

## Effects of dutasteride on the expression of genes related to androgen metabolism and related pathway in human prostate cancer cell lines

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**Summary** Androgens play an important role in controlling the growth of the normal prostate gland and in the pathogenesis of benign prostate hyperplasia, and prostate cancer. Although testosterone is the main androgen secreted from the testes, dihydrotestosterone (DHT), a more potent androgen converted from testosterone by 5 $\alpha$ -reductase isozymes, type I and II, is the major androgen in the prostate cells. The aim of this study is to investigate the cellular and molecular effects of dutasteride, a potent inhibitor of 5 $\alpha$ -reductase type I and type II, in androgen-responsive (LNCaP) and androgen-unresponsive (DU145)

human prostate cancer(PCa) cell lines. The expression pattern of 190 genes, selected on the basis of their proved or potential role in prostate cancerogenesis related to androgen signalling, were analysed using a low density home-made oligoarray (AndroChip 2). Our results show that dutasteride reduces cell viability and cell proliferation in both cell lines tested. AndroChip 2 gene signature identified in LNCaP a total of 11 genes differentially expressed ( $FC \geq \pm 1.5$ ). Eight of these genes, were overexpressed and three were underexpressed. Overexpressed genes included genes encoding for proteins involved in biosynthesis and metabolism of androgen (*HSD17B1*; *HSD17B3*; *CYP11B2*), androgen receptor and androgen receptor co-regulators (*AR*; *CCND1*), and signal transduction(*ERBB2*; *V-CAM*; *SOS1*) whereas, underexpressed genes (*KLK3*; *KLK2*; *DHCR24*) were androgen-regulated genes (ARGs). No differentially expressed genes were scored in DU145. Microarray data were confirmed by quantitative real-time PCR assay (QRT-PCR). These data offer a selective genomic signature for dutasteride treatment in prostate epithelial cells and provide important insights in prostate cancer pathophysiology.

Supplementary Information is linked to web site [http://www.geneticaumana.net/ricerca\\_news\\_scheda.php?id=53](http://www.geneticaumana.net/ricerca_news_scheda.php?id=53)

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### Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related death

in Western European and American males. Approximately 219,000 new cases of prostate cancer and 27,000 deaths are estimated to arise in the USA in 2007 [1].

The mortality in prostate cancer results from metastasis to the bone and lymph nodes and progression from androgen-dependent to androgen-independent disease [2]. Because prostate epithelial cells depend to androgen for growth, function, and survival, androgen-ablation therapy remains the standard treatment for patients with advanced prostate cancer. The major natural potent androgens, in humans and mammals, are testosterone and dihydrotestosterone (DHT) [3]. The testosterone is produced by the testes (95%) and by adrenal glands (5%) [4]. When free testosterone enters in prostate epithelial cells is converted to the more active androgen, DHT, by the 5 $\alpha$ -reductase enzymes (5 $\alpha$ -R). DHT binds the androgen receptor (AR) with up to ten times greater affinity than testosterone and activates gene transcription of androgen regulated genes and cellular proliferation [5]. Two different 5 $\alpha$ -R, type 1 and type 2, have been characterized. Each is encoded by a separate gene (SRD5A1 and SRD5A2) [6]. The 5 $\alpha$ -R type I is the major isoform expressed in tissues, such as liver and skin [6] while 5 $\alpha$ -R type II is expressed mostly in androgen target tissue, including genital skin and prostate. Whereas the major isoform expressed in the prostate stroma is 5 $\alpha$ -R type II, normal and malignant prostate epithelial cells also express 5 $\alpha$ -R type I [6]. Thus, DHT concentration in normal and neoplastic prostatic tissue is affected by varying inputs from both 5 $\alpha$ -R isoforms.

Selective inhibitors of 5 $\alpha$ -R enzyme are finasteride [7, 8] and dutasteride [9]. Finasteride is a specific competitive inhibitor of the 5 $\alpha$ -R type II, both *in vivo* and *in vitro* [7, 8], whereas dutasteride acts on both 5 $\alpha$ -R isoenzymes [9]. An international multicenter, double-blind, placebo controlled chemoprevention study (REDUCE) on 8,000 men taking dutasteride, has been launched on 2004 and results remain to be determined [10]. Therefore, mechanistic investigations *in vitro* on the effects of dutasteride in PCa cells can provide useful findings in order to acquire specific molecular effects of this drug on tumor cell biology. In the present study, we evaluated the expression profile of genes coding for products involved in the biosynthesis and metabolism of androgen, in two different prostate cancer cell lines (LNCaP and DU145) after dutasteride treatment using a low density home-made oligoarray (AndroChip 2). This low-density microarray seems to represent a useful tool to evaluate a selective gene expression pattern generated by a specific compound [11]. Despite the low number of genes studied, AndroChip 2 represents a powerful tool to investigate cellular and molecular effects induced by dutasteride in view of the possible use of this drug for prostate cancer prevention and therapy.

## Materials and methods

### Cytotoxic assay and cell growth

Human prostate cancer cell lines (LNCaP and DU145) were grown in RPMI-1640 (Gibco Invitrogen ,Grand Island, NY) supplemented with 10% of fetal bovine serum (Gibco), 5% of L-glutamine (Gibco) and antibiotics, under standard conditions (37°C temperature, 5% CO<sub>2</sub> in a humidified atmosphere) and treated with varying concentration of dutasteride (1–100  $\mu$ M), obtained from GlaxoSmithKline (Research Triangle Park, NC). For cytotoxicity assays cells were plated in 96-well plates (Falcon, CA) in 100  $\mu$ l of culture medium. Dutasteride was dissolved in DMSO and serially diluted in cell culture medium to the desired concentrations and an equal volume of the diluted solution (100  $\mu$ l/well) was added to the cells. Each treatment was performed in triplicate in three independent experiments. Cells were incubated with drugs for 72 h. To determine the cytotoxic effect at the end of drug incubation, MTS solution (Promega, 20  $\mu$ l/well) was added to the cells. The plates were incubated 2 h at 37°C and then the absorbance at 490 nm was measured using Sirio-S (SEAC, Radim Group). Cell growth inhibition and viability of prostate cancer cells were evaluated following the growth curves by trypan blue exclusion test. Results are expressed as mean  $\pm$  standard deviation (SD) of the percentage of viable cells at each drug concentration compared to the untreated cells.

### Microarray design and printing

To set up a biomarker system for prostate cancer and drug monitoring, we developed a low-density home made oligoarray composed of 190 genes selected on the basis of their proved or potential role in prostate cancerogenesis related to androgen signalling (Andro-Chip2). The genes were subdivided in different classes according to the following criteria: genes regulating the androgen metabolism within the prostate, androgen receptor (AR) and genes that bind to the AR-complex (such as ARA70), genes whose expression is androgen-regulated (ARGs) and involved in pathways associated with androgen-refractory cell growth (Ras-MAPK and PI3K/AKT pathways).

The oligonucleotide set (50 mer, Ocum Biosolutions, Indianapolis, USA) was mechanically “spotted” in triplicate onto UltraGAPS glass slides (Corning, Schiphol-rijk, The Netherlands) using TheRoboArrayer™ (Microgrid Compact Plus, BioRobotics). Printed slides were dried overnight and cross-linked with UV light at 600 mJ using a Strata-linker 2400 (Stratagene, Glenville, VA) and stored in a desiccator at room temperature. Before hybridization,

each slide was incubated in a prehybridization buffer (5× SSC, 0.1% SDS, and 0.1 mg/ml BSA) at 42°C for 45–60 min.

#### RNA extraction, labelling, hybridization

Total RNA was isolated from LnCaP and DU145 cells after treatment with either vehicle alone (DMSO) or dutasteride (10 μM for LnCaP and 40 μM for DU145) at 48 hrs by TRIZOL standard protocol (Invitrogen Corporation, Carlsbad, USA). Small aliquot of RNA was then used for quantification and quality control using respectively a spectrophotometer (Nanodrop, Wilmington, USA) and an agarose gel electrophoresis. Synthesis of the labelled first strand cDNA was conducted using the Superscript Indirect cDNA labelling system (Invitrogen Corporation) with starting material of 10 μg of total RNA. The amino-allyl labelled dNTP mix was added to the reaction to generate amino-allyl labelled second strand cDNA. Following the hydrolysis reaction, single-stranded cDNA probes were purified using a Purification Module (Invitrogen Corporation). Probe mixtures were then evaporated in a vacuum centrifuge, and the cDNA pellet resuspended in 3 μl of water. The dye coupling reactions were performed by mixing the cDNA samples with AlexaFluor Dyes 555 or 647 and were incubated for overnight in the dark. The reactions were purified with a Purification Module (Invitrogen Corporation) to remove the unincorporated/quenched dyes. After the purification, samples were combined for hybridization. The labelled cDNAs were co-hybridized to microarrays in duplicate, with one dye swap. The slides were scanned on the GenePix 4000 B Microarray Scanner (Axon Instruments, Sunnyvale, USA) at the optimal wavelength for the Alexa555 (F532) and Alexa647 (F635) (Invitrogen Corporation) using lasers.

#### Image analysis and processing

The acquired images were analyzed with Genepix Pro 5.0 software (Axon instruments, Union City). Oligonucleotide spots were automatically segmented, total intensities (F635 and F532) and local backgrounds (B635 and B532) were calculated for each spot. The spots were flagged when they exhibited poor hybridization signals or when they were saturated (F635 or F532 median = 65,535). We removed systematic bias in the data by applying the dye-swap normalization [12] that makes use of the reverse labelling in the two microarray replicates. This normalization procedure is well suited to treat low density microarray data, where the majority of the spotted genes are expected to change their expression level. We did not subtracted the local spot background signal from the foreground signal, since the

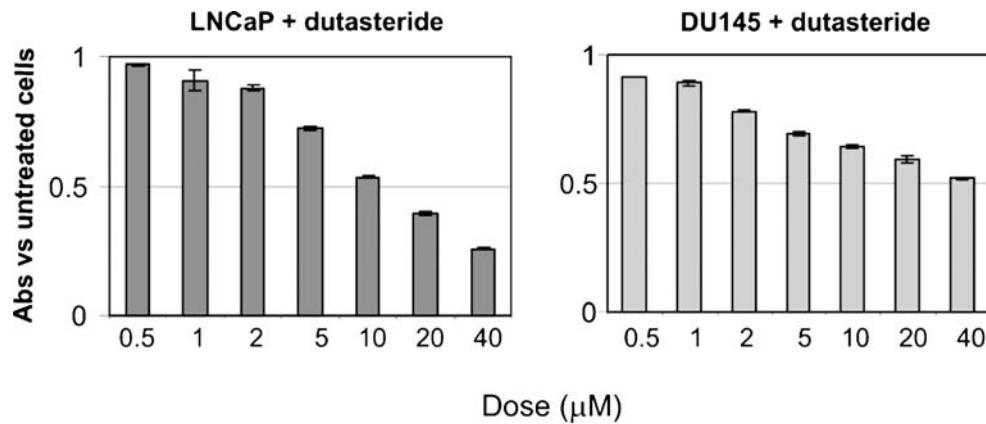
correlation between M vs Mb was very low (below 0.3), as recently suggested by Scharpf et al. [13]. Genes with signal to background ratio below two were filtered, together with genes strongly disagreeing by fold change: if two out of three replicates were over expressed, while the third had a fold change below the cut off-2, the gene was filtered; and similarly for under expressed genes. To establish the significance of observed regulation for each gene, we used t-test with Welsh's correction, and then controlled for the multiplicity of testing. Finally, only genes with a satisfactory effect (absolute value of the fold change at least 1.5) were considered.

#### Validation of relative gene expression by real-time RT-PCR

The absolute fold change values of the differentially expressed genes, reported in Table 1, are always lower than 2. In order to address the question if the microarray experiment has underestimated these values, we have validated the microarray data on selected genes by means of quantitative real-time PCR (QRT-PCR) experiments [14]. The total RNA was reverse-transcribed to cDNA according to the protocol of the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA). The incubation conditions were the following: 10 min to 25°C and 2 h to 37°C. We performed QRT-PCR using the Taqman system (Applied Biosystems.). The expression levels of 11 genes and an internal reference (*β-actin*) were measured by multiplex PCR using Assay-on-Demand™ gene expression products (Applied Biosystems, Foster City, CA USA) labelled with six carboxyfluorescein (FAM) or VIC (Applied Biosystems). We analysed the following genes: Hs00166219\_g1(*HSD17B1*), Hs00970002\_m1 (*HSD17B3*), Hs01597732\_m1, (*CYP11B2*), Hs00765553\_m1 (*CCND1*),

**Table 1** Genes differentially expressed in LNCAp treated with Dutasteride

Gene		Fold change
<b>Up-regulated genes</b>		
<i>HSD17B1</i>	NM_000413	1.5
<i>HSD17B3</i>	NM_000197	1.5
<i>CYP11B2</i>	NM_000498	1.5
<i>AR</i>	NM_000044	2.0
<i>CCND1</i>	NM_053056	1.5
<i>ERBB2</i>	NM_004448	2.0
<i>VCAM1</i>	NM_001078	1.6
<i>SOS1</i>	NM_005633	1.5
<b>Down-regulated genes</b>		
<i>PSA</i>	NM_001648	-2.0
<i>KLK2</i>	NM_005551	-1.8
<i>DHCR24</i>	NM_014762	-1.5

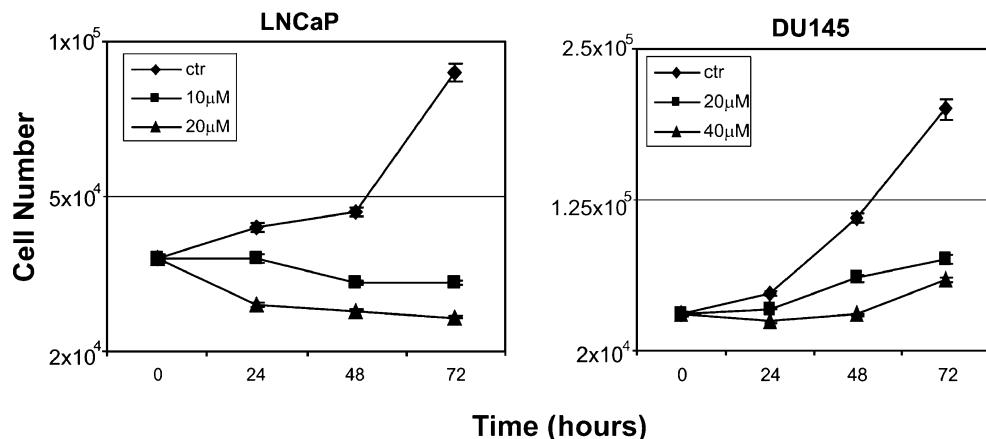
**Fig. 1** MTS assay

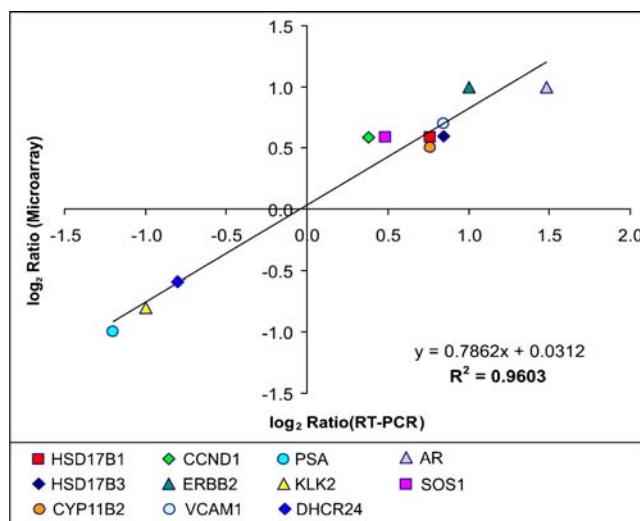
Hs01003372\_m1 (*V-CAM1*), Hs00893133\_m1 (*SOS1*), Hs00171172\_m1 (*AR*); Hs00426859\_g1 (*KLK3*), Hs00170433\_m1 (*ERBB2*); Hs01091576\_m1 (*KLK2*), Hs01092439\_g1 (*DHCR24*). The simultaneous measurement of each gene-FAM and actin-VIC made it possible to standardize the amount of cDNA added per sample. We performed PCRs using the Taqman Universal PCR Master Mix and the ABI PRISM 7000 Sequence Detection System. Each QRT-PCR experiment was performed in triplicate and repeated at least twice. A comparative threshold cycle ( $C_T$ ) was used to determine gene expression relative to a calibrator (RNA from untreated cells). Hence, steady state mRNA levels are expressed as n-fold difference relative to the calibrator. For each sample, our  $C_T$  genes value is normalized using the formula  $\Delta C_T = C_{T\text{gene}} - C_{T\beta\text{actin}}$ . To determine relative expression levels, the following formula was used:  $\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{calibrator}}$  and the value used to plot relative gene expression was calculated using the expression  $2^{-\Delta\Delta C_T}$ .

## Results

### Dutasteride effects on viability and proliferation of LNCaP and DU145 Cells

To evaluate the effects of dutasteride on LNCaP and DU145 cells, a time and dose-response experiment was performed. The effect of dutasteride was dose dependent in both cell line analyzed, LNCaP and DU145 respectively. However, LNCaP cells showed higher sensitivity to dutasteride, reaching an  $IC_{50}$  (inhibitory concentration at 50%) at 10  $\mu\text{M}$  dose, while DU145 cell line showed the same  $IC_{50}$  value at 40  $\mu\text{M}$  (Fig. 1). A dose-dependent inhibition of cell growth was observed in both cell lines analyzed after treatment with dutasteride at the concentration of 10 and 20  $\mu\text{M}$  dose for LNCaP cells and 20 and 40  $\mu\text{M}$  dose for DU145 cells (Fig. 2). No cell cycle perturbation was detected after dutasteride treatment at the concentration used for cell growth inhibition (data not shown). On the basis of cellular

**Fig. 2** Growth curve



**Fig. 3** Pearson correlation analysis between the expression value obtained with QRT-PCR and microarray assays

growth and the MTS assay, we decided to use for microarray experiments, a concentration of 10  $\mu$ M of dutasteride for LNCaP cells and 40  $\mu$ M for DU145 after 48 h of treatment.

#### Gene expression pattern of AndroChip2 in LNCaP and DU145 cell lines after dutasteride treatment

The differential gene expression pattern of LNCaP and DU145 cells treated and untreated with dutasteride were examined by AndroChip2 array ([http://www.geneticaumana.net/ricerca\\_news\\_scheda.php?id=53](http://www.geneticaumana.net/ricerca_news_scheda.php?id=53)). After data normalization, considering only genes showing a fold change (FC) at least equal to  $\pm 1.5$  we identified a total of 11 genes differentially expressed in LNCaP cells (Table 1). Of these 11 genes, 8 were over expressed and 3 were under expressed. The overexpressed genes included genes encoding for proteins involved in biosynthesis and metabolism of androgen (*HSD17B1*; *HSD17B3*; *CYP11B2*), androgen receptor and AR co-regulators (*AR*; *CCND1*), and signal transduction (*ERBB2*; *VCAM*; *SOS1*) whereas all three underexpressed genes (*KLK3*; *KLK2*; *DHCR24*) belonged to the androgen-regulated genes (ARGs) category [15].

To confirm AndroChip2 results, differentially expressed genes were analysed by QRT-PCR. As shown in Fig. 3, Pearson correlation analysis demonstrates a statistically significant correlation between the expression value obtained with QRT-PCR and microarray assays. Differentially expressed genes were also tested by QRT-PCR in DU145 cell line to confirm negative microarrays results. No gene expression alteration was noted in this cell type between dutasteride treated and untreated cells (data not shown).

#### Discussion

The role of DHT in the initiation and maintenance of the abnormal growth of prostate epithelium and the evidence that most prostate cancer cell lines express only the 5 $\alpha$ -R type I (*SRD5A1*) [16, 17] provided a strong biological rationale for the examination of a dual 5 $\alpha$ -R inhibitor as dutasteride. Dutasteride in prostate cells effectively blocks both 5 $\alpha$ -R isoenzymes, strongly reducing the amount of DHT available to bind the AR and direct proliferation [18]. However, clinical benefits of inhibiting both isoenzymes remain to be defined. Therefore, biological effects of dutasteride in cancer cells could provide useful information in the development of clinical treatments based on this drug.

We investigated the cellular and molecular effects of dutasteride, in androgen-responsive (LNCaP) and androgen-unresponsive (DU145) human PCa cell lines using a low density DNA chip (AndroChip2) specifically designed to detect alteration in expression levels of a selected group of genes whose products are involved in the androgen-signalling pathway. Cellular and gene expression analyses have been just used to evaluate the effect of dutasteride in prostate cancer cell lines [19–21]. Accordingly, we confirmed that elevated doses of dutasteride kills both AR-positive androgen-responsive LNCaP and AR-negative androgen-unresponsive DU145 cells, as demonstrated by the reduction of viability and proliferation in both cell lines after drug treatment. Similarly, a differential regulation of gene expression by dutasteride in LNCaP cells was detected. However, the different platforms utilized and the corresponding list of genes analysed restricts the possibility of a comparative analysis. Nevertheless, some confirmatory results appear evident from our study compared to Schmidt et al. [20]. The expression of *KLK3* was downregulated as previously reported [20]. In addition, we found downregulated *KLK2* and *DHCR24*, demonstrating the real effect of dutasteride in reducing the availability of intracellular DHT. In fact, these two genes are expected to be induced in response to androgen [15]. Conversely, we failed in detecting alteration in expression of *FASN*, coding for the fatty acid synthase which is increased during malignant progression of PCa [22]. Discrepancies in microarray analysis are common [23]. Problems encountered include inconsistent sequence fidelity of the spotted microarrays, variability of differential expression, low specificity of oligo array probes, discrepancy in fold-change calculation and lack of probe specificity for different isoforms of a gene could explain discrepancies found [23]. Additionally, we demonstrated that the expression of *HSD17B1*; *HSD17B3*; *CYP11B2*; *AR*; *CCND1*, *ERBB2*, *V-CAM* and *SOS1* were upregulated in dutasteride-treated LNCaP cells compared with non-treated LNCaP cells. However, the treatment with dutasteride induces the overexpression of *AR* and *ERBB2*. This suggests

that dutasteride stimulates LNCaP cells to rapidly respond to the decreased DHT levels by the up-regulating AR transcription. Under androgen-deprived conditions the AR can bind other ligand or function in a ligand-independent manner (“outlaw”receptor) to promote growth and proliferation. Therefore, its expression levels can be critical to cell survival under such conditions [21]. This is confirmed by the overexpression of *ERBB2* that has been demonstrated to be implicated in the activation of the AR pathway by a hormone-independent mechanism [24]. *ERBB2* (also known as HER-2/neu) is a member of the EGF-receptor family of receptor tyrosine kinases which is overexpressed in about 20–30% of breast and ovarian cancers [25]. *ERBB2* has intrinsic tyrosine-kinase activity and can activate the ER in the absence of oestrogenic ligand. Therefore, overexpression of *ERBB2* could lead to oestrogen independent stimulation of ER-mediated signal transduction pathway. Interestingly, in breast cancer, overexpression of *HER-2/neu* correlates with oestrogen independence, probably because *HER-2/neu* activation indirectly leads to phosphorylation and activation of the ER in the absence of oestrogen [26]. Phosphorylation therefore creates an outlaw ER, resulting in the oestrogen-independent growth of breast cancer cells [26]. The AR can be turned into an outlaw receptor by the same mechanism. Craft et al. [27] showed that the overexpression of *ERBB2* in androgen dependent prostate cancer cells is sufficient to confer androgen-independent growth *in vitro* and accelerate progression to androgen-independence in castrate animals. *ERBB2* is therefore able to activate the AR signalling pathway in absence of ligand and in the presence of low levels of androgens, amplifying the AR response. These findings demonstrate that there is cross-talk between the *ERBB2* and AR pathways, and provide a mechanistic insight into the clinical problem of androgen-independent prostate cancer progression.

In view of the fact that DU145 are AR negative, it is interesting to observe that no differential regulation of genes is observed in dutasteride treated cells. In fact, to be responsive to dutasteride, PCa cells require the expression of AR [28]. Our data confirm and extend data from Xu et al. [29] providing a gene expression support to the *in vivo* data. These authors studied the effect of dutasteride on a PC-3 cell line which is AR-negative as DU145 [29]. In an elegant *in vivo* experiment, Xu et al. [28] demonstrated that no response was induced by dutasteride in nude mice bearing androgen-independent PC-3 human prostatic cancer xenografts. These results showed that dutasteride-induced effects are strictly AR-dependent and that the use of this drug could be advantageous only if the prostate cancer is AR positive and sensitive to its signalling. We confirm and extend this observation providing further molecular data that dutasteride effect is AR-dependent. However, AR-dependent cells (i.e. LNCaP) treated with dutasteride,

reduce intracellular DHT concentration but active pathways that confer androgen- independent growth. Thus, further investigation in light of the relationship between intracellular DHT and androgen-independent growth should lead to clinical use of dutasteride as anti-PC agent.

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