Androgen Receptor Expression in Androgen-independent Prostate Cancer Is Associated with Increased Expression of Androgen-regulated Genes

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ABSTRACT

The human prostate cancer (CaP) xenograft, CWR22, mimics human CaP. CWR22 grows in testosterone-stimulated nude mice, regresses after castration, and recurs after 5–6 months in the absence of testicular androgen. Like human CaP that recurs during androgen deprivation therapy, the recurrent CWR22 expresses high levels of androgen receptor (AR). Immunohistochemical, Western, and Northern blot analyses demonstrated that AR expression in the androgen-dependent CWR22 is similar to AR expression in the androgen-dependent CWR22 prior to castration. Expression of prostate-specific antigen and human kallikrein-2 mRNAs, two well-characterized androgen-regulated genes in human CaP, was androgen dependent in CWR22. Despite the absence of testicular androgen, prostate-specific antigen and human kallikrein-2 mRNA levels in recurrent CWR22 were higher than the levels in regressing CWR22 tumors from 12-day castrate mice and similar to those in the androgen-stimulated CWR22. Other AR-regulated genes followed a similar pattern of expression. Differential expression screening identified androgen regulation of α-enolase and α-tubulin as well as other unknown mRNAs. Insulin-like growth factor binding protein-5, the homeobox gene Nkx 3.1, the AR coactivator ARA-70, and cell cycle genes Cdk2 and Cdk4 were androgen regulated in CWR22. In recurrent CWR22, the steady-state levels of all these AR-dependent mRNAs were similar to those in the androgen-stimulated CWR22, despite the absence of testicular androgen. Expression of AR and AR-regulated genes in the androgen-deprived recurrent CWR22 at levels similar to the androgen-stimulated CWR22 suggests that AR is transcriptionally active in recurrent CWR22. Induction of these AR-regulated genes may enhance cellular proliferation in relative androgen absence but through an AR-dependent mechanism. Alternatively, in androgen-independent tumors, induced expression of the AR-regulated gene network might result from a non-AR transcription control mechanism common to these genes.

INTRODUCTION

AR is a member of the subfamily of steroid receptors and the larger family of nuclear receptors that function as transcription factors. AR is activated by androgen, binds specific nucleotide sequences known as androgen response elements, and interacts with other factors to control transcription of androgen-regulated genes, thereby stimulating development and function of the male reproductive tract (1–9). CaP is androgen dependent (10), and its growth is mediated by a network of AR-regulated genes that remains to be fully characterized.

CaP regresses after androgen deprivation but eventually recurs in the absence of testicular androgen. AR protein expression in most androgen-independent CaP is similar to that in androgen-dependent CaP (11). This finding has raised the possibility that AR becomes reactivated and has a role in promoting androgen-independent growth of CaP. Recent reports based on cotransfection experiments in cultured cell lines suggested that certain growth factors and other kinase inducers may activate AR in the absence of ligand (12–15).

We are using the human CaP xenograft, CWR22 (16–18), to investigate the role of AR and AR-regulated genes in the androgen-independent growth of CaP. CWR22 is propagated by s.c. transplantation in male nude mice supplemented with testosterone. This xenograft retains the characteristics of human CaP including dependence on androgen for growth. Androgen-dependent cells undergo apoptosis after androgen withdrawal, and the tumors regress in size. However, a subset of androgen-responsive cells survives androgen deprivation. These surviving cells that populate the recurrent tumor express AR and increased levels of Bcl-2, an apoptosis inhibitor. Four to 6 months after the removal of testicular androgen stimulation, tumor cells undergo recurrent growth. AR expression in the recurrent, androgen-independent CWR22 tumor is similar to that in the androgen-stimulated CWR22. The high level of nuclear AR in recurrent CWR22 suggested that AR might be a factor in stimulating androgen-independent growth. As a first step in investigating this possibility, we used CWR22 to identify androgen-regulated mRNAs and measured the levels of these mRNAs in CWR22 after castration and upon recurrence of tumor growth. The results demonstrated that expression of AR-regulated genes is up-regulated in androgen-independent CWR22, consistent with reactivation of AR. They also suggest that androgen-regulated genes have a role in androgen-independent growth of CaP.

MATERIALS AND METHODS

Transplantation of CWR22 Tumors

Nude mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). CWR22 tumors were transplanted into nude mice containing s.c. testosterone pellets (12.5 mg for sustained release of ~10 μg/day; Innovative Research of America, Sarasota, FL) as described previously (17). Testosterone pellets eliminated the wide variation in serum testosterone of male nude mice and maintained a serum concentration of ~4.0 ng/ml. Intact mice bearing androgen-stimulated tumors and castrated mice (testes and testosterone pellets were removed) carrying regressed or recurrent CWR22 tumors were exposed to methoxyflurane and sacrificed by cervical dislocation. Tumors were harvested rapidly and frozen in liquid nitrogen or fixed in 10% buffered formalin for paraffin embedding. All procedures used were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

RNA Isolation and Northern Hybridization

Total RNA was isolated from CWR22 tumors as described (19). RNA was resuspended in sterile H2O was glyoxylated and fractionated through 1.0% glyoxal. RNA was isolated from CWR22 tumors as described (19). RNA was resuspended in sterile H2O was glyoxylated and fractionated through 1.0% glyoxal.
agarose gels and transferred to Biotrans nylon membrane (ICN Biomedicals, Inc., Aurora, OH). cDNA probes were labeled with [32P]dCTP (Amer sham Corp., Arlington Heights, IL) using the Prime-a-Gen e System (Promega Corp., Madison, WI). Membranes were hybridized in aqueous solution (5X SSC, 5X Denhardt’s solution, 1% SDS, and 100 µg/ml salmon sperm DNA) overnight at 68°C. After washing at 50°C for 1 h in 0.1X SSC, 0.1% SDS, the membranes were exposed to X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −80°C. H2-K2 cDNA was kindly provided by Dr. Charles Y-F. Young (Mayo Clinic, Rochester, MN). 

Southern Hybridization

Library Construction

The RiboQuant Multiprobe RNase Protection Assay System (PharMingen, San Diego, CA) was used to quantitate mRNA transcripts for CDk1–4 and p16. Ten µg of total RNA from each of the CWR22 tumors were hybridized with the radiolabeled hcc-1 multiprobe template set (PharMingen) according to the manufacturer’s instructions. After RNase treatment, protected probes were resolved on 5% polyacrylamide sequencing gels, followed by exposure of the dried gel to BioMax MR film (Kodak) overnight at −70°C.

RESULTS

AR protein expression in CWR22 human CaP xenografts was determined using immunohistochemical and Western blot analyses. AR staining in nuclei of epithelial cells was abundant in CWR22 tumors from androgen-stimulated intact mice (Fig. 1A) and decreased 1–4 days after castration (Fig. 1B) during the period of most rapid apoptosis. At 6 days after castration (Fig. 1C), AR protein was decreased and remained so at 12 days (not shown). In the CWR22 tumor that recurred 5 months after androgen withdrawal (Fig. 1D), the intensity of AR staining and strong nuclear localization was similar to that observed in CWR22 tumors from intact androgen-stimulated mice. Western blotting yielded similar results (Fig. 2). AR in the intact androgen-stimulated CWR22 decreased within 1 day after castration, increased 6 and 12 days after castration, but was less than in the recurrent tumor. In the recurrent tumor, AR protein levels were similar to those in CWR22 from androgen-stimulated intact mice. AR mRNA expression was determined by Northern analysis. A normalized transcript of 9.6 kb was present, and bands of similar intensity were obtained with CWR22 tumors from intact androgen-stimulated mice and recurrent CWR22 tumors from CX mice (Fig. 3).

Differential expression screening of cDNA libraries constructed from CWR22 tumors was performed to identify androgen-regulated genes in human CaP. A total of 1652 plates were screened initially by this method (936 from intact CWR22 library and 696 from the recurrent CWR22 library). A second screening to eliminate false positives resulted in 48 regulated clones. Northern blot analysis confirmed androgen regulation of 24 clones. PSA was identified twice, whereas seven clones had no homology to DNA sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD). The remaining 15 clones had sequence homology to transcripts identified previously. Both α-enolase and α-tubulin showed strong...
Fig. 1. AR protein localization in CWR22 tumors. Immunostaining for AR was performed on paraffin-embedded sections of CWR22 tumors. Nuclei stained for AR in the majority of cells in CWR22 tumors from intact mice (A), whereas AR immunostaining was reduced 1 day after castration (B). AR immunostaining increased slightly at 6 days after castration as compared with 1-day castrates (C). Recurrent CWR22 tumors growing in the absence of testicular androgen expressed nuclear AR at levels similar to those of androgen-stimulated CWR22 tumors from intact mice (D).

up-regulation by androgen, whereas testosterone-repressed prostate message-2 was down-regulated. Vascular endothelial growth factor and mitochondrial hinge protein mRNAs were up-regulated by androgen but <2-fold (data not shown). Several ribosomal protein mRNAs and other unknown transcripts were regulated minimally either in an up or downward direction.

The effects of castration and androgen replacement on mRNAs for a prostate-specific homeobox gene, Nkx3.1, and the human kallikrein gene, hK-2, both of which are androgen-regulated in human prostate (22–24), were demonstrated using Northern hybridization (Fig. 4). IGFBP-5 was androgen up-regulated, whereas other IGFBP mRNAs (IGFBP-2, IGFBP-3, and IGFBP-6) were unchanged (data not shown). We also found that the AR coactivator ARA-70 was androgen up-regulated. As shown in Fig. 4, the levels of all these mRNAs were decreased 6 and 12 days after castration when compared with the levels in CWR22 tumors from androgen-stimulated intact mice. Treatment of the 6- and 12-day CX mice with TP (0.1 mg/animal) for 24, 48, or 72 h increased the level of each of these mRNA transcripts, implicating AR in the control of their expression. In the CWR22 tumor that recurred in the absence of testicular androgen, levels of all these androgen-regulated mRNAs increased compared with castrates and were similar to those in CWR22 tumors from androgen-stimulated intact mice (Fig. 4).

Androgen regulation of genes controlling cell cycle progression in CWR22 tumors was investigated using RNase protection assays to compare the levels of mRNA transcripts encoding the Cdk5 (Cdk 1–4) and the Cdk inhibitor p16 (Fig. 5). Androgen withdrawal from CWR22 tumors for 6 or 12 days resulted in down-regulation of Cdk1 and Cdk2 and up-regulation of p16. Testosterone treatment of 6- or 12-day CX animals resulted in up-regulation within 24 h of Cdk1 and Cdk2 and down-regulation of p16. In recurrent CWR22, Cdk1 and Cdk2 were expressed at levels similar to those of the androgen-stimulated CWR22 from intact mice. However, levels of p16 were variable in 10 different recurrent CWR22 tumors examined. Cdk4 levels did not change appreciably after castration or with testosterone replacement. Ki-67 immunostaining, which correlates well with bromodeoxyuridine incorporation and thymidine labeling (25), was used to estimate cellular proliferation in CWR22 tumors (Fig. 6). The percentage of Ki-67-positive cells decreased from 79.4 ± 3.6 in tumors from intact mice to 0.8 ± 0.5 at 6 days after castration. Injection of TP (0.1 mg) increased Ki-67 immunostaining to 4.5 ± 0.42 within 24 h and to 31.0 ± 6.7% positive within 48 h. CWR22 tumors that recurred in the absence of testicular androgen demonstrated a high percentage of Ki-67-positive cells (67.1 ± 3.3) similar to that of CWR22 tumors from intact mice.
AR IN RECURRENT PROSTATE CANCER

Fig. 3. Northern blot analysis of AR mRNA in CWR22 and recurrent CWR22. Steady-state AR mRNA levels were similar in the androgen-stimulated CWR22 from intact mice and recurrent CWR22 (CWR22R) tumors from CX mice. The position of molecular mass markers (kilobases) are indicated.

Fig. 4. A, Northern blot analysis of androgen-regulated genes in CWR22. Total RNA from CWR22 tumors was analyzed by Northern analysis to examine androgen-regulated changes in expression of several transcripts. All mRNAs examined decreased after castration (6 or 12 days) of mice bearing CWR22 tumors. Treatment of castrated mice with TP for 24, 48, or 72 h restored expression of the mRNAs to levels seen in the intact CWR22. Recurrent CWR22 tumors growing in the absence of testicular androgens expressed the androgen up-regulated mRNAs at levels similar to the mRNA levels in intact CWR22 tumors (non-CX mice containing testosterone pellets). 18S rRNA was used as a control for mRNA loading variability. In B, densitometry results compare integrated band densities for all genes examined in CWR22 tumors 12 days after castration with recurrent CWR22 tumors and represent the means of results from three different tumor RNAs at each time point: bars, SE. The integrated density of 12-day CX samples was arbitrarily set to 1.0 so that fold induction in the recurrent samples can be appreciated.

DISCUSSION

The levels of AR protein found in CWR22 tumors that recurred 5–6 months after castration were similar to AR levels in most androgen-independent CaPs (11, 26) and allowed this xenograft to be used as a model for determining the role of AR in androgen-independent growth. Because AR is a transcription factor essential to the growth of androgen-dependent CaP, reactivation of AR in the absence of testicular androgen may promote growth in androgen-independent CaP. As a first step in testing this hypothesis, we searched for androgen-regulated mRNAs in androgen-dependent CWR22 and measured levels of expression of these androgen-regulated mRNAs in the recurrent CWR22. Several androgen-regulated mRNAs were identified in CWR22 based on decreased steady-state levels after castration and increased levels after testosterone replacement. In the recurrent tumors, the levels of all androgen-regulated mRNAs were higher than in CWR22 tumors after castration and similar to levels in androgen-stimulated tumors from intact mice. This novel observation is consistent with AR induction of gene transcription in androgen-independent CaP. It suggests also that growth of androgen-independent tumor cells is stimulated by a network of AR-regulated genes.

Several factors are known to control AR gene transcription in mammalian cell lines (27–38); however, the specific factors that induce AR expression in CaP remain unknown. Increased AR expression in a significant number of androgen-independent CaPs may result from amplification of the AR gene locus. AR gene amplification was present in ~30% of CaPs that relapsed after androgen deprivation but in none of the original primary tumors (39). In a series of 54 locally recurrent CaPs from patients who survived for 1 year or more on androgen deprivation therapy, comparative genomic hybridization and interphase fluorescence in situ hybridization demonstrated amplification in 15 tumors (40). Amplification was associated with increased expression of AR mRNA and may contribute to the AR expression in recurrent CWR22; however, this remains to be determined.

AR and androgens are essential for development, growth, and function of the prostate. In human and other mammalian species, castration results in apoptosis and loss of androgen-dependent prostate cells. In normal prostate cells that survive androgen deprivation, AR does not become reactivated in the absence of testicular androgen (41). However, in CaP cells, genetic alterations occurring during the months after androgen deprivation might turn on signaling mechanisms and reactivate AR-induced transcription independent of androgen or at a lower concentration of androgen or other steroids (42). AR activation might result from direct effects on AR that alter its structure or phosphorylation state or indirect effects involving protein-protein interactions with AR coactivators or repressors. Modulators of protein kinases A and C might activate AR in the absence of ligand by altering AR phosphorylation (43–46). Other AR-activating pathways might be mediated by transmembrane receptors for growth factors. Studies on CaP cell lines suggested that keratinocyte growth factor, epidermal growth factor, and IGF-I signaling pathways activate AR in recurrent CaPs from patients who survived for 1 year or more on androgen deprivation therapy, comparative genomic hybridization and interphase fluorescence in situ hybridization demonstrated amplification in 15 tumors (40). Amplification was associated with increased expression of AR mRNA and may contribute to the AR expression in recurrent CWR22; however, this remains to be determined.

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Fig. 5. RNase protection assay for Cdk1 and p16 in CWR22 tumors. Cdk1 and Cdk2 mRNAs were androgen up-regulated in CWR22 tumors, whereas Cdk4 mRNA levels were unchanged after castration or testosterone treatment. Cdk1 and Cdk2 mRNA levels in recurrent CWR22 (CWR22R) tumors were similar or higher than those in intact CWR22 tumors. p16 was negatively regulated by androgens and showed variable expression in recurrent CWR22 tumors. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) is included as a loading control. 6d and 12d, 6-day and 12-day, respectively.
the absence of androgen (47). With respect to IGF-I, our results suggest a mechanism whereby its signaling may be enhanced in the recurrent tumor. Because IGFBP-5 is up-regulated by AR and is a positive modulator of IGF-I in some tissues (48), IGFBP-5 may amplify IGF-I activation of AR in the recurrent tumor thereby creating a self-perpetuating autocrine feedback loop at the level of the IGF-I receptor. Moreover, PSA and h-K-2 are AR-regulated members of the human kallikrein family of serine proteases that degrade the IGF-I inhibitor, IGFBP-3 (48). Thus, the increased expression of these proteases in the recurrent tumor could further enhance the activity of IGF-I.

PSA and h-K-2 genes are transcriptionally up-regulated by androgen and contain androgen response elements in their 5′ flanking regions (49–53). Additional upstream PSA gene enhancer elements have been identified that amplify androgen responsiveness in a cell type-specific manner, suggesting that other DNA-binding proteins cooperate with AR in amplifying the transcriptional response (49). Studies on several AR-regulated genes indicated that DNA-binding proteins, some of which are androgen-regulated, cooperate with AR in the induction of gene transcription (3–6, 54–57). However, these interacting factors remain to be characterized, and as yet no factor has been shown to induce transcription of these genes in the absence of AR. This leads to the question: What accounts for the increased expression of PSA and h-K-2 in CaP that recur after androgen deprivation? The increase in serum PSA associated with tumor recurrence during androgen deprivation treatment has often been explained on the basis of increased tumor volume; however, our studies on CWR22 demonstrate that PSA mRNA is increased per tumor weight. In a recent clinical study of 46 patients undergoing treatment for CaP, de Vere White et al. (11) obtained results on AR and PSA similar to our findings in CWR22. In patients who underwent a relatively short period of "combined androgen blockade" for downsizing prior to radical prostatectomy, most residual CaP expressed AR but low to undetectable PSA, whereas CaP that relapsed after a longer period of androgen blockade expressed both AR and PSA, indicating a link between AR and PSA expression in androgen-independent CaP. Factors that modulate PSA expression in androgen-responsive cells may act by enhancing the action of AR. PSA-stimulating activity was identified by Hsieh et al. (58) in conditioned medium of an LNCaP cell subline derived from a s.c. xenograft that exhibited relapsed growth after castration of the host male nude mouse. The conditioned medium was active in the absence of testosterone; however, the LNCaP cells expressing PSA were androgen responsive, suggesting the possibility that AR could have mediated the increase in PSA expression. Similarly, vitamin D (1,25-dihydroxyvitamin D₃) increased PSA secretion and inhibited growth of cultured LNCaP cells through what was believed to be an effect on cell differentiation (59). Moreover, Gleave et al. (60) demonstrated an increase in LNCaP PSA mRNA expression by treatment with transforming growth factor β that was independent of cellular proliferation.

The finding that several AR-controlled genes are up-regulated in androgen-independent CWR22 strengthens the concept that AR has a role in mediating androgen-independent growth and emphasizes further the importance of identifying AR-regulated, growth-promoting genes. ARA-70 was first identified in a thyroid carcinoma as a RET fusion gene in which 790 5′-nucleotides of the ARA-70 gene were fused to the tyrosine kinase domain of the RET oncogene. The full significance of this rearrangement remains to be established; however, the RET gene is mutated frequently in papillary thyroid carcinomas (61). ARA-70 is reported to be an AR coactivator and potentiates the transcriptional activity of AR in DU-145 cells cotransfected with a reporter gene (62). Its up-regulation in recurrent CWR22 could amplify effects of other ligand-independent AR activators.

The Nkx3.1 gene encodes a 234-amino acid homeodomain protein and is a homologue of the Drosophila Nk-3 gene (63). Human and mouse proteins are identical within the homeodomain region. In mouse prostate and seminal vesicle, Nkx3.1 mRNA increases in parallel with androgen stimulation of sexual development, and castration reduces mRNA levels in sexually mature mice (64). Nkx3.1 mRNA was demonstrated in prostate and to a lesser extent in testis in adult human males. It is also expressed in LNCaP cells and up-regulated by androgen. Expression was not detected in the PC-3 or DU-145 androgen-independent cell lines that lack AR (23). The Nkx3.1 gene maps to chromosome 8p2.1, a region commonly mutated in prostate cancer. However, in 50 CaP tissue specimens removed at radical prostatectomy, only one deletion was found, and no sequence-altering mutation was detected in the Nkx3.1 coding region (65). Chromosome 8 alleles are present in both CWR22 and recurrent CWR22 (16), and their Nkx3.1 mRNAs are of equal size. Homeodomain proteins have the ability to route extracellular signals to specific target genes through protein-protein interactions (66–68). Thus, Nkx3.1 may integrate a number of growth-promoting signals in both the androgen-dependent and androgen-independent CWR22.

α-Enolase is the glycolytic enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate, the precursor of pyruvate. Glycolysis is believed to have an important role in DNA synthesis (69). Oxidative decarboxylation of pyruvate to form acetyl-CoA links glycolysis with the citric acid cycle. Human and rat prostate secrete large amounts of citric acid formed by the reaction of acetyl-CoA with oxaloacetate and H₂O. Citric acid production is androgen dependent. In the rat, androgens increase the expression of pyruvate dehydrogenase-E1 that catalyzes the conversion of pyruvate to acetyl-CoA and mitochondrial aspartate aminotransferase that converts aspartate to oxaloacetate. The latter was regulated by a combined increase in transcription rate and mRNA stabilization (70–72).

α-Tubulin and β-tubulin subunits, together with their associated proteins, make up the mitotic apparatus, a microtubule machine for separating chromosomes. α-Tubulin has been considered a constitutive housekeeping gene; however, our data indicate its expression is regulated either directly or indirectly by androgen in CWR22. The relatively slow increase in α-tubulin mRNA in response to androgen

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suggests it is a secondary response gene perhaps controlled by multiple gene products, some of which are androgen-regulated. Androgen regulation of α-tubulin may link AR action to cell division in CaP. Cdk5 together with cyclins control the normal progression of cells through the cell cycle. Abrupt activation of Cdk5s may potentiate the growth of certain cancers (73), including CaP. Cdk2 and Cdk4 were positively regulated by androgen in cultured LNCaP cells in association with increased cellular proliferation (74). Cdk1 and Cdk2 mRNAs in androgen-dependent CWR22 decreased after castration and were up-regulated by androgen replacement. In the recurrent CWR22, Cdk1 and Cdk2 mRNAs were expressed at levels similar to those in androgen-dependent CWR22 tumors from intact mice. Cdk4 mRNA remained unchanged after castration or androgen replacement.

The up-regulation of Cdk1 and Cdk2 mRNAs after testosterone replacement in 6-day CaCIX mice coincided with an increase in cellular proliferation (as assessed by Ki-67 immunohistochemistry). Whereas protein synthesis and subsequent phosphorylation or dephosphorylation is required for Cdk complex formation with cyclins, our data suggest that AR induction of Cdk1 and Cdk2 mRNA expression is an important step in reactivating the cell cycle. Cdk1, which appeared to be strongly androgen-regulated, complexes with cyclin B and is activated by dephosphorylation just prior to mitosis (75). Negative regulation of the Cdk inhibitor p16 in CWR22 would diminish the inhibitory effect of p16, further enhancing the initiation of cellular proliferation. However, p16 mRNA levels were variable in recurrent CWR22 tumors. Chi et al. (76) found similar variability in p16 mRNA expression in 116 human prostate tissue specimens, with 43% of untreated primary CaP showing reduced expression.

Our results indicate that a network of androgen-regulated genes has a role in driving the growth of androgen-independent CaP. Furthermore, they provide a conceptual framework within which to investigate further the role of AR and to identify additional gene products important in the recurrence of CaP after androgen deprivation. The expression of AR and androgen-regulated genes in androgen-independent CaP suggests AR is active in the absence of testicular androgen. Alternatively, a compensatory transcriptional or mRNA-stabilizing mechanism common to androgen-regulated genes might become activated in the absence of androgen stimulation. Pursuit of these possibilities should lead to a better understanding of the transition of CaP from androgen-dependent to androgen-independent growth.

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