Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines

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Abstract. Curcumin, traditionally used as a seasoning spice in Indian cuisine, has been reported to decrease the proliferation potential of prostate cancer cells, by a mechanism that is not fully understood. In the current study, we have evaluated the effects of curcumin in cell growth, activation of signal transduction, and transforming activities of both androgen-dependent and independent cell lines. Prostate cancer cell lines, LNCaP and PC-3, were treated with curcumin and its effects were further analyzed on signal transduction and expression of androgen receptor (AR) and AR-related cofactors using transient transfection assay and Western blotting. Our results show that curcumin down-regulates transactivation and expression of AR, activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and CREB (cAMP response element-binding protein)-binding protein (CBP). Curcumin also inhibited the transforming activities of both cell lines as evidenced by the reduced colony forming ability in soft agar. The results obtained here demonstrate that curcumin has a potential therapeutic effect on prostate cancer cells through down-regulation of AR and AR-related cofactors (AP-1, NF-κB and CBP).

Introduction

Prostate cancer is the most common US male cancer and the second leading cause of male cancer deaths in the United States (1). Despite its pervasive impact, the etiology of prostate cancer and the factors that promote its progression are not well understood (2). To date, there is no secure way to tell whether prostate cancer, once found, requires treatment. Current available treatments often have troubling side effects such as urinary incontinence and erectile dysfunction. There is no effective treatment modality once the cancer has evolved into its hormone refractory stage. Androgen ablation therapy (3) has an initial response but destroys androgen-dependent cells without affecting the continuous growth of the androgen-independent cells. In contrast, the androgen-independent cells survive, resulting in the relapse of the disease and ultimately cause the death of the patient (4). Androgens are believed to promote prostate carcinogenesis, acting via androgen receptor (AR)-mediated mechanisms. Preventive strategies are currently emerging. There is an increase in the usage of nutritional supplements such as soybeans, garlic, green tea, vitamin D_3 etc., to augment the prescribed anti-cancer therapies.

Androgen receptor is a ligand-mediated transcriptional factor that belongs to the superfamily of nuclear receptors (5). It is known that AR activated by a ligand can stimulate or repress the androgen-regulated genes. AR may also be transformed in the absence of androgen in prostate cells (6). Although the mechanism of ligand-independent activation of AR has not been clarified yet, cross-talk between the AR and alternative signal transduction pathways, such as protein kinases, growth factors, or cytokines, has been shown (6-9). Some cofactors and proteins are reported to be interacting with AR, including AP-1 (10-12) and NF-κB (13). Recent studies show that transcriptional interference between AR and AP-1 was largely dependent on the expression of CBP (11), and that CBP is also responsible for mutual transcriptional interference between AR and NF-κB (12).

Curcumin is a natural, non-toxic compound of a plant *Curcuma longa Linn*, which has been traditionally used as a seasoning spice in Indian cuisine and also for the treatment of inflammatory conditions and other diseases because of its anti-inflammatory (14) and antioxidant properties (15). An epidemiological study revealed that low incidence of large bowel cancer in Indians can be in part attributed to the presence of natural additives like curcumin in Indian cookery (16). An animal model study also indicated that dietary administration of curcumin significantly inhibited incidence of colon adenocarcinomas and suppressed the colon tumor volume compared to the control group (17). Curcumin appears to be a potent inhibitor of cell growth or proliferation in a variety of tumor cells (18-25). In addition, previous reports showed that curcumin has a potential role in inhibitory cellular migration or invasion (26) and even metastasis (27). The mechanism of the anti-tumorigenic effect of curcumin has been closely studied by investigators, who have focused particularly on its inhibitory effect on eukaryotic transcriptional factors, such as AP-1 (28,29) and NF-κB (30,31).

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Recent studies have shown that curcumin also significantly inhibits the growth of prostate cancer cells *in vivo* (32) and *in vitro* (33). Curcumin acts as a novel inducer of apoptosis (34) or as potent inhibitors of epidermal growth factor receptor signaling (33) and angiogenesis (32). Treatment of curcumin blocked AP-1 and NF-κB activation in androgen-dependent (LNCaP) and independent (DU-145) prostate cancer cell lines is correlated with the repression of anti-apoptotic gene products and activation of caspases (35). However, the anti-tumorigenic mechanisms of curcumin underlying prostate carcinogenesis are not fully understood. To date, there are no reports describing the association between curcumin and AR activation. In the present study, we have evaluated the effects of curcumin in cell growth, as well as activation of different signal transduction and transforming activities of both androgen-dependent and -independent cell lines.

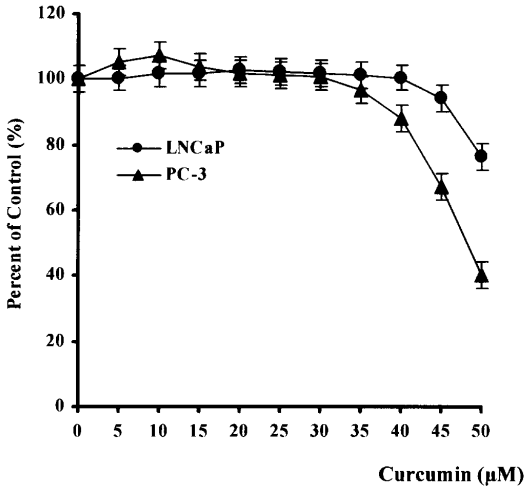

Figure 1. Cell growth following treatment with various doses of curcumin for 24 h in LNCaP (●) and PC-3 cells (▲).

Materials and methods

Cell culture. The androgen-dependent cell line LNCaP and the independent cell line PC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM glutamine, and 5 ml of PSN antibiotic complex (Invitrogen, Carlsbad, CA). Cells were maintained at 37˚C in an atmosphere of 5% CO_2 in air.

Curcumin treatment and cytotoxicity. Cell cultures grown up to 80-90% confluence were exposed to various doses of curcumin (Sigma Chemical, St. Louis, MO) for 24 h. To evaluate toxicity on cultured cells, freshly trypsinized cells ($1x10^5$) were seeded into 35 mm^2 plastic dishes containing 3 ml of medium. The dishes were incubated undisturbed at 37˚C under 5% CO_2 in air. Viable cells were counted on days 3, 5, 7, and 10.

Transient transfection assay. Three types of luciferase reporter plasmids containing androgen responsive elements (ARE) were used for androgen signaling reporter assays. pARE-LUC containing ARE in the upstream of minimal TATA-like promoter were purchased from Clontech (Clontech Laboratories, Palo Alto, CA). p5.3PSAp-LUC encoding 5.3 kb PSA promoter, and the pPB-LUC containing PCR amplified 0.5 kb rat probasin promoter were described previously (36). The pAP-1 (PMA)-TA-LUC and pNF-κB-LUC vectors were also purchased from Clontech. Transient transfections were performed using lipofectamine™ 2000 reagent (Life Technologies, Grand Island, NY). Briefly, for the firefly luciferase reporter assay, the cells were transfected with 0.5 μg of androgen responsive plasmid in combination with 0.05 μg of pTK-Rluc (Promega, Madison, WI) for the internal control. After the 24-h incubation in the presence of various doses of curcumin and/or androgen (R1881, Sigma Chemical, St. Louis, MO), cells were harvested. DNAs were extracted by Dual-Luciferase® reporter assay system (Promega, Madison, WI). Firefly and renilla luciferase activities were both measured concurrently for 12 sec using a luminometer (LUMAT LB9507, Berthold, Germany). Firefly luciferase activities were normalized by renilla luciferase activities of the same lysate. The assays were carried out through quadruplate transfection experiments, and at least three independent values were analyzed to confirm the reproducibility.

Western blot analysis. Cell lysates were collected and estimated using Protein Assay system (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein from each cell line was subjected to SDS-PAGE (10% acrylamide) and transferred onto nitrocellulose membrane. The polyclonal antibodies and working dilution ratios used for immunoblotting were as follows: AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), c-Jun/AP-1 (sc-45; Santa Cruz; 1:2000), NF-κB p-65 (c-20; Santa Cruz; 1:2000), and CBP (C-20; Santa Cruz; 1:500). The membranes were then incubated with horse-radish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham Pharmacia Biotech, Inc. Piscataway, NJ). The expressions of antigen-antibody complexes were detected with an enhanced chemiluminescence kit (Amersham).

Soft agar assay. A cell suspension ($1x10^5$ cells) in 4 ml of 0.35% Noble agar (Difco, Detroit, MI) with medium was overlaid into a 60 mm dish containing a 0.6% agar base. Colonies >0.2 mm in diameter were counted at the 21st day.

Results

Cytotoxicity of curcumin. To estimate the optimal dose for curcumin treatment on prostate cancer cells, the cell growth was determined in the presence of various doses of curcumin (Fig. 1). As the dose of curcumin increased, the cell growth was inhibited in both LNCaP and PC-3 cell lines. The non-toxic maximum doses of cytotoxicity were 40 μM in LNCaP and 30 μM in PC-3 cells, respectively. According to this result, maximum and half non-toxic doses were used for testing further analysis in both cell lines.

Down-regulation of AR signal transduction. As shown in transient transfection assays, the AR transcriptional acitivities were significantly decreased at the maximum non-toxic dose

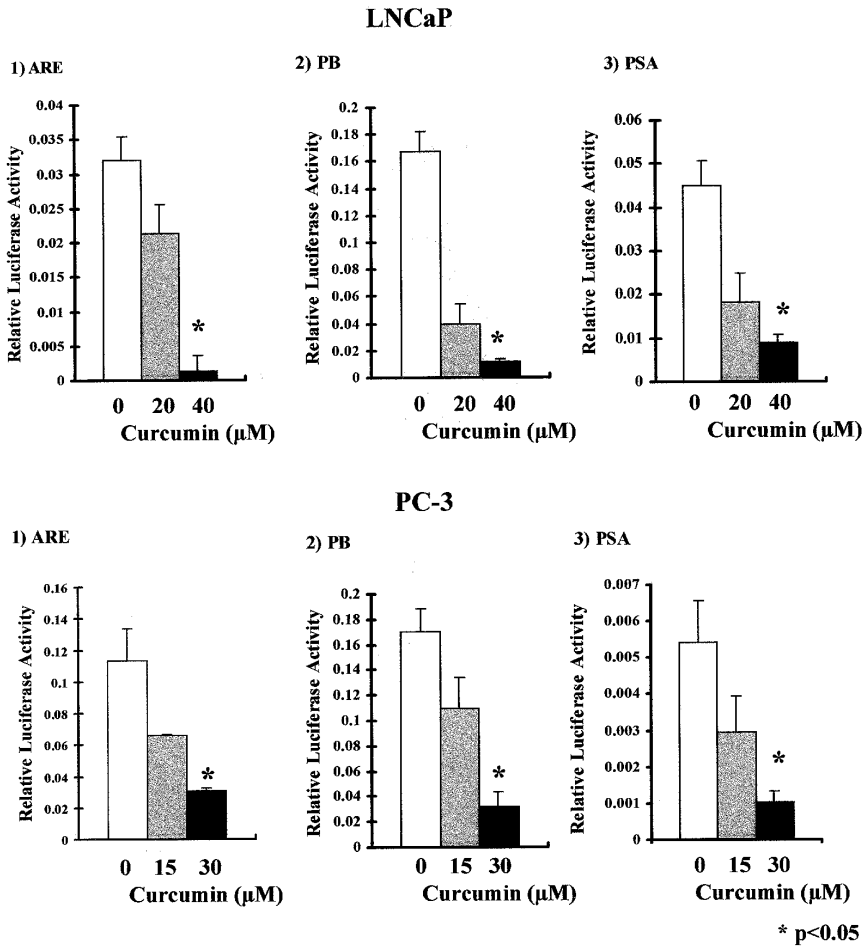

Figure 2. Transient transfection assays. Cells untreated and treated with various doses of curcumin were transfected with 1) ARE, 2) PB, 3) PSA luciferase promoters.

driven by the ARE, PB, and PSA promoters in both LNCaP and PC-3 cells (Fig. 2). When treated with half non-toxic doses of curcumin, the AR transcriptional activities were decreased in both cell lines, but the effect was not significant. To hypothesize that curcumin may regulate the AR transcriptional activity through acting on its ligand-binding site, we examined whether co-treatment with androgen (R1881) can stimulate the AR transcriptional activities in curcumin-treated LNCaP cells (Fig. 3). The result shows that curcumin (30 or 40 μM) had significantly reduced the AR transactivation even when co-treated with R1881, suggesting for either direct or indirect effects on AR mediated transcriptional activities by curcumin. When treated with lower than 20 μM of curcumin, R1881 co-treatment was capable of resuming the reduction of AR transactivation induced by curcumin.

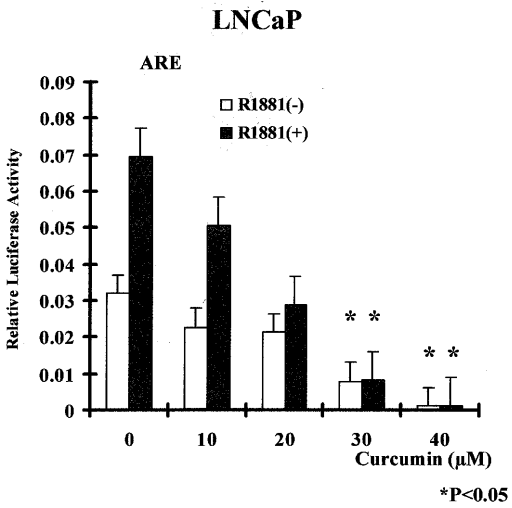

Figure 3. Transient transfection assays. Cells untreated and treated with various doses of curcumin and/or androgen were transfected with ARE promoters.

Down-regulation of AP-1 and NF-κB signal transduction. To confirm the effect of curcumin on other signal transduction pathways, we evaluated the transient transfection assays using AP-1 and NF-κB promoters described in Materials and methods. Transcriptional activities of AP-1 and NF-κB were significantly decreased by the treatment with maximum non-toxic doses of curcumin in both LNCaP and PC-3 cells (Fig. 4). This effect was not observed in the treatment with half non-toxic doses of curcumin.

Reduced expressions in Western blotting. To determine the alteration at protein levels, we evaluated the protein expressions by comparing cell lines which were untreated and treated with

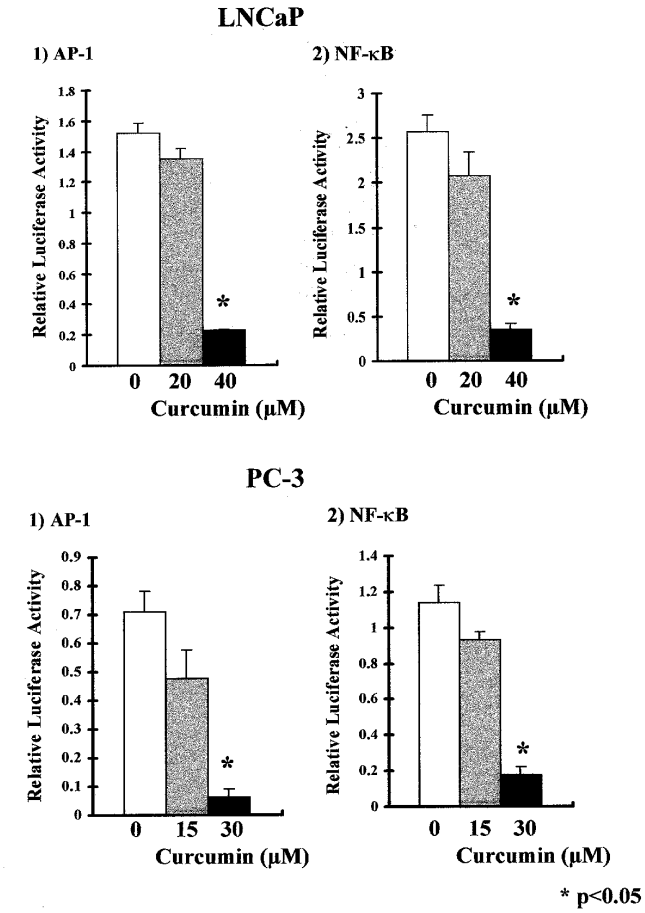

Figure 4. Transient transfection assays. Cells untreated and treated with various doses of curcumin were transfected with 1) AP-1 or 2) NF-κB luciferase promoters.

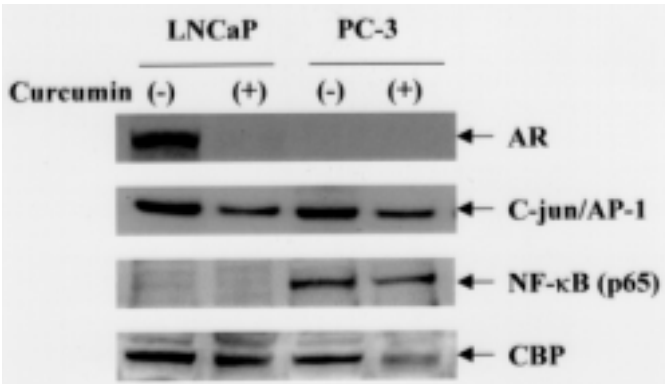

Figure 5. The protein expressions of AR, c-jun/AP-1, NF-κB, and CBP in cells untreated and treated with maximum non-toxic dose of curcumin were detected by Western blotting.

maximum non-toxic curcumin (Fig. 5). The result shows the significant reduction of AR expression in LNCaP cells treated with curcumin. PC-3 was non-evaluable due to lack of AR expression. The expression of c-jun/AP-1 was reduced in each cell line. NF-κB expression was also reduced in curcumin-treated PC-3 cells, whereas NF-κB was undetectable in LNCaP cells. In addition, the down-regulation of CBP protein

Table I. Soft agar colony formation of human prostate cancer cells exposed to curcumin.

Cell line	Curcumin (μM)	No. of colonies in soft agar[a]	Inhibitory effect (%)
LNCaP	0	84	0
	20	53	36.9
	40	8	90.5
PC-3	0	260	0
	15	116	55.4
	30	52.5	79.9

[a]The number was determined through double experiments.

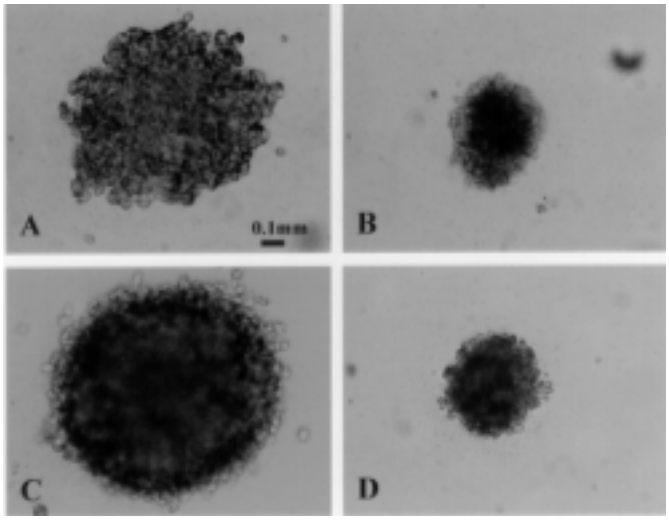

Figure 6. Colonies observed by soft agar assay in LNCaP cells untreated (A), treated with 40 μM of curcumin (B), and PC-3 cells untreated (C), treated with 30 μM of curcumin (D).

expression was detected in the curcumin treated in both cell lines.

Inhibition of transforming activity. The curcumin-treated cells were further characterized in growth properties such as the number of colony-forming ability in soft agar (Table I). The curcumin-treated cells produced smaller number of colonies than those of untreated cells in both cell lines. In addition, both LNCaP and PC-3 cells treated with curcumin produced colonies with significantly smaller size compared to those of the control cells (Fig. 6).

Discussion

Androgen regulates the growth and development of the prostate gland and is necessary for the maintenance of its functions. As a therapeutic modality for prostate cancer, androgen ablation therapy, which attenuates the AR transcriptional

function with either ligand-dependent or independent fashion, can be a powerful tool. In the present study, curcumin has a potential role in suppression of not only protein level but also transcriptional activity of the AR gene. The reduced expression of AR protein induced by the treatment with curcumin was reported elsewhere (34), but to date, there are no reports describing the association between curcumin treatment and AR transactivation. This is the first documented report demonstrating the change of AR transcriptional function with relevance to curcumin.

Earlier works have shown that curcumin could suppress the activation of novel eukaryotic transcriptional factors, such as AP-1 and NF-κB, which are also known as significant cofactors of AR gene activation in prostate cancer (37). Inhibitory effects of curcumin on c-Jun/AP-1 and/or NF-κB activation have been observed in both androgen-dependent (LNCaP) and androgen-independent (DU-145) cells (35) as well as other organ cell lines such as mouse fibroblast cells (28), human leukemia cells (29,30) and human colon epithelial cells (31). On the other hand, AP-1 and NF-κB are known to be potent inhibitors interacting with AR (10,12,13). If curcumin initially acts on AP-1 or NF-κB, the reduction of each factor consequently leads to work toward recovery of AR attenuation. However, in fact, the AR transcriptional function and protein expressions are still reduced by curcumin. One of the candidate factors or substances to help in the resolution of such controversy may be CBP. CBP is an important co-regulator that interacts with AR (11,12,38). In addition, the AR is inhibited by increased expression of either AP-1 (10) or NF-κB (13) through their crucial competition for limited cellular amounts of CBP (11,39). Although further analysis is needed, the data in the current study suggest that CBP can be one of the key factors which regulate AR through interacting with other cofactors.

The optimal dose for the present study was determined by analysis of cell growth under treatment with various concentrations of curcumin. We defined the maximum dose which does not begin to inhibit cell growth as a maximum non-toxic dose. Maximum non-toxic doses of LNCaP and PC-3 cells were 40 and 30 μM, respectively. These values are almost compatible with those reported in prior studies, 20-50 μM (33,34). In our experiments, curcumin required a 30-40 μM dose to attenuate AR gene transcriptional activity, therefore we evaluated protein expression level using this dose in Western blotting and found that AR transcriptional activities were mediated through the AR binding site. But when treated with half non-toxic dose (15-20 μM), either LNCaP or PC-3 cells also exhibited down-regulation of various AR-related promoter activation, although its effect was not significant. Dorai *et al* reported AR protein was strikingly repressed in LNCaP cells even when treated with 20 μM of curcumin (34), implying only 15-20 μM may be enough to suppress AR protein expression even in our system. Other signal transduction pathways, such as AP-1, NF-κB, and CBP can be down-regulated at the same maximum non-toxic dose. It has been reported by other groups that curcumin suppressed the function of AP-1 at the similar concentration (50 μM) in DU-145 cells, but it required larger concentration (75 μM) to suppress NF-κB activation (35). In our study, another androgen-independent cell line PC-3 required a smaller dose to down-regulate NF-κB transactivation, although the change of protein expression at that dose was not significant. When treated with a higher dose of curcumin, PC-3 cells may be working as a complete inhibitor to NF-κB gene activation and expression as well.

Colony formation in soft agar is known to be a hallmark of malignant phenotype. Curcumin was capable of suppressing not only the number of colonies but also the size of the colony in soft agar assay. In addition, using a cell aggregation assay, another sensitive and rapid method to detect the ability of malignant cell phenotypes (40), cell aggregates of both prostate cancer cells treated with curcumin showed significantly smaller size and number compared to those of untreated cells (data not shown). Although we did not evaluate the direct effect of curcumin against tumors *in vivo*, Dorai *et al* have reported that curcumin could cause a marked decrease in the extent of LNCaP cell proliferation implanted into nude mice, and in the microvessel density of the LNCaP tumors *in vivo* (32). Analysis of anti-cancer activity of curcumin is still in progress in our system.

The results obtained here demonstrate that curcumin, a known non-toxic dietary ingredient, has a therapeutic effect on prostate cancer cells through down-regulation of AR gene expression and activation. Alternative AR-related signal transduction pathways are also down-regulated, suggesting that CBP may have a key role in comprehensive interference between AR and AR-related cofactors. The potent role of curcumin that can abrogate cell survival will help to elucidate the anti-tumorigenic mechanism of androgen-independent cells. In addition to its activity as a chemopreventive agent, the potential therapeutic role of curcumin in hormone refractory and advanced prostate cancer is worthy of further evaluation and clinical trials.

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