

Identification of μ -Crystallin as an Androgen-Regulated Gene in Human Prostate Cancer

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BACKGROUND. Androgen receptor (AR) signaling is implicated in prostate cancer progression. Therefore, identification of AR downstream genes is potentially important for selection of novel markers and therapy targets in prostate cancer.

METHODS. Expression of a thyroid hormone T3-binding protein μ -crystallin (CRYM) mRNA and protein in cell lines was evaluated by real-time PCR and Western blot, respectively. CRYM expression in vivo was analyzed in patients' samples by immunohistochemistry. The effects of androgen and T3 on proliferation of MDA PCa 2b cells were assessed by ³H-thymidine uptake assay.

RESULTS. CRYM expression was detected in AR-positive LNCaP and MDA PCa 2b cells. In MDA PCA 2b cells, CRYM was regulated by androgens. Androgen-induced CRYM expression was diminished by antiandrogens or AR siRNA. Inhibition of transcription by α -amanitin caused a reduction in CRYM mRNA. The lack of CRYM expression was noted in LAPC-4 cells and in AR-negative prostate cancer cell lines PC-3 and DU-145. CRYM protein was increased in cancer tissue and decreased in samples from patients after hormonal therapy. In samples from patients with therapy-refractory cancer CRYM was not detectable. We also found that androgens and T3 have additive effects on stimulation of MDA PCa 2b cells proliferation.

CONCLUSION. CRYM is a novel androgen-regulated gene whose expression is elevated in prostate cancer but down-regulated in castration therapy-resistant tumors. *Prostate* 69:1109–1118, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; μ -crystallin; androgen receptor

INTRODUCTION

Prostate cancer is the most common malignancy among men in Europe [1] and in the United States [2]. The growth and development of prostate gland are regulated by androgenic hormones that are also implicated in carcinogenesis. The action of androgens is mediated by androgen receptor (AR) that is the main transcription factor in prostate cells. Upon ligand binding, AR is activated and translocates to the nucleus where AR dimers bind to androgen response elements on promoters of androgen-regulated genes. As a consequence, the transcription of downstream genes occurs.

Several mechanisms implicated in prostate cancer progression are AR-related. The main and most

successful therapy for non-organ-confined tumors is androgen ablation [3]. This therapy improves quality of life but is still palliative. Despite a favorable initial response, castration-resistant disease develops after 3–5 years. These tumors are heterogenous and still

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contain active AR signaling [4–6]. Thus, identification of novel androgen-regulated genes implicated in prostate cancer development and progression is of great interest. It could give a possibility to validate potential markers for prostate cancer diagnosis and discrimination between tumor stages. Differential expression of these genes in androgen-dependent and castration-resistant tumors can make them potential therapeutic targets.

μ -Crystallin (CRYM) is a cytoplasmic protein first identified in the kangaroo lens and in the mammalian eye [7]. CRYM plays a role in visual function and is involved in macular degeneration of the retina [8]. A similar role has also been proposed for nuclear 3,5,3'-triiodo-L-thyronine (T3) receptor [9]. CRYM has been shown to be a NADPH-dependent binding protein of T3 whose action on target cell is mediated through nuclear receptors and a number of thyroid hormone-binding proteins present in cells, particularly in the endoplasmic reticulum [10], mitochondria [11], nuclear envelope, and cytoplasm. Overexpression of CRYM increases the uptake and decreases the cellular efflux of T3 [12]. The mechanism of CRYM action involves the formation of dimers in the cytoplasm and binding NADPH. NADPH-activated CRYM binds T3 and increases hormone concentration in the cytoplasm. Dissociation of NADPH enables the release of T3. Free T3 can transactivate expression of genes, whereas the action of CRYM-bound T3 is suppressed [13].

One objective of the present study was to characterize CRYM as an androgen-regulated gene in prostate cancer cell lines. For the first time we show that androgens are able to upregulate CRYM at RNA and protein levels. Application of antiandrogens bicalutamide and hydroxyflutamide (OHF) blocked the androgen-induced CRYM expression indicating AR involvement in this process. A similar effect was observed with AR downregulation by siRNA. Expression of CRYM was not detected in AR-negative cell lines. Additionally, expression of CRYM at various tumor stages in patients' samples was evaluated.

MATERIALS AND METHODS

Reagents

The synthetic androgen methyltrienolone (R1881) was purchased from R&D Systems (Minneapolis, MN) and dissolved in absolute ethanol. Bicalutamide was a kind gift from Astra Zeneca (Macclesfield, United Kingdom). OHF was purchased from Sigma–Aldrich (Vienna, Austria). Stock solutions of antiandrogens were prepared in DMSO. T3 was purchased from Sigma–Aldrich and dissolved in NaOH. α -Amanitin and cycloheximide were also purchased from Sigma–Aldrich.

Cell Culture

The LAPC-4 prostate cancer cell line was a kind gift from Dr. Charles Sawyers (formerly at University of California, Los Angeles, CA). The cells were cultured in IMDM medium (PAA Laboratories, Pasching, Austria) containing 15% HyClone FCS (Szabo Scandic, Vienna, Austria), 100 U/ml penicillin, 100 U/ml streptomycin solution, and 10 nM R1881.

The MDA PCa 2b prostate cancer cell line was obtained from Szabo Scandic. The cells were grown in F-12 medium (Sigma–Aldrich) supplemented with 20% FCS, 100 U/ml penicillin, 100 U/ml streptomycin solution, 25 ng/ml of cholera toxin (Sigma–Aldrich), 10 ng/ml epidermal growth factor (Strathmann Biotech, Hannover, Germany), 5 μ M phosphorylethanolamine, 100 pg/ml hydrocortisol, and insulin/transferin/selenid mix (Invitrogen, Leek, The Netherlands).

DU-145, PC-3, and LNCaP prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). DU-145, PC-3, and PC-3-AR cells were grown in RPMI 1640 (HyClone, Logan, UT). LNCaP cells were grown in MCDB 131 medium (PAA Laboratories). The media were supplemented with 10% FCS and penicillin/streptomycin solution. All cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Real-Time PCR

MDA PCa 2b cells were incubated for 4, 8, 16, 24, 48, and 72 hr in the absence or presence of R1881 (1 nM) or T3 (30 ng/ml). RNA was isolated according to the manufacturer's instructions (Qiagen Kit, Hilden, Germany). The procedure of cDNA reverse transcription with Super Script III RT (Invitrogen) was based on the manufacturer's protocol. Real time-PCR was performed with the CRYM TaqMan Gene Expression Assays according to the manufacturer's instructions (Applied Biosystems, Brunn am Gebirge, Austria).

Transfections

Single-stranded RNAs were purchased from Gene-Express (Vienna, Austria). Hybridization of sense and antisense single stranded RNA (100 μ M each) was performed for 1 hr at 37°C following denaturation in annealing buffer (20 mM KCl, 6 mM HEPES–KOH, pH 7.5, 0.3 mM MgCl₂) for 1 min at 90°C [14]. A 21-nucleotide double-stranded siRNA duplex was generated against the amino-terminus of the AR (5'-AAG CAG CAG CAG CAG CA G CAG-3') and will be referred to as AR_{CAG} siRNA. siRNA against luciferase (luc siRNA) was generated to 5'-CGU ACG CGG AAU ACU UCG ATT-3' sequence and functioned as a nonspecific control siRNA for the RNAi experiments.

On day zero, MDA PCa 2b cells were seeded at an initial density of 5×10^5 cells onto 6-well plates and incubated for 24 hr in culture medium. On day 1, the cells were serum-starved and transfected with luc or AR_{CAG} siRNAs at a final concentration of 10 nM using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After 48 hr, the second transfection was performed. Then the cells were treated with R1881 (1 nM) for 72 hr and harvested for Western blot or subjected to ³H-thymidine uptake assay.

Western Blot Analysis

MDA PCa 2b cells were seeded in culture media and grown to subconfluence. After 24 hr incubation, fresh serum-free medium was added. Cells were incubated with R1881 (1 nM) or T3 (30–50 ng/ml) for 72 hr and then harvested and lysed with European Organization for Research and Treatment of Cancer (EORTC) lysis buffer. The lysed cells were centrifuged and the supernatant (cytosolic fraction) was collected. The amount of protein was measured by the Bradford method [15] and 20–50 µg of protein were used for SDS-PAGE analysis. Polyacrylamide Bis-Tris gel (Invitrogen) was run in 1× MOPS buffer (Invitrogen). Transfer of the proteins to the PVDF membrane (Invitrogen) was done in 1× transfer buffer (Invitrogen) containing 1% methanol. Membrane was then blocked in Starting Block buffer (Pierce Biotechnology, Rockford, IL, USA) for 1 hr and incubated with either mouse monoclonal CRYM antibody (Abnova, Taipei City, Taiwan), mouse monoclonal AR antibody (BioGenex, Haag, Netherlands), mouse monoclonal β-actin antibody (Upstate Cell Signaling Technology, Beverly, MA), or mouse monoclonal α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The fluorescent signals were read with Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE).

Patients' Samples

Tissue microarrays were performed with samples from prostate cancer patients. Expression of CRYM was compared between normal, prostate intraepithelial neoplasia, low, and high grade cancer areas. Prostates from patients who underwent cystectomy were used to validate the expression of CRYM in benign tissues. Four tissue arrays were performed with prostate samples derived from eight patients before androgen ablation and eight patients who underwent androgen ablation therapy. Six prostate samples used for tissue array were derived from therapy-refractory tumors. The procedure concerning patients' data protection was according to regulations of Medical University of Vienna.

Immunohistochemistry

For histological analysis and immunohistochemistry, tumors were fixed with neutral buffered 4% paraformaldehyde. The tissues were then embedded in paraffin. The sections (5 µm) were stained either with hematoxylin and eosin or processed further. Immunohistochemical staining for CRYM was performed using the ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. As a negative control, staining was performed on selected sections without the primary antibody.

Image Acquisition and Protein Quantification In Vivo

Samples were analyzed with a Zeiss AxioImager Z1 microscope system with CCD camera and an automated acquisition system TissueFAXS™ (TissueGnostics, Vienna, Austria). The percentage of CRYM-positive cells was determined and depicted as scattergrams. Pictures were digitalized, analyzed, and protein expression was quantified. Statistical flow analysis was done using HistoQuest™ software (TissueGnostics).

³H-Thymidine Uptake Assay

MDA PCa 2b cells were seeded at a density of 10^4 cells/well onto 96-well plates in regular culture media. The next day media were changed and supplemented with 5% steroid-free FCS, 100 U/ml penicillin, and 100 U/ml streptomycin and cells were incubated with increasing doses of R1881 (0.1–1 nM) or T3 (10–50 ng/ml) for 5 days. After incubation, the mix of 1 µl of ³H-thymidine (1 µCi/well) and 49 µl of serum-free medium/well was added. Cells were then harvested after 24 hr and DNA was fixed on filter plates (UniFilter, Perkin Elmer, Boston, MA). Chameleon 5025 liquid scintillation counter (HVD Life Sciences, Vienna, Austria) was used to read the filter plates.

Statistical Analysis

The SPSS 12.0 program was used for statistical evaluation and the Mann–Whitney *U*-test was used for the assessment of statistical significance.

RESULTS

CRYM Expression in Prostate Cancer Cell Lines

To identify new AR targets with relevance in prostate cancer, literature search regarding gene expression profiles in normal tissue and prostate cancer specimens was performed. Previous results were published by Mousses et al. [16] who showed that CRYM mRNA decreased in therapy-refractory tumors

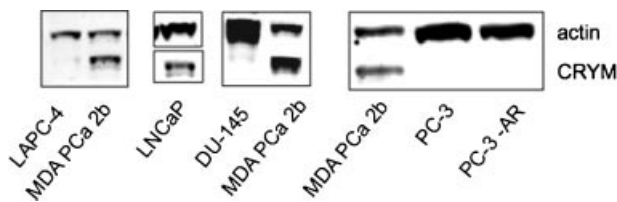


Fig. 1. Expression of CRYM in AR-positive prostate cancer cell lines LAPC-4, MDA PCa 2b, and LNCaP as determined by Western blot. In contrast, AR-negative cell lines DU-145 and PC-3 are also CRYM-negative. PC-3 cells stably transfected with wild-type AR do not express CRYM either.

as compared with primary tumors. To evaluate CRYM expression in prostate cancer cell lines, Western blot analyses of CRYM in AR-positive and -negative cells were performed. Constitutive expression of CRYM was observed in LNCaP and MDA PCa 2b cells. Interestingly, in AR-positive LAPC-4 and PC-3 cells stably transfected with the wild-type AR cDNA CRYM was not detected. AR-negative DU-145 and PC-3 cell lines do not express CRYM (Fig. 1).

CRYM RNA Levels in Prostate Cancer Cell Lines

Detection of CRYM in AR-positive prostate cancer cell lines raised a question whether its expression is regulated by androgens. Therefore, MDA PCa 2b cells were incubated for 4, 8, 16, 24, 48, and 72 hr with R1881 (1 nM). Expression of CRYM in MDA PCa 2b cells upon exposure to R1881 reached the maximal level after 48 hr (Fig. 2A). In order to confirm the involvement of AR in regulation of CRYM expression, the cells were treated with R1881 and bicalutamide. The upregulation of CRYM mRNA after R1881 treatment was diminished by bicalutamide (Fig. 2B). Application of the transcription inhibitor α -amanitin entailed the strongest effect on reduction of R1881-induced CRYM RNA after 48 hr of incubation (Fig. 2B).

Additionally, we investigated the effect of thyroid hormones on CRYM transcription because CRYM has been identified as a NADPH-dependent T3 binding protein. MDA PCa 2b cells were exposed to increasing concentrations of T3 (30–50 ng/ml) for 4, 8, 16, 24, 48, and 72 hr. CRYM mRNA levels slightly increased after 30 ng/ml T3 treatment for 24 or 48 hr (Fig. 2C) and there was no significant regulation of CRYM expression when the cells were subjected to 50 ng/ml T3 treatment (data not shown).

CRYM Protein Levels in Prostate Cancer Cell Lines

To test whether increased CRYM mRNA in response to R1881 and T3 is consistent with the protein levels, Western blot analyses were performed. We noted up to 2.5-fold induction of CRYM protein after R1881 (1 nM)

incubation for 72 hr in MDA PCa 2b cells (Fig. 3A). In LNCaP cells, CRYM protein was detected, although it was not regulated by androgens (data not shown). Exposure of MDA PCa 2b cells to T3 (30 ng/ml) did not exert any effect on CRYM expression (data not shown).

The increase of CRYM in response to R1881 raised a question whether this process is AR-dependent. We applied AR_{CAG} siRNA to downregulate expression of the receptor. MDA PCa 2b cells were transfected for 48 hr with AR_{CAG} siRNA (10 nM) followed by 72 hr incubation with R1881 (1 nM). AR protein was significantly reduced what resulted in blockade of R1881-induced CRYM expression by 113.26% (Fig. 3A). To further study the role of AR in regulation of CRYM expression, we used antiandrogens. In the presence of bicalutamide (5 μ M) or OHF (5 μ M), R1881-induced CRYM was significantly downregulated by 81.58% and 136.86%, respectively (Fig. 3B). Application of the inhibitor of translation cycloheximide had no effect on R1881-induced CRYM expression (data not shown).

Expression of CRYM in Prostate Tissue Patients' Samples

To characterize CRYM expression pattern in prostate cancer, immunohistochemical studies were performed with patients' material. In non-malignant tissue obtained from patients who did not receive a treatment, 12.18% cells were CRYM-positive. CRYM expression decreased to 0.84%-positive cells in patients who underwent androgen ablation therapy. Interestingly, the highest CRYM expression (19.62%) in non-malignant tissue was detected in samples from patients with therapy-resistant tumors (Fig. 4). The same trend with decreasing expression of CRYM was observed in prostate intraepithelial neoplasia tissue from patients who underwent androgen ablation therapy (34.69%) in comparison to samples from untreated patients (49.3%) (Fig. 4). CRYM was detected in cancer tissue samples from untreated patients with the expression of 79.4% and its levels decreased to 40.62% in patients who received androgen ablation therapy. In therapy-refractory tumors CRYM expression was not detectable (Fig. 4).

Effects of T3 and R1881 on Proliferation of MDA PCa 2b Cells

In order to better characterize the role of thyroid hormones in prostate cancer cells proliferation, experiments with T3 were performed. We employed MDA PCa 2b human prostate cancer cells whose proliferation is induced by androgens [17]. Proliferation of MDA PCa 2b cells was significantly stimulated by increasing doses of T3 (10–50 ng/ml) (Fig. 5A). We also confirmed that R1881 (0.1–1 nM) elevated the growth rate of MDA

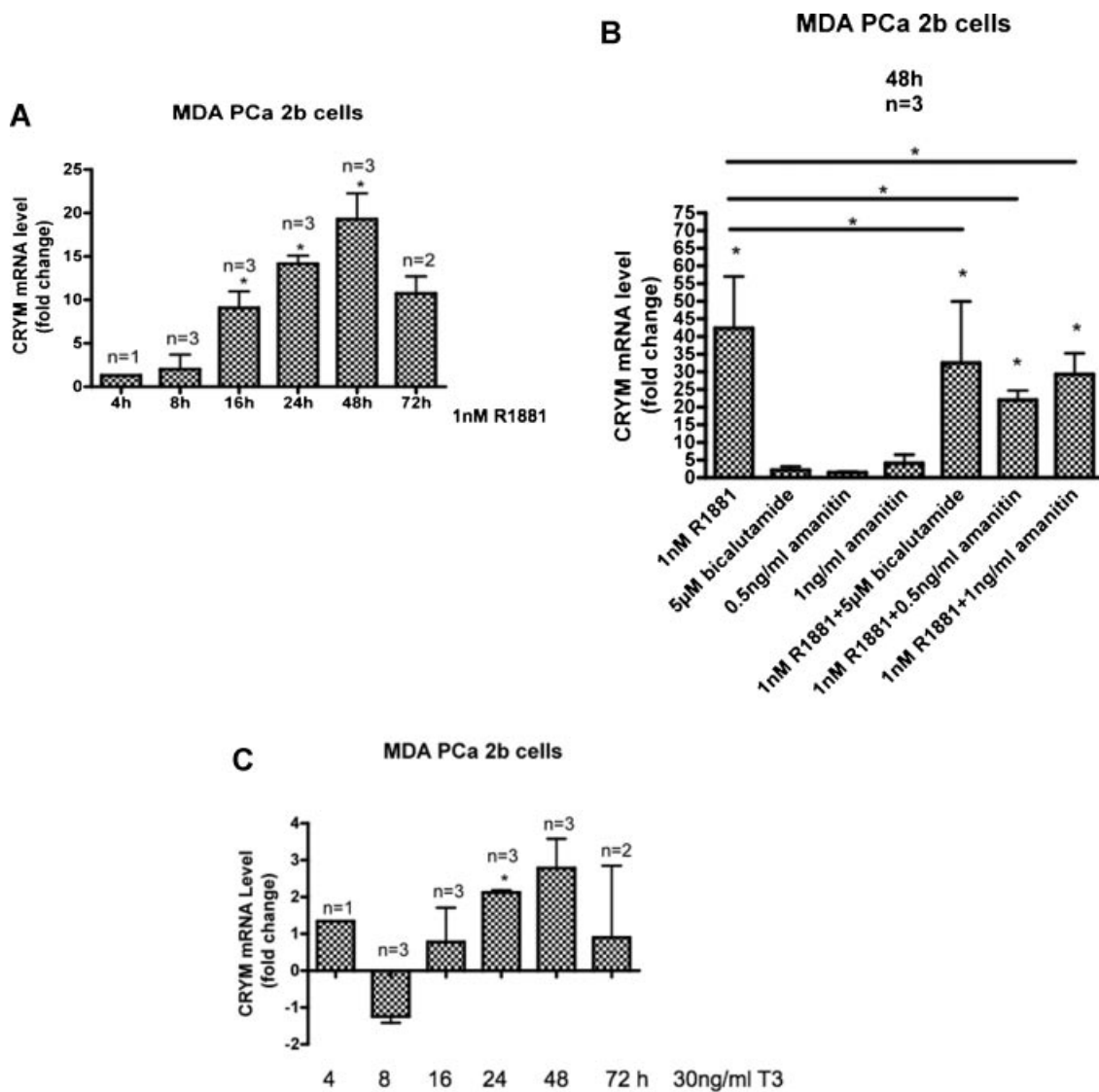


Fig. 2. CRYM mRNA expression after androgen or T3 treatment in the presence or absence of an antiandrogen or α -amanitin treatment as determined by real-time PCR. **A:** Time-dependent increase of CRYM mRNA in MDA PCa 2b cells upon R1881 treatment in comparison to untreated cells. **B:** Reduction of R1881-induced CRYM mRNA expression in MDA PCa 2b cells upon bicalutamide or α -amanitin treatment. **C:** Regulation of CRYM mRNA levels in MDA PCa 2b cells by T3. Data represent the mean \pm SD, $n = 3$. * $P < 0.05$ versus control, Mann–Whitney U -test.

PCa 2b cells with the maximal effect at a concentration of 0.1 nM (Fig. 5A). To examine the effect of cotreatment with androgens and thyroid hormones, MDA PCa 2b cells were incubated with R1881 in combination with T3. Additive effect of both compounds on proliferation was observed with lower R1881 concentration (0.1 nM) and increasing T3 concentrations (10–50 ng/ml) (Fig. 5B).

DISCUSSION

AR is a transcription factor that mediates the action of androgens during the growth and development of prostate gland. It also plays a pivotal role in prostate

cancer progression. The disease that is initially androgen-dependent becomes resistant to androgen ablation therapy. Thus, characterization of AR signaling and expression of its downstream genes could be relevant to understanding the mechanisms underlying cancer progression. Androgens stimulate not only the expression of proteins required for the production of seminal fluid but also elevate levels of molecules involved in cellular metabolism [18,19]. CRYM is such a component of thyroid hormone signaling identified as a T3-binding protein. CRYM levels are decreased in therapy-refractory tumors comparing with benign tissue [16]. Our in vitro studies showed that CRYM is expressed in two

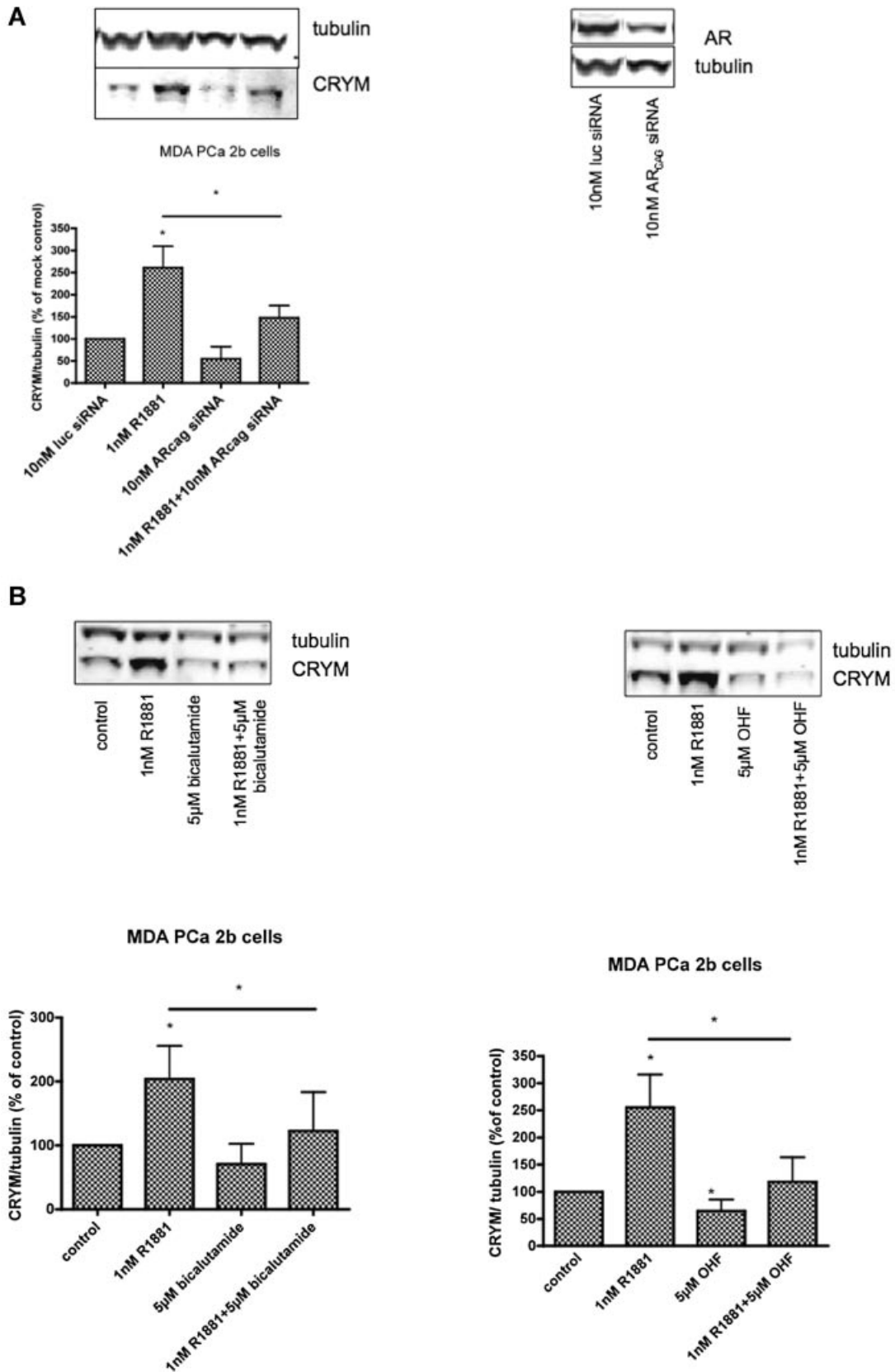


Fig. 3. A: Downregulation of R1881-induced CRYM expression by AR_(CAG)siRNA. **B:** Diminished CRYM expression after treatment with either antiandrogen bicalutamide or OHF. Data represent the mean ± SD, n = 3. *P < 0.05 versus control, Mann–Whitney U-test.

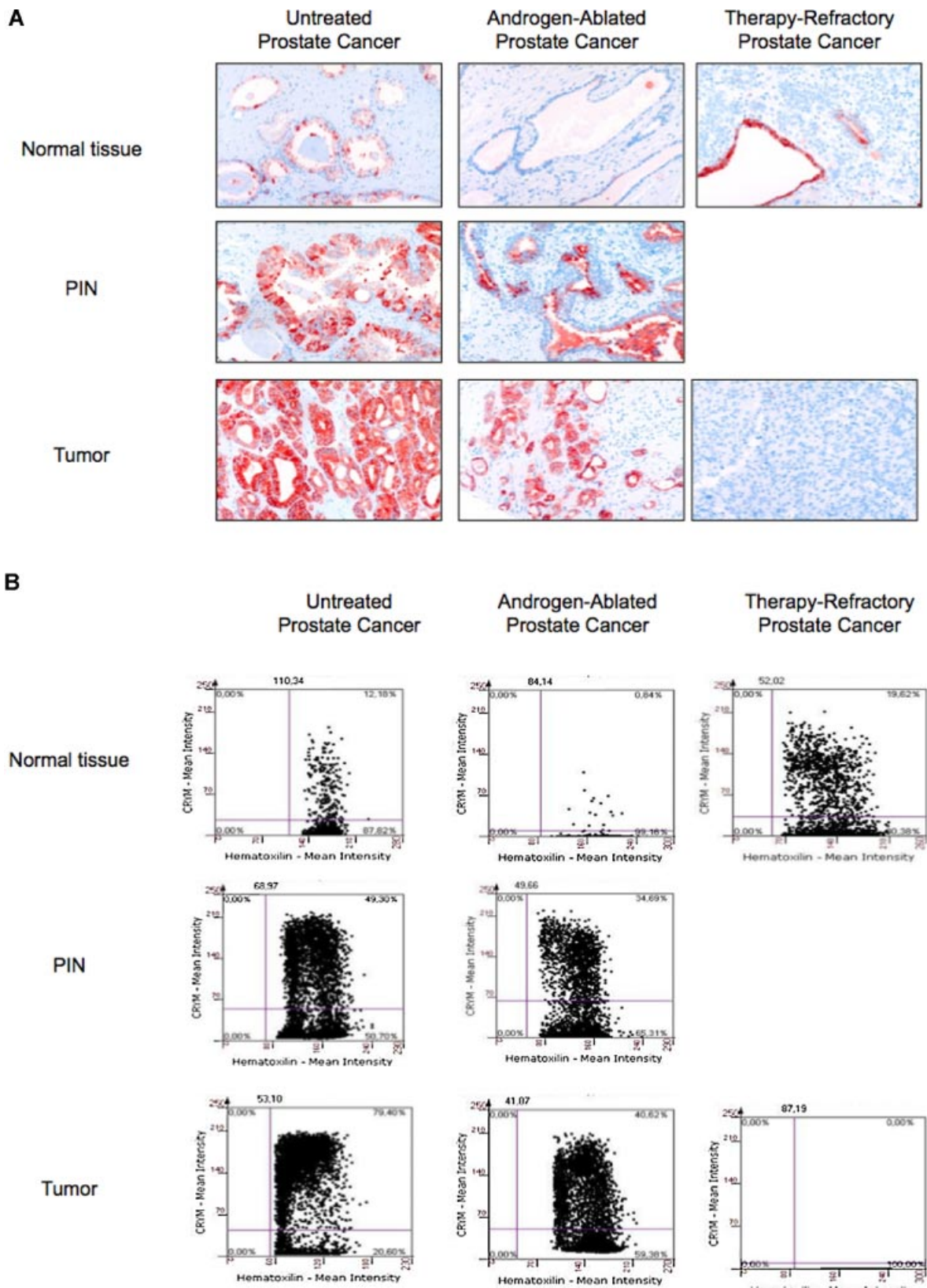


Fig. 4. Expression of CRYM in normal, prostate intraepithelial neoplasia (PIN), and cancer tissue in samples obtained from prostate cancer patients. CRYM immunohistochemistry was performed using the ABC staining kit according to the manufacturer's instructions. The percentage of CRYM-positive cells was determined and depicted as scattergrams. CRYM is not expressed in castration therapy-refractory tumors.

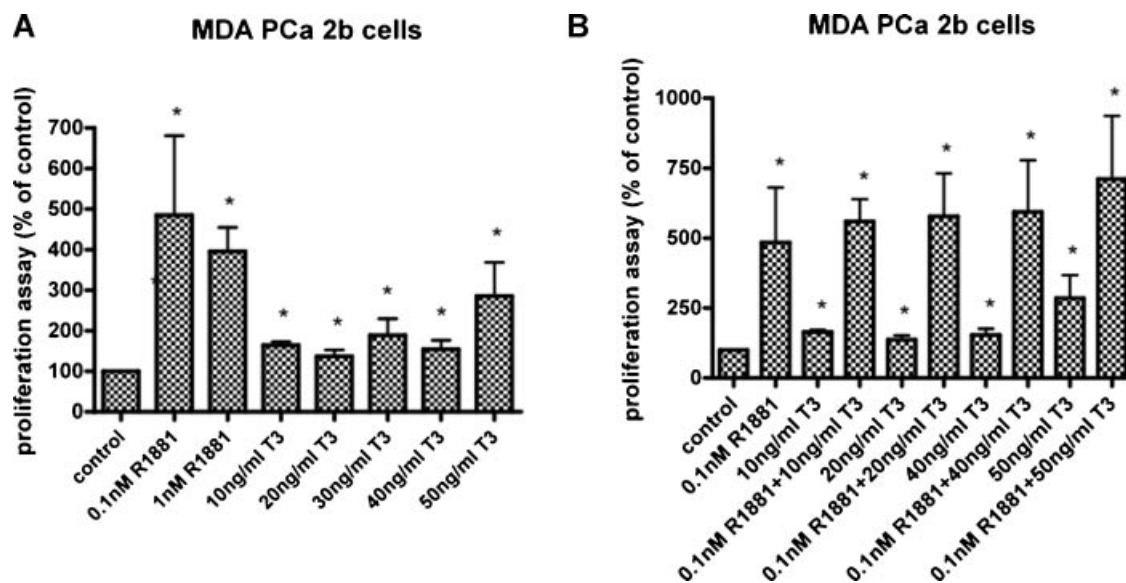


Fig. 5. Regulation of proliferation of prostate cancer cells MDA PCa 2b after androgen or T3 treatment. Proliferation was determined by ^3H thymidine uptake assay. **A:** Increased proliferation of MDA PCa 2b cells after R1881 or T3 treatment. **B:** Additive effect of combined treatment with 0.1 nM R1881 and increasing doses of T3 (10–50 ng/ml T3) on MDA PCa 2b cells proliferation. Data represent the mean \pm SD, $n = 3$. * $P < 0.05$ versus control, Mann–Whitney U -test.

cell lines LNCaP and MDA PCa 2b expressing mutated AR [20,21]. LAPC-4 cells that express wild-type AR [22] are CRYM-negative. It seems that expression of CRYM is cell type-specific and depends on numerous factors regulating transcription. For example, expression of hTERT gene was inhibited by wild-type AR, whereas the mutated AR (T877A) abrogated this inhibitory mechanism in prostate cancer cells [23]. The fact that androgen regulated CRYM expression in MDA PCa 2b but not in LNCaP cells may be somehow surprising because androgenic stimulation of AR activity in the MDA PCa 2b cell line is decreased [21]. However, AR coactivator expression may differ between MDA PCa 2b and LNCaP cells. Studies regarding recruitment of coactivators to wild-type AR could probably explain the lack of CRYM expression in LAPC-4 cells. In concordance with previous data, CRYM was not detected in AR-negative prostate cancer cell lines.

Induction of CRYM mRNA levels after androgen treatment was consistent with increased expression of the protein. Reduction of androgen-induced CRYM mRNA by the transcription inhibitor α -amanitin and the lack of effect of the translation inhibitor cycloheximide indicated that androgens regulated CRYM expression at the transcriptional level. Androgen-stimulated CRYM was reduced by two antiandrogens, bicalutamide and OHF. Downregulation of AR with siRNA also resulted in the reduction of androgen-induced CRYM. This evidence strongly suggested that the regulation of CRYM expression requires functional AR.

Zhang et al. [24] demonstrated that T3 stimulated proliferation of AR-positive prostate cancer cell line LNCaP but had no or marginal effect on the expression of prostate-specific antigen and human kallikrein 2, two androgen-regulated genes. In our study the treatment of MDA PCa 2b cells with T3 caused enhanced proliferation and this response was even stronger in combination with androgens. However, we did not observe increased expression of CRYM after T3 treatment. Thus, we assumed that androgens and T3 exert similar effects on cell cycle regulators but there is a number of genes differentially regulated by two hormones. In future studies, it may be determined whether downregulation of CRYM has a specific effect on T3-regulated proliferation of prostate cancer cell lines.

An interesting issue regarding AR signaling and CRYM expression is associated with hair growth. It is known that development of hair is regulated by androgens and the highest accumulation of CRYM in mouse skin has been reported during anagen, an active phase of hair growth [25].

CRYM has been identified in our studies as one of the androgen-regulated genes. The report of Mousses et al. [16] showed decreased mRNA levels of CRYM in therapy-refractory specimens compared to normal tissue. It is in accordance with our immunohistochemistry results where the lack of CRYM expression was obvious in samples from patients with castration therapy-resistant tumors. Our histopathological studies revealed increased expression of CRYM in cancer

tissue. In patients who underwent androgen ablation therapy decreased CRYM levels in comparison to untreated patients were observed. This finding provides additional support for the conclusion that functional AR signaling is required for regulation of CRYM expression.

CRYM can be implicated in development of other tumors in addition to prostate cancer. Almost all cancer cell lines examined for CRYM showed its expression, with the exception of rat hepatoblastoma dRLh-84 cells [26]. The expression of CRYM was elevated in non-small cell lung carcinoma [27], whereas brain tumors revealed low expression levels [28].

In summary, the most important finding of this work is the identification of CRYM as an androgen-regulated gene. In future studies, expression of CRYM could be correlated with patients' survival or time to progression towards therapy resistance. Further experiments to explore potential significance of CRYM silencing in prostate cancer progression are therefore justified.

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