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·*Original Article*·

Novel flutamide regulated genes in the rat ventral prostate: differential modulation of their expression by castration and flutamide treatments

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Abstract

Aim: To identify flutamide regulated genes in the rat ventral prostate. **Methods:** Total RNA from ventral prostates of control and flutamide treated rats were isolated. Differentially expressed transcripts were identified using differential display reverse transcriptase polymerase chain reaction. The effect of castration on the expression of flutamide-regulated transcripts was studied. **Results:** We have identified β2-microglobulin, cytoplasmic FMR1 interacting protein 2 and pumilio 1 as flutamide induced and spermine binding protein and ribophorin II as flutamide repressed targets in the rat ventral prostate. Although flutamide treatment caused an induction of pumilio 1 mRNA, castration had no effect. **Conclusion:** Castration and flutamide treatments exert differential effects on gene expression. Flutamide might also have direct AR independent effects, which might have implications in the emergence of androgen independent prostate cancer and the failure of flutamide therapy. *(Asian J Androl 2007 Nov; 9: 801–808)*

Keywords: androgens; antiandrogen; castration; differential display reverse transcriptase polymerase chain reaction; flutamide; prostate

1 Introduction

Androgens are essential in the development and maintenance of the prostate, and play an important role in the malignant growth of the prostate. Flutamide is an antiandrogen and frequently used as therapeutic agent in prostate cancer. Although flutamide therapy leads to the elimination of androgen dependent prostate cancer cells, it fails to arrest the androgen independent growth of cells. The precise molecular switch underlying the development of androgen independent prostate cancer (AIPC) is poorly understood. However, possible mechanisms of acquisition of an androgen independent state have been proposed, including mutations in the ligand binding regions of androgen receptor (AR), amplification of AR and altered expression of AR coregulators [1]. The emergence of AIPC has also been attributed to the multifocality and heterogeneity of prostate tumors [2]. Various foci in a tumor may vary in terms of their androgen dependence and flutamide treatment may select the growth of androgen independent foci. Lee *et al.* [3] demonstrate that hydroxyflutamide (an active metabolite of flutamide) can induce mitogen activated protein kinase (MAPK) cascade in AR negative DU145 cells, leading to enhanced Cyclin D1 expression and cell proliferation. It was also shown that these flutamide effects could be blocked by an inhibitor or a neutralizing antibody against the epidermal growth factor receptor. The data suggest the possi-

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bility of AR independent effects of flutamide in prostate cells and a direct role of flutamide in AIPC. Interestingly, Wang *et al.* [4] showed that flutamide increases the expression of the cell cycle related gene CDKN1A (a cyclin dependent kinase inhibitor that blocks cell cycle progression) in LNCaP prostate carcinoma cells. These data taken together imply that flutamide exerts complex cell type dependent effects on gene expression. Hence, extensive studies on flutamide effects on gene expression in the prostate cells are warranted.

Identification of androgen-regulated genes, especially those that have roles in proliferation or apoptosis, has the potential of offering alternative therapeutic targets. Traditionally, a large number of androgen-regulated genes have been identified using the rat castration model and prostate cancer cell lines. However, very few studies have used AR blockade with clinically used antiandrogens such as flutamide or bicalutamide. Therefore, a comprehensive knowledge of the effects of flutamide at the level of gene expression in the prostate is lacking. In the present study, we aimed at obtaining insights into the effects of flutamide on gene expression using the rat prostate model.

2 Materials and methods

2.1 Animals and hormonal manipulations

The present study used 60–70-day-old Wistar male rats obtained from the Central Animal Facility at the Indian Institute of Science. The rats were maintained according to the institutional guidelines with access to rat chow and water *ad libitum*. Orchidectomy was performed under ether anesthesia, via the scrotal route and maintained for 1, 3 or 5 days. Sham castrated rats maintained for 5 days served as controls. Testosterone propionate and 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) were given to day 2 castrated rats by daily intra-peritonial injections of 1 mg/kg body weight in propylene glycol as vehicle for 3 days. Flutamide (Sigma-Aldrich, St. Louis, MO, USA) was given intraperitonially at a daily dose of 50 mg/kg body weight in peanut oil (for differential display reverse transcriptase polymerase chain reaction [DD-RT-PCR]) or 50 mg/kg body weight in 100% DMSO once every 12 h for 5 days.

2.2 RNA extraction

RNA was extracted from frozen tissues using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The total RNA was further purified using RNeasy Kit (Qiagen GmbH, Hilden, Germany). RNA concentration was determined by measuring the absorbance at 260 nm and the quality assessed by agarose-formaldehyde gel electrophoresis.

2.3 DD-RT-PCR

The DD-RT-PCR protocol described by Wan and Erlander [5] was used with minor modifications. Briefly, 500 ng of total RNA from the tissues was reverse transcribed with anchored primer E1T12MT (5'-CG*GAATTC*GGTTTTTTTTTTTTVT-3'; V = A, G or C) or E1T12MA (5'-CG*GAATTC*GGTTTTTTTT-TTTTVA-3'; V = A, G or C) in separate reactions. The resultant cDNAs were diluted 20 times in water and 1 μL was used as template for PCR using 500 ng of anchor primer (corresponding primer used during cDNA synthesis) and 500 ng of arbitrary primer AP2 (5'-CGT*GAATTC*GGACCGCTTGT-3') or AP3 (5'-CGT*GAATTC*GAGGTGACCGT-3') in the presence of 1 μCi of α-^{32}P dCTP (3 000 Ci/mmole, Perkin Elmer Life Sciences, Boston, MA, USA). The PCR conditions were 94ºC for 20 s, 42ºC for 20 s and 72ºC for 30 s for 40 cycles. Then, 1 μL of PCR product was mixed with 1 μL of sequencing loading dye (95% formamide, 10 mmol/L EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), heated at 80ºC for 5 min, chilled on ice and resolved in a 6% polyacrylamide gel containing 7 mol/L urea, transferred into 3MM Whatman (3MM CHR) paper, dried under vacuum at 80ºC and exposed to an X-ray film. The differentially expressed cDNAs were excised from the gel and transferred to 100 μL of water and the DNA was eluted by boiling for 15 min and centrifugation at 10 621 × *g* for 5 min. The cDNA in the supernatant was reamplified using the same combination of anchored and arbitrary primers and sequenced in an automated sequencer (Applied Biosystems, Foster City, CA, USA).

2.4 Semi-quantitative RT-PCR

Using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), 500 ng of total RNA was reverse transcribed. cDNA equivalent to 10 ng of total RNA was used as template for PCR reactions using DyNAzyme II Master Mix (FINNZYMES, Espoo, Finland) with 1 μCi of α-^{32}P dCTP (3 000 Ci/mmole, Perkin Elmer Life Sciences, Boston, MA, USA) and gene specific primers (Table 1) in a final volume of 20 μL.

PCR reactions were carried out at cycle numbers in the linear range of product amplification. 5 μL of the PCR products were analyzed on 6% non-denaturing PAGE in 1 × TBE buffer. The gels were dried at 80ºC under vacuum and exposed to X-ray films.

2.5 Northern blot analysis of β2-microglobulin

Using a Random Primer Labeling Kit (Bangalore Genei, Bangalore, Karnataka, India) 25 ng of β2-microglobulin cDNA (reamplified F1 fragment) was labeled with α-^{32}P dCTP (specific activity 3 000 Ci/mmole, Perkin Elmer, Boston, MA, USA), and purified using Sephadex-G50 spin columns. Then, 15 μg of RNA was resolved on 1% agarose-formaldehyde gel and blotted onto a nylon membrane (Biodyne B Membrane; Pall Corporation, East Hills, NY, USA). Prehybridization, hybridization and washing steps were performed as described previously [6].

2.6 Western blotting

Rabbit anti-β2m antibody (used in 1 : 2 500 dilution) was a kind gift from Dr C.R. Wang (Department of Pathology, University of Chicago, USA). Anti-β actin monoclonal antibody was from Sigma-Aldrich (St. Loius, MO, USA) and horseradish peroxidase (HRP)-tagged goat anti-rabbit and goat anti-mouse IgGs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Tissues were homogenized in RIPA lysis buffer. The supernatants were stored in –70ºC in small aliquots. 50 μg protein was resolved in a 10% denaturing SDS-PAGE followed by electrophoretic transfer to Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked in 5% skimmed milk, incubated with primary antibody for 1 h, and washed and incubated with appropriate secondary antibody for 1 h. The blots were washed again and the HRP tagged secondary antibody was detected using Enhanced Chemiluminescence plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden) followed by exposure to X-ray films (Kodak, Rochester, NY, USA).

3 Results

3.1 Identification of flutamide regulated genes by DD-RT-PCR

To identify genes regulated upon androgen receptor blockade in the rat ventral prostate, DD-RT-PCR was performed with total RNA isolated from ventral prostates of vehicle treated and flutamide-treated rats. Using combinations of two anchored primers and two arbitrary primers, several differentially expressed fragments were identified. Among several flutamide regulated transcripts, we chose eight (F1–F8) for further analysis (Figure 1 and Table 2). The sequences of these fragments were used to search for homologies with the existing

Table 1. Primer sequences of flutamide regulate genes for validation. F and R denote forward and reverse primers; *CycA*, cyclophillin A; *PC1*, prostatein C1; *CYFIP2*, FMR1 interacting protein 2; *Pum1,* pumilio 1; *RibII*, ribophorin II; PCR, polymerase chain reaction.

Gene	Primer sequences	Amplicon (bp)	No. of cycles	PCR conditions
CycA	F: 5'-ACACGCCATAATGGCACTGG-3'	105	25	95ºC (30 s)
	R: 5'-ATTTGCCATGGACAAGATGCC-3'			55ºC (30 s)
				72ºC (30 s)
PC1	F: 5'-AGCACCATTAAGCTGAGCCTGTGT-3'	324	20	95ºC (30 s)
	R: 5'-ATCGTAGTAGAAATCGATCTCAGG-3'			63ºC (30 s)
				72ºC (30 s)
CYFIP2	F: 5'-TGACCTGCTGACCAAGGAGC-3'	334	30	95ºC (30 s)
	R: 5'-CTGCCTCTGCACCTTCAGCA-3'			60ºC (30 s)
				72ºC (30 s)
Pum1	F: 5'-CGCCAGCTTGTCTTCAACGA-3'	330	25	95ºC (30 s)
	R: 5'-CTGAACCACATGGTTGCCAT-3'			58ºC (30 s)
				72ºC (30 s)
RibII	F: 5'-ACATTCATCGCAGACAGCCAT-3'	293	25	95ºC (30 s)
	R: 5'-ACCACATCAGCCACGTTCCAG-3'			62ºC (30 s)
				72ºC (30 s)

sequences in the NCBI database using BLAST and their identities are shown in Table 2. F3 and F5 transcript sequences did not produce any significant hits. F8 was identified as spermine binding protein (SBP), which is a known androgen induced gene in the ventral prostate [7], suggesting at the outset, the reliability of our DD-RT-PCR protocol. Others showed identities to cytoplasmic FMR1 interacting protein 2 (*CYFIP2*), pumilio 1 (*Pum1*), ribophorin II (*RibII*) and β2-microglobulin (*β2m*) (Table 2).

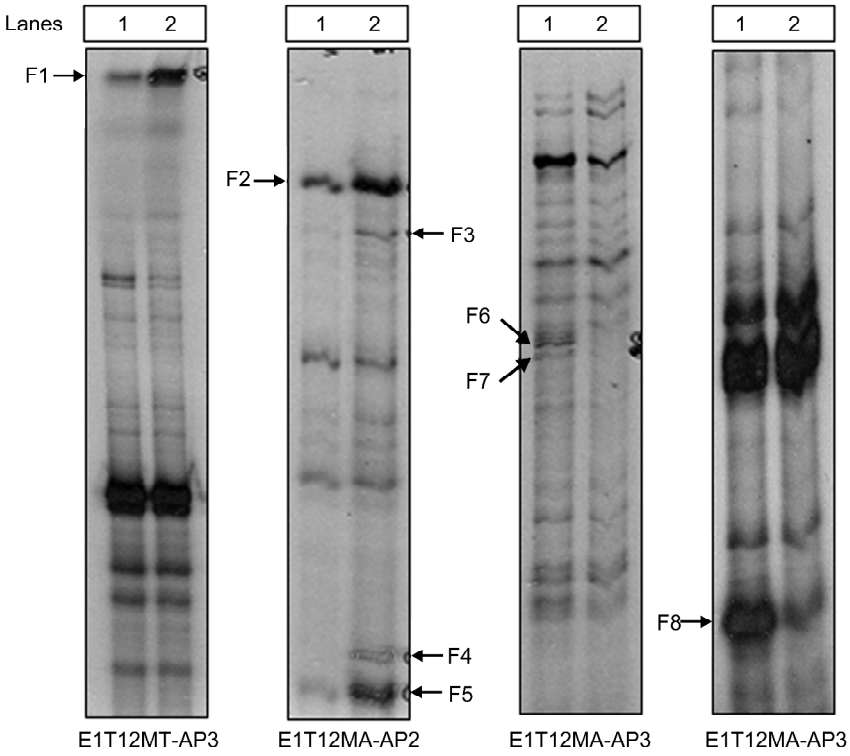

Figure 1. Differential display reverse transcriptase polymerase chain reaction analysis of differentially expressed mRNAs in ventral prostates of flutamide treated rats. The panels show differentially expressed transcripts obtained with combinations of anchored and arbitrary primers indicated at the bottom of each panel. The differentially expressed transcripts chosen for further analysis (F1–F8) are indicated by arrows. Lanes: 1, day 5 vehicle treated; 2, day 5 flutamide treated.

Table 2. Identities of flutamide regulated transcripts in the rat ventral prostate. *Percent identities of some fragments show deviation from published sequences due to sequencing errors.

Fragment	Regulation	Sequenced clone/Fragment	Acc. No.	Gene name (% identity)*
F1	Up	F1 PCR product	NM_012512	Rat β2m mRNA (97%)
F2	Up	F2 PCR product	XM_342928	Rat Pum 1_predicted mRNA (98%)
F3	Up	F3 PCR product	No significant hits	
F4	Up	F4 PCR product	BC028941	Mouse cytoplasmic FMR1 interacting protein 2 mRNA (97%)
F5	Up	F5 PCR product	No significant hits	
F6	Down	F6 PCR product	BC060556	Rat ribophorin II mRNA (99%)
F7	Down	F7 PCR product	NM_031698	Rat ribophorin II mRNA (99%)
F8	Down	F8 PCR product	NM_013024	Rat spermine binding protein mRNA (91%)

3.2 β2m is an androgen repressed gene

Using Northern and Western blotting we studied the effects of flutamide and castration on the expression of β2m. As shown in Figure 2, castration induced β2m mRNA gradually from day 1 to day 5 post-castration. Compared to sham castrated controls, there was a 6.03 ± 0.48-fold induction of β2m mRNA at day 3 and 11.62 ± 0.46-fold induction of β2m mRNA at day 5 post-castration (mean ± SEM, n = 2). Testosterone supplementation reversed the effects of castration on the expression of β2m mRNA (Figure 2A). Flutamide treatment caused a 3.77 ± 0.42-fold induction of β2m mRNA over the control (mean ± SEM, n = 2) (Figure 2B), which was lesser in extent compared to that observed in day 5 castrated animals. Induction of β2m protein expression by castration, as analyzed by western blotting, correlated with the induction of mRNA and was reversed by testosterone supplementation. The induction of β2m protein over control was also observed upon flutamide treatment, albeit to a lesser extent compared to castration (Figure 2C).

3.3 Regulation of CYFIP2, Pum1 and RibII by castration and flutamide

We compared the effect of flutamide and castration on CYFIP2, Pum1 and RibII mRNA expressions using semiquantitative RT-PCR. As expected, both flutamide and castration caused downregulation of prostatein C1 mRNA (Figure 3). Flutamide induced the steady-state levels of CYFIP2 and Pum1 mRNAs but downregulated

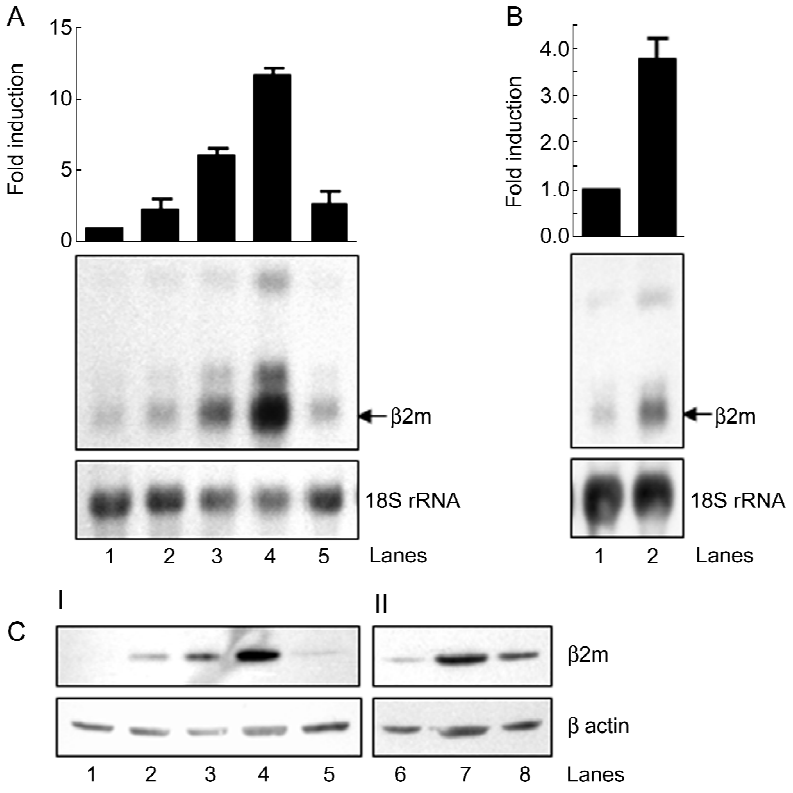

Figure 2. Analysis of β2-microglobulin (β2m) expression in the rat ventral prostate. (A): Northern blot and analysis of the effect of castration. Lanes: 1, day 5 sham castrated; 2, day 1 castrated; 3, day 3 castrated; 4, day 5 castrated; 5, testosterone supplemented. (B): Northern blot analysis of the effect of flutamide treatment. Lanes: 1, day 5 sham castrated; 2, day 5 flutamide treated. For quantification of blots in (A) and (B), the band intensities obtained for β2m were normalized against those obtained for 18S rRNA. The normalized values obtained for day 5 sham castrated controls were assigned the value of 1 and those obtained for other treatments have been plotted as fold induction. Bars represent mean ± SEM (n = 2). (C): Western blot. I: Effect of castration and testosterone supplementation. Lanes: 1, day 5 sham castrated; 2, day 1 castrated; 3, day 3 castrated; 4, day 5 castrated; 5, testosterone supplemented; II: Effect of castration and flutamide treatments. Lanes: 6, day 5 sham castrated; 7, day 5 castrated; 8, day 5 flutamide treated.

the levels of RibII mRNA (Figure 3). Therefore, the results are in agreement with our DD-RT-PCR analysis. Castration induced CYFIP2 and downregulated RibII mRNA expressions, in a manner similar to the effect of flutamide treatment. Interestingly, although flutamide induced the levels of Pum1 mRNA, castration had no effect (Figure 3). The induction and downregulation of CYFIP2 and RibII mRNAs, respectively, by castration were also reversed by testosterone but not by estrogen supplementation (Figure 3).

4 Discussion

The emergence of an androgen independent state is a major hurdle in the effective treatment of prostate cancer. Although it is likely that flutamide treatment selects the AIPC cells, we hypothesized that flutamide might also have a direct role in the emergence of an androgen independent state. Our hypothesis is largely based on the demonstration by Lee *et al.* [3] that hydroxyflutamide can induce the MAPK pathway in AR negative cells. Comprehensive studies on the effects of flutamide on prostate cells are, therefore, relevant in this context. The present work offers insights into the effects of flutamide at the level of gene expression on prostate cells by way of identifying novel flutamide regulated genes in the rat ventral prostate using DD-RT-PCR. To our knowledge, this is the first attempt to identify novel flutamide regulated genes.

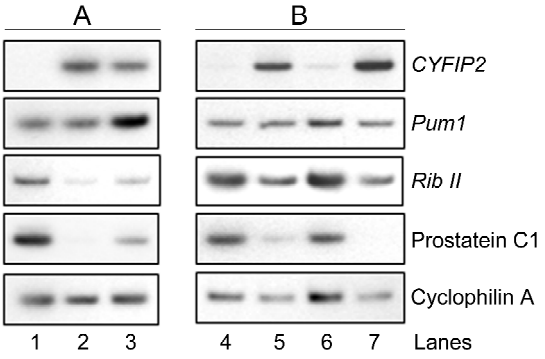

Figure 3. Semi-quantitative reverse transcriptase polymerase chain reaction analysis of flutamide regulated genes. (A): Comparison of the effects of castration and flutamide treatments. (B): Effect of castration and testosterone or estrogen supplementation. Lanes: 1, day 5 sham castrated and vehicle treated; 2, day 5 castrated; 3, day 5 flutamide treated; 4, day 5 sham castrated; 5, day 5 castrated; 6, testosterone supplemented; 7, estrogen supplemented. Cyclophilin A was used as an internal control.

We have shown for the first time that androgen represses the expression of β2m mRNA and protein in the rat ventral prostate. The physiological relevance of induction of β2m expression in the rat ventral prostate post-castration is currently not clear. β2m has been shown to exert a mitogenic effect on PC3 cells by antagonizing TGF-β1 action [8]. Hence, the significance of induction of β2m in the context of castration induced expression of TGF-β1 concomitant to prostate epithelial cell death [6, 9] needs to be addressed. Increased expression of major histocompatibility class 1 (MHC I)/β2m and soluble β2m in tissue fluids is an established phenomenon in cases of viral infection and tissue degeneration [10, 11]. The increased cell surface expression of MHC I/β2m leads to stimulation of T cell response and cytolytic activity to eliminate degenerating cells. The increased soluble β2m might function to induce proliferation of adjacent non-degenerating cells as a part of the tissue repair process. In a microarray study to identify androgen regulated genes in the rat ventral prostate, MHC I antigen gene was identified as an androgen repressed gene [12]. Infiltration of immune cells in the rat ventral prostate following castration is also known [13]. Therefore, induction of MHC I/β2m in the rat ventral prostate could signal cytotoxic T cells to eliminate degenerating cells of the prostate following castration. β2m has been shown to induce proliferation of PS-1 prostate stromal cells [8]. The induced β2m following castration is likely to influence the proliferation of prostatic stromal cells or adjacent basal epithelial cells, which remain unaffected by castration.

CYFIP2 plays a role in development and maintenance of dendritic spines and neuronal plasticity and is involved in fragile X mental retardation syndrome [14]. The significance of CYFIP2 mRNA induction in the rat ventral prostate upon castration or flutamide treatment is certainly not obvious. Investigations into the expression and androgen regulation of its interacting proteins, such as FMRP, FXR1 and FXR2, might provide clues into the possible roles of these proteins in normal or involuting rat ventral prostate. Interestingly, FMRP is known to be associated with actively translating ribosomes [15]. In addition, recent data indicates that FMRP might inhibit translation of mRNA [16]. Being an interacting partner of FMRP, CYFIP2 might be involved in the translational shut-off of a wide array of secretory proteins expressed in the prostate epithelium following castration and

flutamide treatments. Induction of p53 has been shown in the rat ventral prostate following castration [17]. Interestingly, CYFIP2 is known to be a p53 inducible protein [18]. It is possible that CYFIP2 might have a role in castration-induced cell death of the prostate epithelium.

Pum1 is a member of an evolutionarily conserved PUF (for Pumilio and FBF) family of RNA binding proteins [19]. In drosophila, the Pum is involved in translational repression of maternal hunchback mRNA, which is necessary for proper posterior segmentation and abdomen formation. It represents a well-characterized model of translational repression [20]. Our data suggests induction of Pum1 by flutamide. It is likely that Pum1 is involved in translational repression of target mRNAs in the rat ventral prostate and is a subject of future investigations.

For secretory epithelial cells, such as the ones present in the prostate, the cellular processes involved in protein synthesis, processing, trafficking and secretion will be highly active. Because the differentiated function of the prostate is androgen dependent, it follows that these cellular activities and participating molecules therein will be androgen dependent. We show RibII as an androgen regulated gene in the rat ventral prostate. RibII is a component of the oligosaccharyl transferase, which is involved in transfer of high mannose oligosaccharide to the nascent polypeptide chains and, therefore, has a role in protein processing. DAD1, which is also a component of the oligosaccharyl transferase has been shown to be androgen induced in the rat ventral prostate [12]. Taken together, these data suggest the androgen dependence of the expression of the oligosaccharyl transferase complex in prostate cells and a role for androgen in protein glycosylation.

We have demonstrated that castration and flutamide treatments cause differential effects. In the present study, three distinct patterns of differential modulation of gene expression hitherto unreported in the published literature have been observed: (i) dowregulation by both castration and flutamide treatments (RibII); (ii) upregulation by both castration and flutamide treatments (β2m, CYFIP2); and (iii) upregulation by flutamide but not by castration (Pum1). The similar and differential patterns of modulation of gene expression are suggestive of the following possibilities. First, the observed regulation of a given gene following castration need not necessarily be a result of androgen ablation. Instead, it could be a consequence of enrichment of stromal cells following castration. Alternatively, it could represent secondary effects of cell death. Second, actions of androgen at the level of gene expression could be mediated via mechanisms independent of the canonical AR. We have shown that β2m is induced by both castration and flutamide treatments. However, the level of induction by flutamide is only partial (compared to castration). Because flutamide does not cause apoptosis of the prostate epithelium it is likely that the greater induction of β2m by castration compared to flutamide could be a combined effect of loss of androgen signaling and castration-induced cell death. Finally, regulation of Pum1 by flutamide alone suggests that flutamide might have independent effects on gene expression.

Therefore, we propose that interpretation of genes as "androgen regulated" based solely on the effects of castration and testosterone reversal could be misleading. This has a lot of bearing with regards to the search for novel androgen regulated genes as therapeutic targets for prostate cancer. Our data also suggest that flutamide might have independent effects on the level of gene expression. Such independent actions of flutamide could impact tremendously on the outcomes of antiandrogen therapy of prostate cancer. Further investigation might help in better understanding the failure of flutamide therapy and its role in androgen independence. Detailed studies on the regulation, function and upstream and downstream targets of novel genes identified in the present study might provide valuable insights into mechanisms by which androgens control various cellular processes.

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