

ORIGINAL ARTICLE

Importance of the intracrine metabolism of adrenal androgens in androgen-dependent prostate cancer

K Suzuki¹, T Nishiyama¹, N Hara¹, K Yamana¹, K Takahashi¹ and F Labrie²

¹Division of Urology, Department of Regenerative and Transplant Medicine, Course of Biological Function and Medicine Control, Niigata University Graduate School of Medical and Dental Science, Niigata, Japan and ²Laboratory of Molecular Endocrinology and Oncology, Laval University Hospital Research Center (CRCHUL) and Laval University, Quebec City, Quebec, Canada

The metabolic pathways of androgens and processes by which androgens induce re-growth after androgen deprivation therapy in prostate cancer have not been fully elucidated. In this study, finasteride decreased PSA secretion in medium containing testosterone, androstenedione, androstenediol and dehydroepiandrosterone, whereas dihydrotestosterone (DHT)- and hydroxyflutamide-induced PSA production was not inhibited by finasteride in LNCaP-FGC cells. The present data show that adrenal androgen precursors do not directly interact with androgen receptors (ARs) but are converted to DHT via the intraprostatic metabolic pathways, resulting in the induction of LNCaP activity. This is the first report confirming this mechanism experimentally and also suggest the use of combined therapies that target ARs and prevent the formation of DHT within prostate cancer cells to achieve optimal therapeutic efficacy.

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Introduction

As Huggins and Hodges¹ observed that disseminated prostate cancer reacts favorably to castration or to the administration of estrogens, first-line hormonal therapy has been used to impair the production or activity of androgens, or both. At present, androgen deprivation therapy (ADT), consisting of testicular androgen blockade, the use of antiandrogens, or the combination of both (combined androgen blockade) is the standard therapy for metastatic prostate cancer. In such cases, the response rate of ADT is up to 80% with monotherapy and at more than 90% with combination therapy. However, at the metastatic stage, prostate cancer becomes resistant to ADT. If we are to improve significantly survival, new therapeutic strategies designed to avoid the emergence of resistant phenotypes must be developed.

The mechanisms of development of resistance to treatment or androgen independence have been the subject of many investigations. The AR signaling pathway might still be active during the early stage of transition to 'androgen independence' of prostate cancer cells. In fact, cancer cells adapt themselves to grow in a

low androgen environment by sensitizing the AR signaling pathway. Prostate cells can amplify and overexpress the AR gene, amplify and overexpress AR coactivators, mutate AR to become activated directly by steroids other than dihydrotestosterone (DHT) such as precursor adrenal androgens, and activate AR by growth factors and cytokines.^{2–5} Phosphorylation of AR or AR-associated proteins occurs as observed for the estrogen receptor.^{6,7}

As mentioned above, many investigators have paid attention to the AR hypersensitivity-related mechanisms of development of androgen independence. On the other hand, steroid metabolic enzymes are still expressed in prostate cancer cells, although at the androgen-independent stage, the enzyme activity has been reported to be dramatically changed,⁸ thus suggesting that steroidogenic processes of androgen metabolism are also important for the development of androgen independence. These two processes, namely AR hypersensitivity-related development mechanisms and changes in the androgen steroidogenic processes are not mutually exclusive and could simultaneously operate in prostatic cells. However, which process is the predominant pathway in the development of androgen independence remains to be determined.

The aim of the present study was to clarify the mechanism of the early stage transition to a mixed androgen-independent and androgen-sensitive status of prostate cancer cells. We first elucidated the intracellular metabolism and action of androgens, especially adrenal androgens, using LNCaP-FGC cells as model.

Correspondence: Dr K Suzuki, Division of Urology, Department of Regenerative and Transplant Medicine, Course of Biological Function and Medicine Control, Niigata University Graduate School of Medical and Dental Science, Asahimachi 1-757, Niigata 951-8510, Japan.
E-mail: kazuyas@med.niigata-u.ac.jp

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2 Materials and methods

Human prostate tumor LNCaP-FGC (American Type Culture Collection (ATCC), Manassas, VA, USA), PC3 (ATCC) and DU145 (ATCC) cells were grown in Roswell Park Memorial Institute-1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (CSL Ltd, Victoria, Australia), 1% MEM non-essential amino acid (Gibco), 1% MEM sodium pyruvate solution 100 mM (Gibco) and 90 µg/ml kanamycin. The prostate epithelial cell line, PrEC (Clonetics, Biowhitaker, San Diego, CA, USA) was grown in PrEBM (Clonetics). Cells were maintained in an atmosphere of 5% CO₂ and 95% air at 37°C. LNCaP-FGC cells, on the other hand, were plated at a concentration of 5 × 10⁵ cells per 25 cm² flasks (Falcon Labware, Lincoln Park, NJ, USA) and cultured in phenol-red free medium with 5% charcoal-dextran stripped of FBS 24 h before the experiment. Cells were treated with DHT, testosterone, androstenedione, androstenediol, dehydroepiandrosterone (DHEA), hydroxy-flutamide (all purchased from Sigma Chemical, St Louis, MO, USA) in the presence or absence of 5α-reductase (5-R) inhibitor finasteride (Merck Research Laboratory, Rahway, NJ, USA). Cells were also treated with finasteride alone. The cells were kept for 7 days after culture in the experimental media. The media were collected to determine PSA levels. The PSA levels in the culture media were determined by commercially available solid-phase radioimmunoassay using the Tandem PSA Test (Beckman Coulter Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Total cellular RNA from the cell lines was isolated using an ISOGEN RNA isolation kit (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. RNA was subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis as described earlier.⁹ To synthesize first-strand cDNA, 1 µg of the total RNA was reverse-transcribed using a first-strand cDNA Synthesis Kit (Roche Diagnostics, Tokyo, Japan). The first-strand DNA was then amplified by PCR using the primers shown in Table 1. PCR was performed in a 50 µl reaction volume-containing buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.4; 2.5 mM MgCl₂ and 200 mg/ml gelatin), with 200 µM of each dNTP, 400 nM of each outer primer and 2.5 units of AmpliTac Gold (Roche Diagnostics, Tokyo, Japan). PCR consisted of 35 cycles of denaturation (94°C, 30 s), annealing (50, 60, 60, 58, 55 and 60°C for 5-R type 1, 2, 17β-hydroxysteroid dehy-

drogenase (17-HSD) type 2, 3, 5 and 3β-hydroxysteroid dehydrogenase (3-HSD) type 1 respectively, 45 s) and extension (72°C, 60 s) followed by a final 10 min extension at 72°C. The PCR products were size fractionated by 3% agarose gel electrophoresis. DNA bands were visualized with an ultraviolet transilluminator (Spectoline, Funakoshi, Tokyo, Japan).

Statistical comparisons were made using the Kruskal-Wallis test with SPSS 10.0 J software for Windows. $P < 0.05$ was considered significant. The results are expressed as mean ± s.e.m.

Results

We first studied the effect of DHT, testosterone, androstenedione, androstenediol, DHEA and hydroxy-flutamide on PSA secretion in LNCaP cells. At 10⁻¹⁰, 10⁻⁹ and 10⁻⁸ M, DHT increased PSA secretion 7 days after treatment by 1.28-, 2.2-, 3.58-fold, respectively ($P = 0.016$) (Figure 1a). The same doses of testosterone (Figure 1a), androstenedione, androstenediol, and DHEA (Figure 1b) increased PSA production by 1.6-, 2.95-, 3.37-fold ($P = 0.016$), 1.15-, 2.05-, 3.09-fold ($P = 0.016$), 1.69-, 3.12-, 4.82-fold ($P = 0.016$) and 1.19-, 1.51-, 2.71-fold ($P = 0.019$) in a dose-dependent manner, respectively. Treatment with the antiandrogen hydroxy-flutamide at 10⁻¹⁰, 10⁻⁸, 10⁻⁶ M resulted in a 1.46-, 2.86-, 3.78-fold dose-dependent increase in PSA production in conditioned media, respectively ($P = 0.019$, Figure 1c). Although these androgens and hydroxy-flutamide had some effect on cell growth, no statistically significant difference was found (data not shown). PSA production with conditioned medium was normalized to the cell numbers.

The 5-R inhibitor finasteride was first used to block the conversion of testosterone into DHT. Treatment with 10⁻⁸, 5 × 10⁻⁸ and 10⁻⁷ M finasteride alone had no significant effect on PSA secretion in the media after 7 days, of incubation (Figure 2a). On the other hand, treatment with 10⁻⁸, 5 × 10⁻⁸ and 10⁻⁷ M finasteride resulted in 95.5, 89.4 and 80.7% ($P = 0.034$) dose-dependent inhibition of 10⁻⁸ M testosterone-induced PSA secretion, respectively. However, the same doses of finasteride treatment had no significant effect on DHT-induced PSA secretion (Figure 2b). These results suggest that testosterone-induced PSA production up to a large

Table 1 Different sets of primers used in the PCRs

RNA transcript	Primer 5'-3'	Position in cDNA	PCR fragment size (bp)
5-R1	TGC GAG GAG GAA AGC CTA TG (F)	347-366	308
	GCC ACA CCA CTC CAT GAT TTC (R)	654-634	
5-R2	CAT ACG GTT TAG CTT GGG TGT (F)	456-476	315
	GCT TTC CGA GAT TTG GGG TAG (R)	770-750	
17-HSD2	AGT TGC TTC CAT CCA ACC TGG A (F)	851-872	405
	TTC CAT TGC CTA GGT GGC CTT T (R)	1256-1235	
17-HSD3	CGA GCA TAT TAA AGA AAA ACT TGC AGG CTT (F)	387-416	378
	AGA TAC TTT GTC ATT GCA GTC GAG A (R)	764-740	
17-HSD5	CCA GGT GAG GAA CTT TCA CCA A (F)	381-402	112
	TGG CCA ATC CTG CAT CCT T (R)	492-474	
3-HSD1	GCA CCC TGT ACA CTT GTG CCT T (F)	664-685	255
	GGT GAG GCG TGT CAT CTG AGAT (R)	918-897	

Abbreviations: F, forward; R, reverse; 5-R, 5α-reductase; 17-HSD, 17β-hydroxysteroid dehydrogenase; 3-HSD, 3β-hydroxysteroid dehydrogenase.

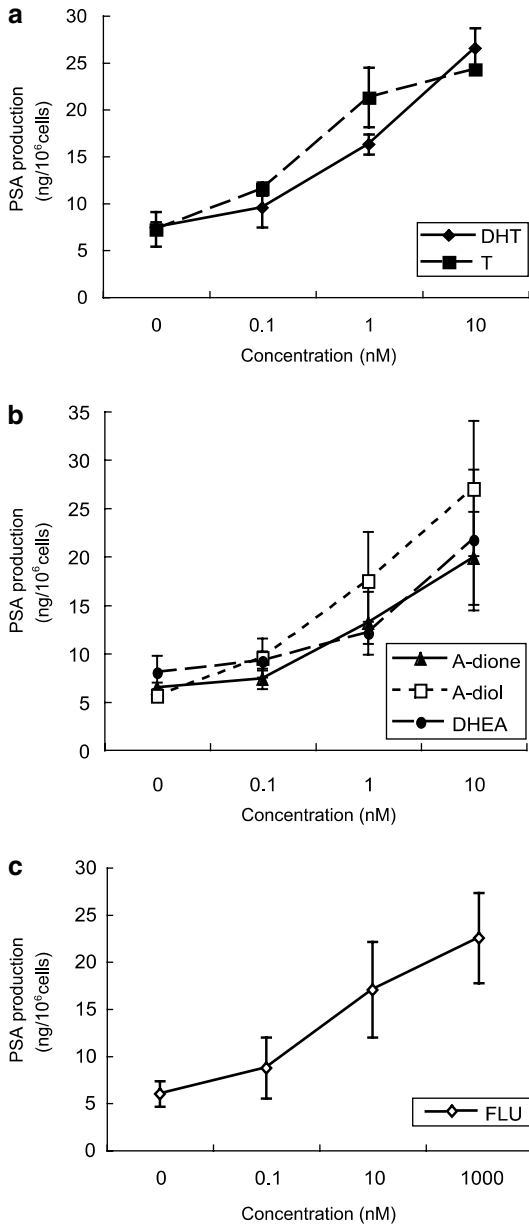


Figure 1 Effect of androgens and their precursors as well as of the anti-androgen hydroxy-flutamide on PSA production in LNCaP cells: 24 h after 5×10^5 LNCaP cells were seeded, cells were treated with the indicated concentration of DHT, testosterone (a), androstenedione, androstenediol, DHEA (b), or hydroxy-flutamide (c), and cultured for 7 days. PSA levels in the treated media were then measured by solid-phase RIA methods. PSA production with conditioned medium was normalized to the cell numbers. The results are expressed as means \pm s.e.m.

extent, is mediated by conversion to DHT in LNCaP cells instead of a direct interaction of testosterone with AR.

Treatment with 10^{-8} , 5×10^{-8} and 10^{-7} M finasteride added to a fixed concentration of 10^{-6} M hydroxy-flutamide had no significant effect on PSA secretion (Figure 3a). As shown in Figure 3b, treatment with 10^{-8} , 5×10^{-8} and 10^{-7} M finasteride added to a fixed concentration of 10^{-8} M androstenedione, androstenediol or DHEA resulted in 98.1, 93.1, 83.9 ($P = 0.031$), 93.3, 88.5 and 82.3% ($P = 0.029$) and 83.1, 69.5 and 49.3% ($P = 0.016$) dose-dependent inhibitions of PSA secretion in the

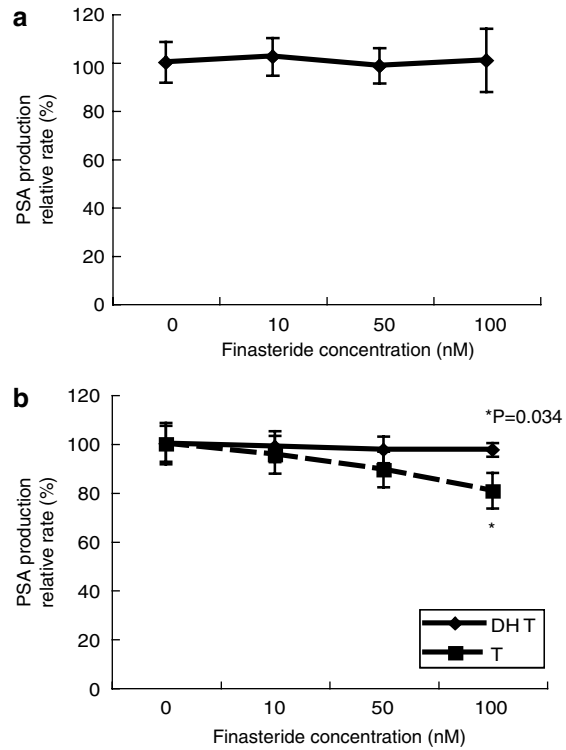


Figure 2 Inhibition of testosterone-induced PSA accumulation in LNCaP cell medium by finasteride: LNCaP cells were incubated with 10^{-8} M DHT or testosterone in the absence or presence of finasteride for 7 days. DHT-induced PSA production was not inhibited by finasteride.

media, respectively. These results indicate that androstenedione and androstenediol but mostly DHEA induce PSA secretion in LNCaP cells after transformation into DHT.

5-R type 1 and 2, 17-HSD types 2, 3, and 5, and 3-HSD type 1 expressions were detected in LNCaP cells. As shown in Figure 4, mRNA expression for 5-R types 1, 2, 17-HSD types 2, 3, 5 and 3-HSD type 1 was detected as specific single bands (308, 315, 405, 378, 112 and 255 bp, respectively) in LNCaP-FGC, PC-3, DU145 and PrEC cells.

Discussion

The present data show that precursor adrenal androgens exert a stimulatory effect on PSA secretion in LNCaP cells following enzymatic conversion into DHT with subsequent binding to the AR. This is the first report that describes the major importance of the steroidogenic process from DHEA over the direct binding of potential androgens to hypersensitive AR in the action of precursor adrenal androgens in prostate cancer cells. This steroidogenic process should be taken into account in addition to the AR hypersensitivity-related development mechanisms leading to resistance to antiandrogen treatment in prostate cancer.

PSA has been used as parameter to study androgen action in prostate cancer. LNCaP cells are the most frequently studied AR-positive prostate cancer cell line

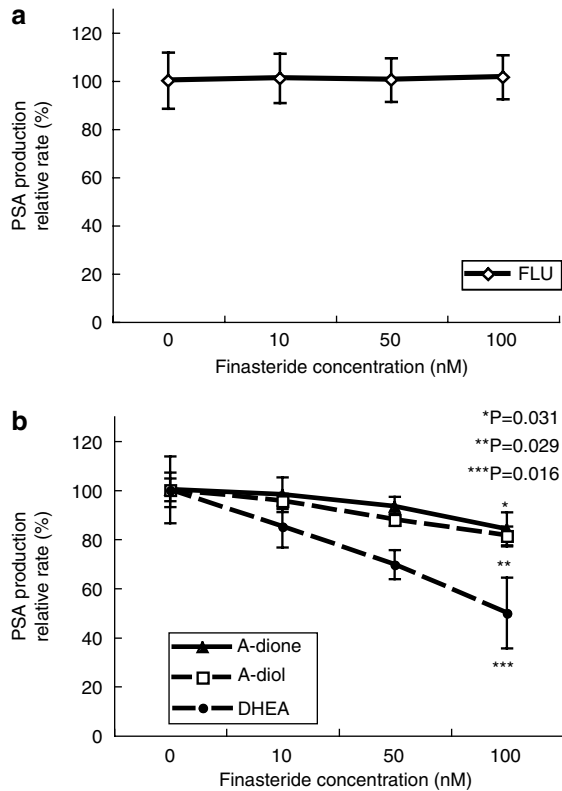


Figure 3 Inhibition of precursor adrenal androgen-induced PSA accumulation in LNCaP cell medium by finasteride: LNCaP cells were incubated with 10^{-8} M androstenedione, androstenediol, DHEA, or 10^{-6} M hydroxy-flutamide in the absence or presence of finasteride for 7 days. PSA production induced by hydroxy-flutamide was not inhibited by finasteride, but was inhibited with androstenedione, androstenediol and DHEA used as stimulators.

with a Thr877Ala point mutation in the AR gene. These cells were established from a tumor that progressed after castration and flutamide therapy. These cells have a mutated AR that responds not only to androgens but also to flutamide with increased cellular proliferation and elevated expression of PSA.¹⁰ LNCaP-FGC cells continuously grown in steroid-free medium did not die and the cells remained responsive to androgen.¹¹ These findings indicate that LNCaP-FGC cells are in an early period of mixed androgen independence and androgen-sensitive state. In order to evaluate the effect of androgens, anti-androgen and 5-R inhibitor on the activity of LNCaP cells, the present study investigated the levels of PSA production in conditioned media.

Prostate cells contain a variety of steroid-metabolizing enzymes required for the local formation of active androgens from precursor steroids provided by the adrenals. The main enzymes involved in local steroid metabolism are 17-HSDs, 3-HSDs and 5-Rs. At present, nine different 17-HSD isoenzymes, namely types 1, 3, 5, 7 (reductive enzymes), 2, 4, 8, 10 and 11 (oxidative enzymes) have been characterized in humans.¹² In the prostate, androstenedione is converted to testosterone by 17-HSD type 5 enzyme.^{12,13} In prostate cancer specimens, a reduced expression of 17-HSD type 2 mRNA has been detected¹⁴ and the expression levels of the 17-HSD type 3 gene were significantly higher than those in nonmalignant

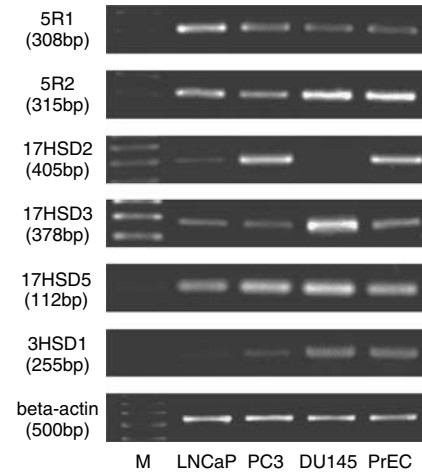


Figure 4 RT-PCR detection of 5 α -reductases (5-R), 17-HSD and 3-HSD mRNA in prostate cancer cell lines and prostate epithelial cell lines: reverse transcription and amplification were performed as described in Materials and methods. A 500 bp fragment of the human β -actin gene was amplified as a positive control.

tissues.¹⁵ For 5-R, it is now clear that not only 5-R type 1 but also the type 2 isozymes are expressed in the human prostate and the inhibition of both is more effective in lowering DHT than the inhibition of a single isozyme.¹⁶

In prostate cancer, 5-R type 1 protein levels have been reported to be increased¹⁷ whereas type 2 immunostaining has been found to be decreased.¹⁸ Previous reports have shown that LNCaP cells contain only 5-R type 1.¹⁹ In the present study, we detected 17-HSD types 2, 3 and 5, 3-HSD type 1 and both 5-R types 1 and 2 mRNA expressions in LNCaP-FGC cells. Such data indicate that enzymes of androgen metabolism are well expressed in LNCaP-FGC cells.

Although finasteride mainly inhibits 5-R type 2, a high dose of finasteride can inhibit the 5-R type 1 enzyme in LNCaP cells.²⁰ In fact, Finasteride has a K_i of 3.25×10^{-7} M for the type 1 isozyme and 1.2×10^{-8} M toward type 2 5-R.²⁰ In the present study, concentrations of 10^{-7} – 10^{-8} M finasteride alone (without androgens) did not have an inhibitory effect on PSA production of LNCaP cells. PSA secretion induced by a fixed concentration of testosterone was decreased by finasteride in a dose-dependent manner although secretion induced by the same concentration of DHT did not have an inhibitory effect. These data also show that concentrations of 10^{-7} – 10^{-8} M finasteride block type 2 5-R activity in LNCaP cells.

Previously, we indicated that serum levels of adrenal androgens in prostate cancer patients remained at about 60% following castration and flutamide, as opposed to testosterone levels that fell to about 2.7%.²¹ Plasma levels of the sulfate form of DHEA in adult men are about 100–500 times higher than those of testosterone in the circulation in adult men.²² It is believed that these large amounts of precursor adrenal androgens participate in prostate cancer recurrence after ADT. The transition of prostate cancer to androgen insensitivity can result from many different adaptive survival mechanisms. Point mutation in the steroid-binding domain of the AR gene, such as Thr877Ala, decreases binding specificity and

- 9 Tomita Y, Bilim V, Hara N, Kasahara T, Takahashi K. Role of IRF-1 and caspase-7 in IFN-gamma enhancement of Fas-mediated apoptosis in ACHN renal cell carcinoma cells. *Int J Cancer* 2003; **104**: 400–408.
- 10 Berns EM, de Boer W, Mulder E. Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor containing human prostate tumor cell line LNCaP. *Prostate* 1986; **9**: 247–259.
- 11 van Steenbrugge GJ, van Uffelen CJ, Bolt J, Schroder FH. The human prostatic cancer cell line LNCaP and its derived sublines: an *in vitro* model for the study of androgen sensitivity. *J Steroid Biochem Mol Biol* 1991; **40**: 207–214.
- 12 Soronen P *et al*. Sex steroid hormone metabolism and prostate cancer. *J Steroid Biochem Mol Biol* 2004; **92**: 281–286.
- 13 Pelletier G, Luu-The V, Li S, Labrie F. Localization of type 5 17beta-hydroxysteroid dehydrogenase mRNA in mouse tissues as studied by *in situ* hybridization. *Cell Tissue Res* 2005; **320**: 393–398.
- 14 Elo JP *et al*. Characterization of 17beta-hydroxysteroid dehydrogenase isoenzyme expression in benign and malignant human prostate. *Int J Cancer* 1996; **66**: 37–41.
- 15 Koh E, Noda T, Kanaya J, Namiki M. Differential expression of 17beta-hydroxysteroid dehydrogenase isozyme genes in prostate cancer and noncancer tissues. *Prostate* 2002; **53**: 154–159.
- 16 Lazier CB, Thomas LN, Douglas RC, Vessey JP, Rittmaster RS. Dutasteride, the dual 5alpha-reductase inhibitor, inhibits androgen action and promotes cell death in the LNCaP prostate cancer cell line. *Prostate* 2004; **58**: 130–144.
- 17 Bonkhoff H, Stein U, Aumuller G, Remberger K. Differential expression of 5 alpha-reductase isoenzymes in the human prostate and prostatic carcinomas. *Prostate* 1996; **29**: 261–271.
- 18 Thomas LN *et al*. Differential alterations in 5alpha-reductase type 1 and type 2 levels during development and progression of prostate cancer. *Prostate* 2005; **63**: 231–239.
- 19 Negri-Cesi P *et al*. Presence of 5alpha-reductase isozymes and aromatase in human prostate cancer cells and in benign prostate hyperplastic tissue. *Prostate* 1998; **34**: 283–291.
- 20 Tian G. *In vivo* time-dependent inhibition of human steroid 5 alpha-reductase by finasteride. *J Pharm Sci* 1996; **85**: 106–111.
- 21 Nishiyama T, Hashimoto Y, Takahashi K. The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clinical Cancer Research* 2004; **10**: 7121–7126.
- 22 Bartsch W, Klein H, Schiemann U, Bauer HW, Voigt KD. Enzymes of androgen formation and degradation in the human prostate. *Ann NY Acad Sci* 1990; **595**: 53–66.
- 23 Culig Z *et al*. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol Endocrinol* 1993; **7**: 1541–1550.
- 24 Palmberg C *et al*. Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. *J Urol* 2000; **164**: 1992–1995.
- 25 Culig Z, Comuzzi B, Steiner H, Bartsch G, Hobisch A. Expression and function of androgen receptor coactivators in prostate cancer. *J Steroid Biochem Mol Biol* 2004; **92**: 265–271.
- 26 Labrie F, Dupont A, Bélanger A. Complete androgen blockade for the treatment of prostate cancer. In: de Vita VT, Hellman S, et Rosenberg SA (eds). *Important Advances in Oncology*. J.B. Lippincott: Philadelphia, 1985; pp 193–217.
- 27 Prostate Cancer Trialists' Collaborative Group. Maximum androgen blockade in advanced prostate cancer: an overview of the randomised trials. *Lancet* 2000; **355**: 1491–1498.
- 28 Labrie F *et al*. GnRH agonists in the treatment of prostate cancer. *Endocr Rev* 2005; **26**: 361–379.
- 29 Bennett CL *et al*. Maximum androgen-blockade with medical or surgical castration in advanced prostate cancer: a meta-analysis of nine published randomized controlled trials and 4128 patients using Flutamide. *Prostate Cancer Prostatic Dis* 1999; **2**: 4–8.
- 30 Akaza H *et al*. Superior anti-tumor efficacy of bicalutamide 80 mg in combination with a luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist monotherapy as first-line treatment for advanced prostate cancer: interim results of a randomized study in Japanese patients. *Jpn J Clin Oncol* 2004; **34**: 20–28.
- 31 McConnell JD *et al*. Finasteride, an inhibitor of 5 alpha-reductase, suppresses prostatic dihydrotestosterone in men with benign prostatic hyperplasia. *J Clin Endocrinol Metab* 1992; **74**: 505–508.
- 32 Thompson IM *et al*. The influence of finasteride on the development of prostate cancer. *New England J Med* 2003; **349**: 215–224.
- 33 Leibowitz RL, Tucker SJ. Treatment of localized prostate cancer with intermittent triple androgen blockade: preliminary results in 110 consecutive patients. *Oncologist* 2001; **6**: 177–182.