments for LAPC-AD). Significant differences between HPCs derived from metastases and the PrEC cells are marked by ∗ ($P < 0.050$) and between the PrSC cells are marked by † ($P < 0.050$). Significance differences between the parental line and the androgen-deprived subclones are denoted by ‡ ($P < 0.050$).

onstrated an increase of tCho with malignant transformation and the data on tCho obtained here for HPCs are similar to results obtained for mammary epithelial cells. However, prostate epithelial cells did not exhibit a switch from "high GPC and low PC" to "low GPC and high PC" after malignant transformation as observed for human mammary epithelial cells (7) and for immortalized, oncogene-transformed rat Schwann cells (25), suggesting that the pathways resulting in the alteration of the individual Cho compounds after malignant transformation may not be identical for mammary and prostatic epithelial cells.

Although the precise mechanisms underlying the alteration in phospholipid metabolism after malignant transformation are yet to be delineated, several possible explanations exist. At least two of these explanations may be ruled out from the cell culture conditions used in our study. For instance, it has been suggested that rapidly proliferating tissues such as tumors have increased membrane/fatty acid requirements, which may be responsible for the high Cho phospholipid metabolite levels in cancer *versus* healthy tissues (reviewed in Ref. 26). However, this explanation is the least plausible in our study. Because of the culture conditions, both normal prostate cell lines had DTs comparable with the HPC tumor cells. Alterations in concentrations of Cho compounds have also been observed as attributable to extra- and intracellular acidosis (27). Again this was largely ruled out in our study because of the frequent changing of culture medium that prevented any acidification of the medium.

The concentration of free Cho in the cell culture medium used was within a fairly narrow range (~20–30 μmol/liter) and high enough to avoid any effects attributable to Cho deprivation. The most plausible explanations for the alteration of Cho metabolites must therefore relate to pathways of Cho transport, incorporation and utilization. Of these, an increased Cho kinase activity and Cho transport (28–30) as well as increased phospholipase activity (31, 32) appear to be the most plausible. Although none of these mechanisms have been investigated in the HPCs studied here, there is some evidence for increased Cho transport and Cho kinase activity in breast cancer cells (28) and after oncogenic transformation (33–35). Similarly, Narayan *et al.* (36) have shown that, whereas phosphatidylcholine content in the membrane remained constant with malignant progression, the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine was altered between benign prostatic hyperplasia and prostate cancer. The total content of palmitic acid was increased, whereas arachidonic acid was decreased in prostate cancer tissues compared with benign prostatic hyperplasia tissues. The decrease in arachidonic acid in the membrane phospholipids of prostate cancer was also confirmed in a study by Faas *et al.* (31), where a 2-fold increase in phospholipase A2 and a 4- to 12-fold higher fatty acyl-CoA lysophosphatidylcholine acyltransferase activity were observed in malignant *versus* benign human prostatic tissue.

Both androgen-dependent cell lines exhibited changes in Cho metabolites after androgen deprivation. Long-term androgen deprivation of the androgen-dependent LNCaP line (LN series) seemed to induce a more malignant phospholipid phenotype with higher levels of Cho compounds. The LN95, 96, 97, and 98 androgen-deprived cells also form xenograft tumors in nude mice more readily than the parental LNCaP cells, and can do so without Matrigel coinjected with the cells (37). The androgen-deprived LNCaP subclones also demonstrated increased colony formation in soft agar assays relative to the parental LNCaP cells. In contrast, androgen-deprivation of LAPC-4 cells over a shorter time period resulted in a decrease of GPC and tCho. Although the effects of chronic androgen deprivation are to be determined for LAPC-4 cells, these findings are consistent with results from Agues *et al.* (38) which indicate that androgen deprivation in prostate cancer produces cell cycle arrest rather than apoptosis, resulting in the emergence of androgen-independent sublines. These results also suggest that, although the initial Cho levels in prostate cancer cells seem to be high irrespective of androgen status, after androgen deprivation, the AR-positive lines showed a significant change in Cho metabolites. These data suggest that androgen deprivation therapy may be evaluated clinically by ^{1}H MRSI. It is also possible that differences in the AR between the cell lines may contribute to the different response in phospholipid metabolism after androgen deprivation. Sequence analyses of the LNCaP cell AR revealed one point mutation in codon 868 (threonine→alanine substitution) in exon 8 located at the COOH-terminal end of the steroid-binding domain (39, 40). The mutant receptor displayed increased binding affinity for progesterone and estradiol. In addition, these ligands activate transcription at concentrations which would be inactive with the wild-type AR. In contrast, the AR of LAPC-4 contains wild-type sequences in the DNA and ligand-binding domain (11). In view of these differences in response to androgen deprivation, the impact of the point mutation of the AR in the LNCaP cell line on the phospholipid profile and prostate cancer progression deserves additional investigation.

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