

Increased Akt Signaling Resulting from the Loss of Androgen Responsiveness in Prostate Cancer

J. Dulińska-Litewka^{*1}, J.A. McCubrey² and P. Laidler¹

¹Chair of Medical Biochemistry, Jagiellonian University Medical College, 31-034 Kraków, ul. Kopernika 7 POLAND; ²Department of Microbiology & Immunology, Brody School of Medicine, 600 Moye Boulevard, East Carolina University, Greenville, NC 27834, USA

Abstract: The mechanisms responsible for the switch of prostate cancer from androgen-sensitive (AS) to androgen-insensitive (AI) form are not well understood. Regulation of androgen receptor (AR), through which androgens control the expression of genes involved in prostate cells proliferation, migration and death also involves its cross-talk with the other signaling pathways, transcription factors and coregulatory proteins, such as β -catenin. With the aim to determine their possible contribution in triggering the switch from AS to AI form, which occurs upon androgen deprivation therapy - AR, Akt and β -catenin expression were knocked-down with respective siRNAs. Treatment of LNCaP prostate cells with siRNA for AR significantly reduced their proliferation (45-70%), expression of nuclear β -catenin, cyclin-D1, cyclin-G1, c-Myc as well as activity of metalloproteinases (MMPs) -2,-7,-9 and cell migration. Surprisingly, after longer (over 72 hrs) silencing of AR in LNCaP cells, elevated levels of p-Akt were detected and enhanced proliferation as well as expression of nuclear β -catenin, cyclin-D1, c-Myc and activity of MMPs were observed. Such effects were not observed in either PC-3 or DU145 AI cells. However, silencing of Akt and/or β -catenin in those as well as in LNCaP cells led to their decreased proliferation and migration. Our findings suggest that in prostate cancer cells, either AR or Akt signaling prevails, depending on their initial androgen sensitivity and its availability. In AI prostate cancer cells, Akt takes over the role of AR and more effectively contributes through the same signaling molecule, β -catenin, to AI cancer progression.

Keywords: Akt, androgen-insensitive, androgen-sensitive, androgen receptor, β -catenin, metalloproteinases, prostate cancer, cyclin-D1, c-Myc, siRNA.

1. INTRODUCTION

Prostatic adenocarcinoma is one of the most frequently diagnosed malignancies and the second leading cause of cancer death amongst men in the developed world [1]. It has a high tendency to metastasize, making it very difficult to treat if not detected early. However, invasive or even micrometastatic disease presents a clinical challenge, as these tumors respond poorly to standard cytotoxic regimens that act through genomic insult. Therefore, prostate cancer patients are often treated based on a unique characteristic of prostatic tissue, in that it is expressly dependent on androgen whose biological effects are mediated through the androgen receptor (AR), which regulates target gene transcription with respect to its development, growth, and survival [2, 3]. Virtually all prostate cancer patients respond well when first treated with androgen ablation. However, resistance to hormonal blockade ultimately results in the recurrence of highly aggressive and metastatic prostate cancer that is androgen-independent.

Tumoral development results from a multistep process that leads successively to the formation of low- and high-grade prostatic intraepithelial neoplasia, which is mainly under androgenic control [4]. Following androgen deprivation, the androgen dependence of prostate tissue is manifested by rapid cellular apoptosis, involution and progression to the regressed state [5]. As a result, androgen ablation induces initial tumor regression, but, after a limited time, the cancer relapses as an androgen-independent (AI) disease [6]. The mechanisms involved in the escape of prostate cancers from androgen control include mutation and over expression of the AR gene or ligand-independent activation by other signaling pathways [7]. In effect most, but not all, AI prostate cancer cells still express AR as well as the androgen-inducible prostate-specific antigen (PSA), suggesting that these cells maintain an active AR signaling pathway [8, 9]. Previous studies have demonstrated the value of using PSA immunohistochemistry in the diagnosis of metastatic prostate cancer [10]. However, recently it was shown that there

seemed to be no correlation between AR and PSA expression, strongly suggesting that the retained PSA expression may be driven by non-AR mediated mechanisms in late-stage prostate cancer [11].

In comparison to many types of cancer, prostate cancer takes longer to invade the adjacent tissues and spread to distant sites in the body. However, it is a life-threatening disease and no effective treatment is currently available. Therefore, it is important to understand the molecular mechanisms behind the transition of prostate cancer from AS to AI state in order to search for new targets to treat advanced disease more efficiently. Some of the molecular players have been identified, but the process itself is far from clear.

Our study describes a molecular mechanism very likely involved in prostate cancer progression from AS to AI state. It includes the identification of a mechanism bypassing the principal role of AR in prostate cancer progression and has been supported by experimental studies presented in this report.

It is well documented that the progression of many types of cancer is accompanied by or linked to increased expression of N-cadherin. It seems to be a case for prostate cancer as well. The commonly studied AS prostate cancer cell line LNCaP express E-cadherin but no N-cadherin at the mRNA and protein levels, while malignant AI prostate cell line PC-3 expresses no E-cadherin and shows unanimous presence of N-cadherin. The gain of N-cadherin expression in prostatic cells has been shown to be important in regulation of cell migration, invasion, proliferation and survival [12]. Jennbacken suggested that the expression of N-cadherin was influenced by androgen deprivation and was associated with metastasis in prostate cancer [13]. They observed that expression of N-cadherin increased in the absence of androgens in LNCaP-19 primary tumors and metastases and also *in vitro*, but not in PC-3 tumors, indicating a possible involvement of the AR in the regulation of N-cadherin. Its expression was also associated with metastasis and Gleason score and was increased in castration-resistant tumors in patients with established metastases. This might indicate that castration induces molecular alterations in tumor cells, resulting in a more invasive and metastatic phenotype [13].

*Address correspondence to this author at the Chair of Medical Biochemistry Jagiellonian University Medical College, ul. Kopernika 7, 31-034 Kraków, Poland; Tel/Fax: +48124223272; E-mail: mblitewk@cyf-kr.edu.pl

As cadherins are crucial for regulation of β -catenin, the possible effect of AR silencing on β -catenin function was also investigated. β -catenin, originally identified as a component of cell-cell adhesion structures, interacts with the cytoplasmic domain of E-cadherin and has a second function in the canonical Wnt (wingless/int) cascade that links E-cadherin to α -catenin, which in turn mediates anchorage of the E-cadherin complex to the cortical actin cytoskeleton [14, 15].

The important kinase involved in modification and therefore the fate of β -catenin is Akt. In PC-3 cells, a loss of adhesion and viability were associated with a loss of Akt phosphorylation at Ser⁴⁷³, which may explain the phenotype observed in PC-3 cells, critically dependent on PI3K signaling [16]. Assuming the roles of Akt and β -catenin in prostate cancer, we decided to knock out them selectively or together in AS and AI prostate cancer cells with the aim to elucidate the mechanism that might be responsible for the switch of prostate cancer from androgen-sensitive to androgen-insensitive. Our findings suggest that in prostate cancer cells either AR or Akt signaling prevails depending on androgen sensitivity and availability. We demonstrate that in AS prostate cancer cells, AR signaling recruits β -catenin through which it affects the expression of crucial mediators of cell behavior. It seems that in AI prostate cancer cells, Akt takes over the role of AR and signals through the same signaling molecule, β -catenin, resulting in highly invasive androgen independent cancer progression.

2. MATERIALS AND METHODS

2.1. Cell Lines

The following established cell lines or primary cells were used: LNCaP - androgen-sensitive, brain metastasis; and PC-3, androgen-insensitive, lymph nodes metastasis, and DU145 - androgen-insensitive, metastatic prostate carcinoma from brain metastasis, human prostate cancer cell lines (American Type Culture Collection, Manassas, Virginia, USA), CA-K - primary prostate cancer cells and BPH-K - primary benign prostate hyperplasia cells derived as described previously [9]. Treated and untreated cells were maintained in RPMI-1640 medium (Sigma, Poland) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 μ g/ml streptomycin. When steroid hormone (dihydrotestosterone, testosterone) was added to medium charcoal-stripped fetal calf serum (SF - steroid free) was used. In some cases, the cells were treated with testosterone or dihydrotestosterone (Sigma; 1-10nM) for 1 to 5 days.

2.2. Tissue Samples and Isolation of Cells

Tissue samples - 20 prostate cancer, 15 hyperplastic and 7 normal were collected at the time of surgery from the Department of Surgery Military Hospital, Krakow (consent of Bioethics Committee n° KBET/36/B/2010). Among prostate cancer samples there were 8 well-differentiated (Gleason score 2-4), 9 moderately differentiated (Gleason score 5-7) and 3 poorly differentiated adenocarcinomas (Gleason score 8-10). All prostate tissue samples used in the study were histopathologically confirmed (Department of Pathology, Jagiellonian University Medical College, Krakow, Poland). The median age of patients with prostate cancer was 68.5 years (range 38-84). The preparation of tissue slices and the isolation of cells from them was performed as previously described [17, 18]. Cells were released by overnight treatment of tissue slices in 0.125% trypsin solution [9].

2.3. siRNA Transfection

Prostate cells were grown until 60% confluence and then transfected using Lipofectamine-2000 according to the manufacturer's protocol with three different 21bp double-stranded siRNA mole-

cules specifically targeting the AR- ID# 289124, ID# 146896, ID# 4096; Akt - ID# 103719, ID# 103718, ID# 118270; β -catenin - ID#146154, ID#146156, ID#146155 or a control non-silencing sequence (Ambion, Life Technologies, Poland). All cells were transfected with 80 nM siRNA. After 24 h, the medium was replaced with fresh medium and cells were grown for an additional 48h period (72h post-transfection, 96h or 120h) prior to isolation of RNA and protein for further analysis.

2.4. Cytotoxicity of siRNA

The cells were seeded in triplicate into 96-microwell plates at a density of 1.5×10^3 cells/well and incubated with or without the specific siRNA (AR, Akt, β -catenin) for 48, 72, 96 or 120h. Negative and positive controls were also included. Colorimetric assays were performed according to manufacturer's instructions (Cytotoxicity Detection Kit, Roche Diagnostics, Poland) and as described elsewhere [18]. Absorbance of the colored product - formazane - was measured at 492 nm by an ELISA reader (Synergy HT, BIO-TEK, USA). No cytotoxic effects of the used concentrations of siRNAs on any of the studied cell lines were observed.

2.5. Cell Proliferation (ELISA, BrdU)

Cells were seeded in triplicates into 96-microwell plates at a density of 1.5×10^3 cells/well and cultured in medium with or without the different siRNAs for AR, Akt and β -catenin in the presence of positive and negative controls for various periods of time (24-120h). ELISA BrdU (Roche) colorimetric immunoassay tests were performed as described previously [18]. The absorbance was measured at 450 nm on an ELISA plate reader.

2.6. Cell Migration and Invasion Assay

Cell migration and invasion assays were performed using conventional Boyden transwell methods according to the manufacturer's protocol (BD BioCoat Tumor Invasion System No. 354166, Poland). Quantitation of invasive cells was achieved by post invasion cell labeling with calcein and measuring the fluorescence of invading cells.

2.7. Zymography

Gelatinolytic activities of metalloproteinases MMP-2 and MMP-9 were evaluated from the conditioned medium. Proteins were separated on 10% polyacrylamide gels containing 0.1% gelatin (Sigma, Poland) under nonreducing conditions. After electrophoresis, the gel was washed two times for 30 min in 2.5% TritonX-100. After 48 h incubation at 37°C in buffer (50 mM Tris pH 7.5; 10 mM CaCl₂; 0.15 mM NaCl) the gel was stained with 1% Coomassie blue R250 for 1h and its excess was eluted with methanol (50%)/acetic acid (10%) solvent. Gelatinolytic activity was observed as clear areas in the gel.

2.8. Preparation of Nuclear and Cytoplasmic Cell Lysates

Nuclear and cytoplasmic extracts were prepared using the Cell-Lytic NuCLEAR Extraction Kit (Sigma, Poland) according to producer's protocol and equal amounts of protein were used in immunoblot analysis.

2.9. Western Blot Analysis

Cells were lysed in sample buffer (0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol). Cell lysates containing equal amounts of protein were separated on 10% SDS-PAGE gels, and subsequently transferred onto a PVDF membrane. Antibodies against: Akt, phospho-Akt(S473), AR, β -catenin, phospho- β -catenin(S552), c-Myc, cyclin D1, E-, N-cadherin, p21, p27

(all Lab-JOT - Cell Signaling Technology, Poland), β -actin (Sigma, Poland), PSA (DAKO, Denmark) and MMP-2, -7, -9 (Santa Cruz, USA) were used to detect indicated proteins. Total protein loading was determined by probing the membranes for β -actin. Bands were visualized using alkaline phosphate-coupled secondary anti-mouse or anti-rabbit antibody (Sigma, Poland). Finally, immunoreactions were visualized by NBT/BCIP staining (Roche, Poland).

2.10. RNA Extraction, cDNA Synthesis and RT-PCR Analysis

RNA extraction, preparation of cDNA and RT-PCR reaction were carried out as previously described [19]. The temperature profile of RT-PCR amplification consisted of activation of Taq polymerase at 95°C for 4 min, denaturation of cDNA at 95°C for 30–45 s, applying primer annealing temperature for 30–40 s, elongation at 72°C for 40–60 s for the following 35 cycles and completion of the process by the extension step for 10 min. PCR protocols were optimized for each studied gene according to the specific annealing temperature (Table 1). The PCR reaction products were separated electrophoretically on 2% agarose gel and visualized with ethidium bromide.

2.11. Real-Time PCR

In parallel, cDNA was synthesized using 1000 ng of aliquots of total RNA primed with Superscript II reverse transcriptase (Invitrogen, Poland) and oligo d(T) primers (Sigma) in 20 μ l reactions volume in a thermocycler DYAD (MJ Research, Inc., Waltham, MA, USA) After reverse transcription, cDNA was dissolved in water (2:3) Next, 5 μ l of cDNA solution was added to the RQ-PCR reaction. Specificity was determined by BLAST searches. Real-time PCR (RQ-PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems, foster City, USA) and TaqMan probes. Specific primers and probes for *MMP9* gene and the *ABL* control gene were purchased from TIB MOLBIOL (Poznań, Poland) and Applied Biosystems (Applied Biosystems, Cheshire, UK), respectively: *ABL* - forward primer ENF1003 5'TGGAGATAA CACTCTAAGCATAACTAAAGGT3', reverse primer ENR1063 5'GATGTAGTTCTTGGG ACCCA3', molecular probe ENP1043 Fam - CCATTTTGGTTTGGGCTTCACACCATT-Tamra; *MMP9* - (forward, 5'AATCTCTTCTAGAGACTG GGAA GGAG3'; reverse, 5'AGCTGATTGACTAAAGT AGCTG GA3'. The standardized protocol for the comparative threshold cycle (Ct)

cycle (Ct) method was used as described in the Europe Against Cancer program. Amplification was performed using recommended standard conditions [20]. RQ-PCR results were expressed using the Δ CT method and relative RNA expression was calculated according to Macabeo-Ong *et al.* [21]. All experiments were performed in triplicate, and mean values were calculated. Negative controls lacking template RNA were always included in each experiment.

2.12. The Limitations of the Study

In order to demonstrate differences in expression of the investigated genes, the authors used research material originating from prostate tissue biopsy specimens (normal and cancer tissues with various tumor stages – Gleason score). In view of the limited amount of material with a normal phenotype only 7 tissues was employed in the investigations.

2. 12. Statistical Analysis

All data of the proliferation and cytotoxicity experiments represent the average of five wells in each experiment (n=4). Data was expressed as mean \pm standard deviation. In cases of RT-PCR and western blotting, each analysis was performed in triplicate. The significance of the differences between mean values were calculated using Student's t Test (≤ 0.05).

3. RESULTS

To assess the actual changes in expression of AR, Akt, β -catenin, E- and N- cadherins, cyclin-D1, c-Myc, as well as activity of MMPs and cell cycle inhibitors p21^{Cip-1} and p27^{Kip-1}, we used established prostate cancer cell lines: LNCaP (androgen-sensitive AS), PC-3 and DU145 (both androgen-insensitive AI), as well as primary cells derived from tissue specimens, one prostate cancer (CA-K) and one benign prostate hyperplasia line (BPH-K) [9] and respective tissues of their origin. Elevated expression of PSA, β -catenin, cyclin-D1 and c-Myc and decreased or lack of expression of E-cadherin was observed in AI cells and in cancer tissues Fig. (1). Expression of AR also depended on the stage of tumor, but there was no typical correlation between the degree of malignancy and expression of AR (data not shown). However, the observed alternations in expression of AR confirmed the lack of an obvious direct relationship between the kind of tissue/cell (normal, hyper-

Table 1. Primers Used for RT-PCR Analysis

Primer Protein	Sequences	Conditions
GAPDH	5' CACCGCCTCGGCTTGTCACAT 3'; 5' CTGCTGTCTTGGGTGCATTGC3';	59°C, 30 s
PSA	5'TTGTCTTCTCACCTGTCC3'; 5'TGCTCTTGATCCACTTCCGGTA3'	59°C, 40 s
AR	5'TGCAACTCCAGGATGCTCTACTT3'; 5'ATTCGGACACCACTGGCTGTACA 3'	55°C, 45 s
N-CADHERIN	5'GTGCCATTAGCCAAGGAATTCAGC3'; 5'CGAGGATACTACCTTGTCTTGGCG 3'	68°C, 40 s
E-CADHERIN	5'GCCAAGCAGCAGTACATTCTACACG3'; 5'GTCGTCTTTCACGTGCTCAAAATCC 3';	68°C, 40 s
β -CATENIN	5'-GCTGATTGATGGAGTTGGA3'; 5'TTCACTCAAGAACAAGTAGC3'	55°C, 120 s
CYCLIN D1	5'TGTTCTGGCCTCTAAGATGA3'; 5'GCTTGACTCCAGAAGGGCTT3'	60°C, 60 s
C-MYC	5'CAAGAGGCGAACACACAACGTCT3'; 5'AACTGTTCTCGTCTTCCGCAA3'	55°C, 60 s
MMP2	5'GTGCTGAAGGACACACTAAAGAAGA3'; 5'TTGCCATCCTTCTCAAAGTTGTAGG3'	59°C, 60 s
MMP9	5'AGATTCGACTCTCCACGCA - 3'; 5'CTGAGAACCAATCTACCGACA - 3'	60°C, 60 s
MMP7	5'TCGATGAGGATGAACGCTGGACGG3'; 5'TGCTAAATGGAGTGGAGGAACAGTGC3'	60°C, 60 s

plastic, cancer stage) and the level of this receptor additionally reflecting the variation in its mRNA/protein stability [Fig. (1). A and B lane AR]. The observed different expression of selected proteins in various cell lines and tissues indicated phenotype alterations due to cancer progression. The cell lines PC-3, CA-K and cancer tissue CA did not express E-cadherin, while we noted there was expression of N-cadherin both at mRNA and protein level in these samples. Additionally, in the same cells and respective tissues samples, expression of β -catenin, cyclin D1 and c-Myc showed similar increasing trends. Expression of AR in AI cells was not observed Fig. (1).

We previously reported on the differential response of prostate cells to treatment with peroxisome proliferator-activated receptor gamma (PPAR γ) ligands (fatty acids, ciglitazone) or kinetin riboside in respect to their androgen sensitivity [9, 18, 22]. We were also able to show the strong effect of T and DHT on the expression of genes/proteins related to proliferation and apoptosis in AS human prostate carcinoma cells. Therefore, we sought to determine whether cell behavior could be modulated in response to silencing AR activity.

Cells were treated with DHT or T under previously described conditions [18]. AS LNCaP cells stimulated with T or DHT showed elevated proliferation Fig. (2A) and high levels of nuclear β -catenin and AR Fig. (2B) as well as migration Fig. (2C-D). No such effects were observed with AI DU145 and PC-3 cells. Interestingly, in LNCaP cells, increased β -catenin levels in nucleus were accompanied by increased levels of AR Fig. (2B). The observations on the interactions between β -catenin and AR suggest a possible mechanism of the cross talk between β -catenin and AR signaling pathways [23]. It also seems that DHT has a stronger impact on AR-mediated proliferation and the transport of β -catenin to nucleus as compared to testosterone [Fig. (2A) and data not shown]. There-

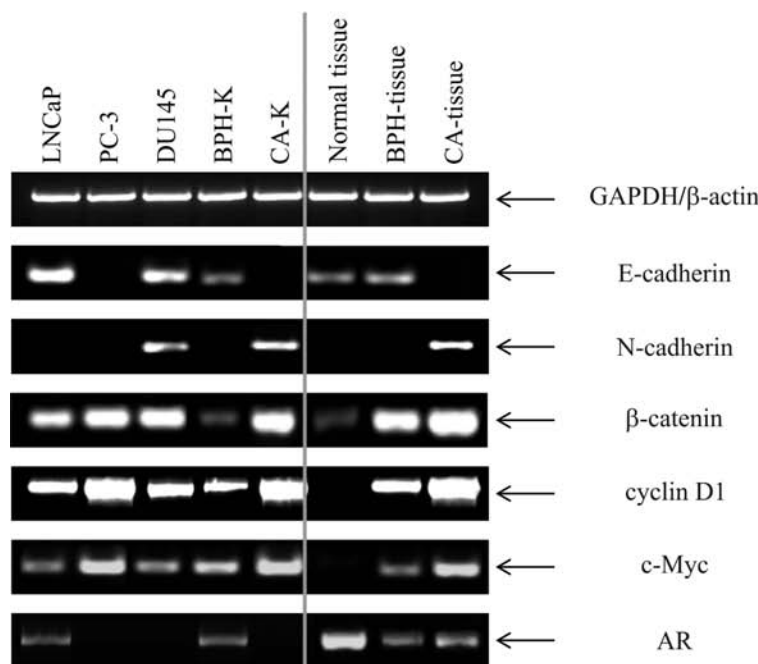
fore, DHT was used in further studies as the AR ligand when its effects on AR-mediated proliferation and migration were investigated. DHT significantly influenced the migration and MMPs activity in LNCaP cell line Fig. (2).

Because MMPs are involved in tumor metastasis, we compared the constitutive levels of MMP gene expression in prostate cancer cell lines with different metastatic potentials Fig. (2C). A significantly higher level of MMP-9 gene expression was observed in PC-3 and DU145 cells compared with LNCaP cells. MMP-9 activity in LNCaP cell line increased after treatment with DHT Fig. (2C).

Treatment of LNCaP prostate cells with siRNA for AR significantly reduced proliferation (45-70%), expression of cell cycle proteins - cyclin D1 and cyclin G1, c-Myc, cell migration and activity of MMPs after 72hrs Fig. (3A-E). We observed that LNCaP cells transfected with siRNA specific for AR completely lost the ability to migrate through Matrigel-coated Boyden chambers when compared with respective control cells Fig. (3B). In parallel, increased expression of E-cadherin, p21^{Cip-1} and p27^{Kip-1} in LNCaP cells were observed Fig. (3D). In contrast, neither proliferation nor migration were affected by PC-3 and DU145 cells after transfection with siRNA specific for AR.

mRNA and protein analysis revealed that LNCaP cells expressed E-cadherin but not N-cadherin Fig. (1). The gain of N-cadherin expression in prostate has been shown to be important with respect to regulation of cell migration, invasion, proliferation and survival [19]. AR knockdown led to increased expression of E-cadherin in LNCaP cells. A similar observation was recently made in BPH-K primary cells [9]. As cadherins are crucial for regulation of β -catenin, the possible effect of AR silencing on β -catenin function was investigated. The results confirmed the participation of AR

A. RT-PCR



B. WESTERN BLOT

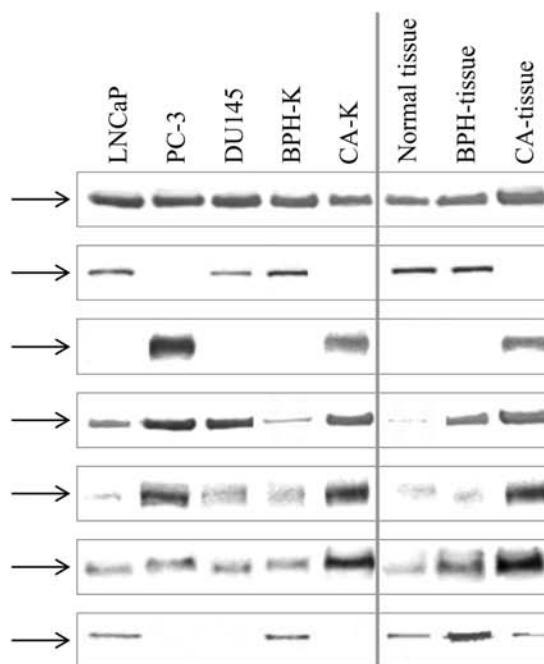


Fig. (1). Expression of E-, N-cadherin, β -catenin, cyclinD1, c-Myc and AR in various human prostate cells and tissues at mRNA (Panel A) and protein (Panel B) levels. RT-PCR analysis. The cells and tissues examined were: LNCaP (AS), PC-3 and Du145 (AI), BPH-K (primary benign prostate hyperplasia cells), CA-K (primary prostate cancer cells) derived as described previously [16] and their respective BPH and CA, as well as, normal prostate tissues. The upper lanes show the levels house-keeping genes GAPDH or β -actin under the same culture conditions.

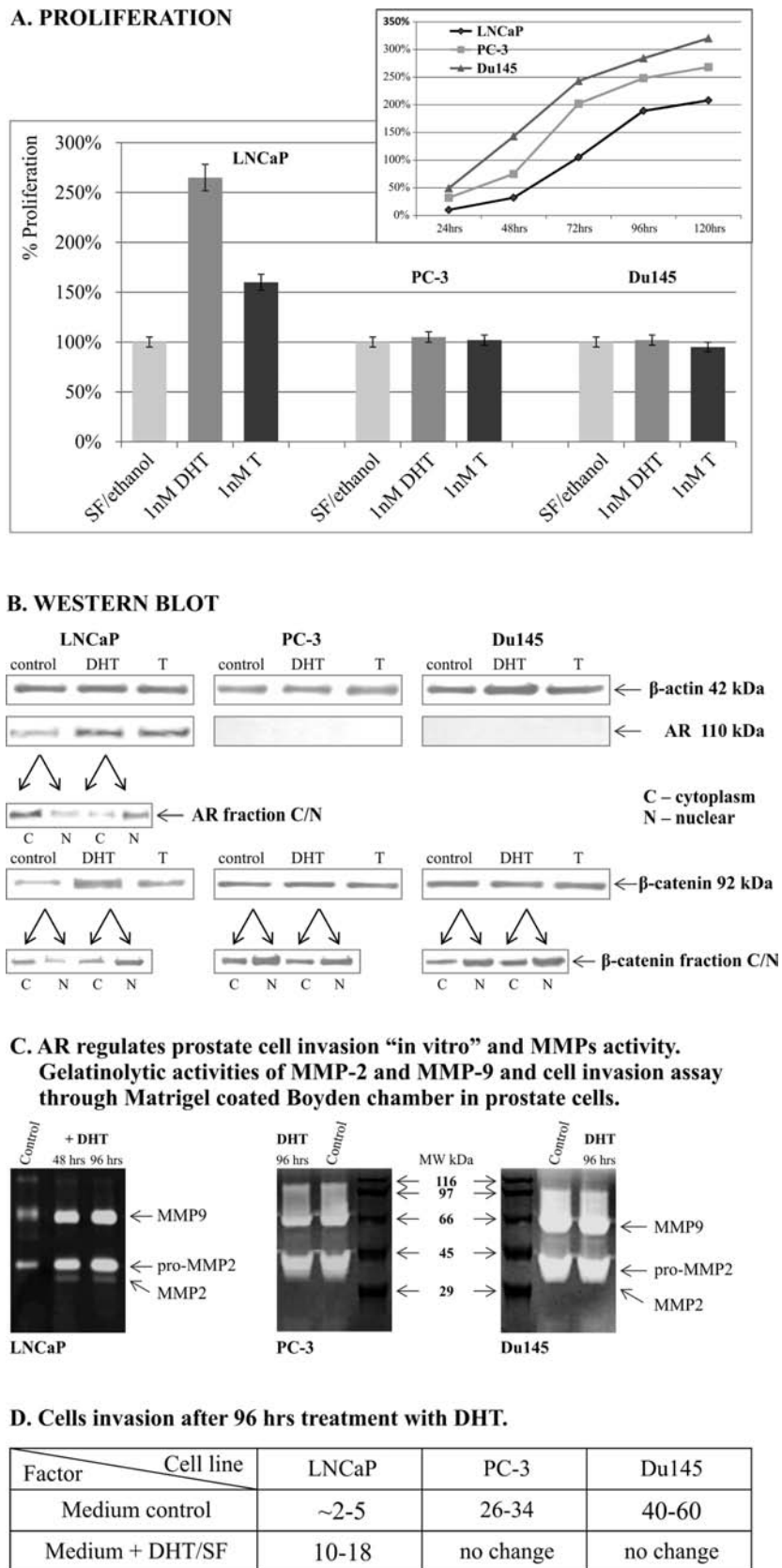
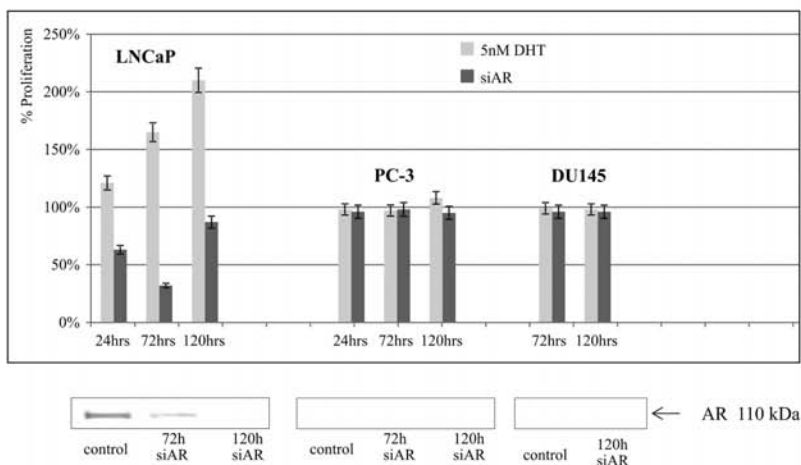


Fig. (2). The effect of androgen [1nM dihydrotestosterone (DHT) or 1nM testosterone (T) treatment of LNCaP, PC-3 and DU145 prostate cancer cells on: Panel A. proliferation, the graph insert in the right upper quadrant shows the proliferation of cells over time; Panel B. Total cell protein extracts were analyzed for AR by western blot analysis. β -catenin cytoplasmic and nuclear expression was examined by western blot analysis. Cytoplasm and nuclear protein extracts were prepared as described in M&M. Panel C. Zymography - Gelatinolytic activities of MMP-2 and MMP-9. Panel D. cell invasion assay through Matrigel coated Boyden chamber. Table presents quantification of cell invasion which are presented as the mean \pm SD from three independent experiments.

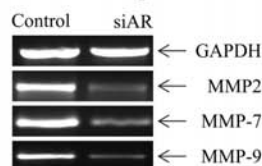
A. PROLIFERATION



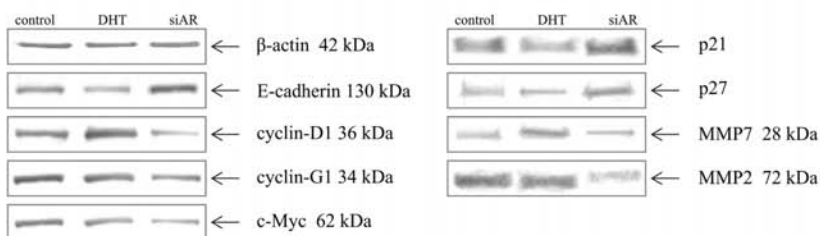
B. MIGRATION – Boyden chamber/Matrigel

Factor \ Cell line	LNCaP	PC-3	DU145
Medium (control)	~2-5	26-34	40-60
siAR	Not detected	26-35	38-42

C. MMPs expression in LNCaP



D. Western blot LNCaP



E. Western blot – nuclear translocation of β-catenin

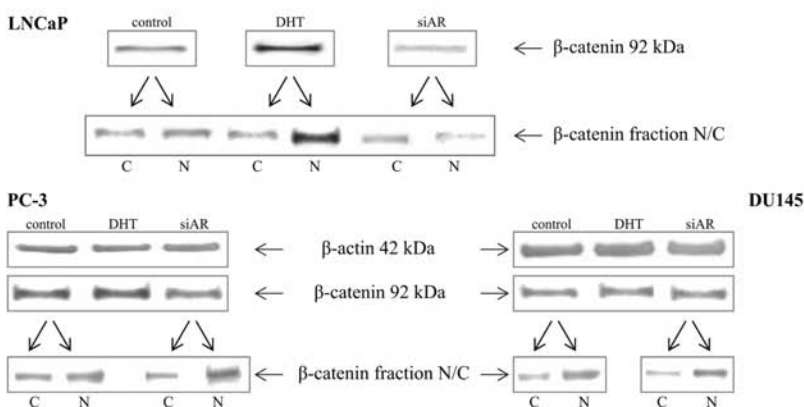


Fig. (3). The effects of AR knock-down in LNCaP, PC-3 and DU145 prostate cancer cells were determined by various approaches. In panel A, cell proliferation was measured over a 24-120hrs time period. In panel B, cell invasion assays through Matrigel coated Boyden chamber are presented in a table which quantifies the extent cell invasion. In Panel C, MMPs expression was examined at the mRNA level in LNCaP cells. In Panel D, the expression of: MMP7 and MMP2, cyclin D1, cyclin G1, c-Myc and E-cadherin, p21^{Cip-1} and p27^{Kip-1} in the LNCaP cell line were examined at the protein level. In panel E, β-catenin expression and its nuclear translocation was determined. Total cell lysates as well as cytoplasmic and nuclear fractions were analyzed for β-catenin expression by western blotting. Representative results of at least four independent experiments are presented.

in the translocation of β -catenin, as DHT treatment increased the nuclear levels of β -catenin while silencing of AR led to decreased levels of nuclear localized β -catenin Fig. (3E). As might have been expected, we did not detect a significant difference between control and AR siRNA transfected cells, with respect to β -catenin localization, in the case of the AI DU145 and PC-3 cells Fig. (3E). Surprisingly, after AR silencing for a period longer than 96 hrs in LNCaP cells, we observed increased levels of proliferation Fig. (3A) as well as elevation of p-Akt levels and MMPs activity Fig. (4). These effects were not observed in either PC-3 or DU145 cells Fig. (4A). Additionally we observed changes in the levels of β -catenin phosphorylation at Ser 552 after longer periods (120h) of AR silencing in LNCaP cells. Significantly higher levels of β -catenin phosphorylation after 120 hrs of AR knock-down were detected in these cells. In addition, we noted significantly increased migration of LNCaP cells treated for 120h with AR siRNA through Matrigel-coated Boyden chambers compared to untreated cells Fig. (4B).

To confirm the presumed role of Akt in prostate cancer progression as the main mediator of β -catenin function in AR knocked-down cells, Akt expression was also silenced. In this case, the important effects of Akt knock-down were observed in PC-3 and DU145 cells, as siRNA specific for Akt caused inhibition of proliferation (50-60%) in AI cell line and surprisingly only 10-20% in AS LNCaP cells line Fig. (5A). Silencing of Akt gene caused inhibition of both of Akt and β -catenin expression in AI PC-3 and DU145 cells. In contrast, Akt silencing in AS LNCaP cells did not result in a change in β -catenin expression Fig. (5B). Treatment of Akt-depleted LNCaP cells with DHT led to an increase of proliferation and β -catenin expression as well as its translocation to the nucleus. The results indicated that translocation of β -catenin to the nucleus depended more on AR than Akt activity in AS cells. In contrast, translocation of β -catenin to the nucleus in AI cells depended mainly on Akt activity Fig. (5).

Our results indicated that proliferation in prostate cancer cell lines depends both on AR and Akt regulation. To confirm these results, we knocked-down AR and Akt simultaneously. Simultaneous silencing of AR and Akt resulted in 70-85% inhibition of proliferation in AS LNCaP cells, whereas simultaneous silencing of AR and Akt resulted in 50-70% and 55-60% in PC-3 and DU145 inhibition of proliferation in the AI cell lines, respectively Fig. (6A). Silencing of both AR and Akt genes caused a higher degree of inhibition of Akt expression and β -catenin nuclear levels in AS as compared to AI cells PC-3 and DU145. In addition, decreased expression of cyclin D1 and increased levels of p21^{Cip-1} were observed Fig. (6). These results indicated that both AR and Akt regulate prostate cancer and one of their main targets is β -catenin. Therefore, to confirm that both AR and Akt use β -catenin as an important factor involved in the regulation of gene transcription, we also silenced β -catenin. β -catenin knock-down caused significant inhibition of proliferation Fig. (7A) in prostate cell lines and was sufficient to down-regulate the expression of cyclin D1, c-Myc and to increase p21^{Cip-1} expression, but the most potent effects were observed in LNCaP cells Fig. (7B). Similar decreases in the expression of MMPs and the migration of cells were observed in all lines when either siRNA for AR+ siRNA for Akt or siRNA for β -catenin were used Fig. (8A, B).

4. DISCUSSION

The Role of the Androgens and AR Expression in Prostate Cancer Progression

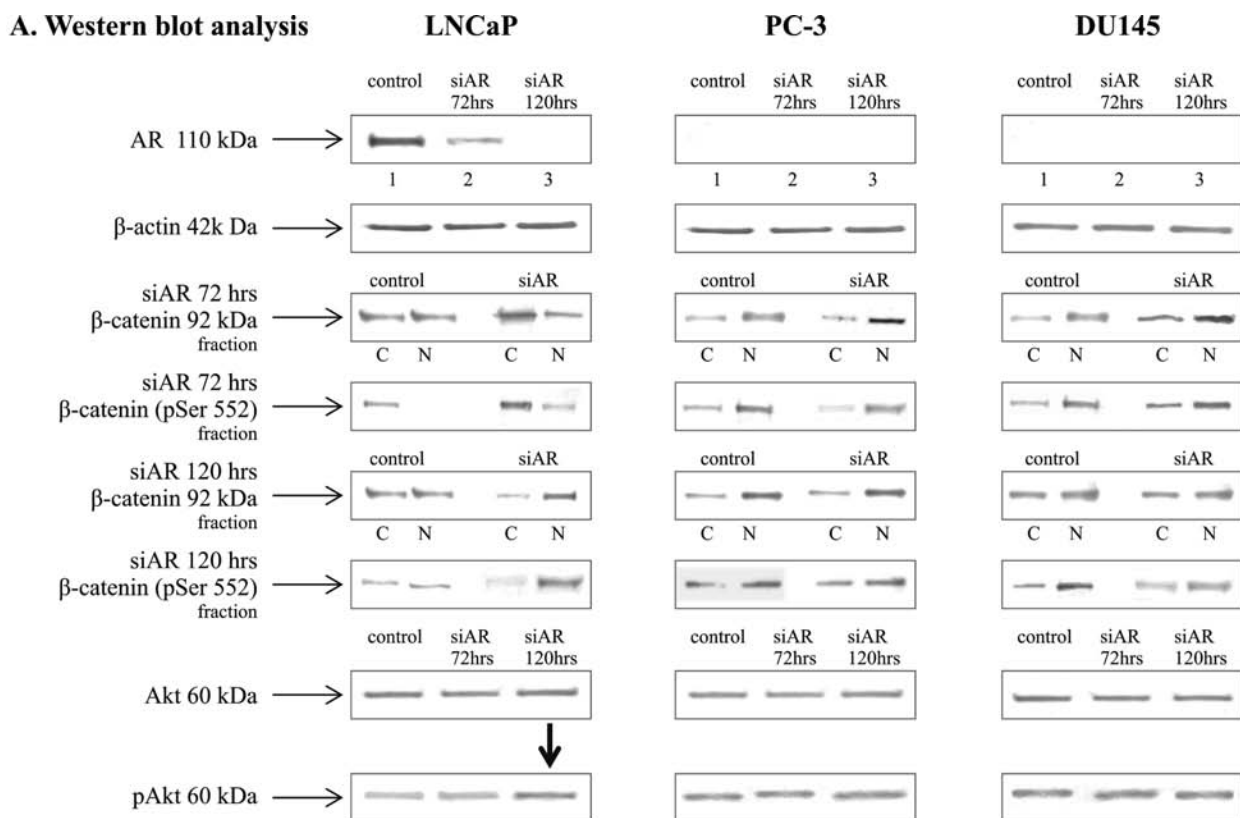
The regulation of gene expression by steroid hormones, in particular androgens, plays an important role in the development of normal prostate and strongly influences the pathogenesis of steroid dependent prostate cancers. In the process of tumor progression, fundamental changes in the expression of proteins involved in cell

signaling are observed. Only a small number of studies have attempted to explain the mechanism of prostate cancer with respect to the regulation of cell signaling pathways and their cross-talk on AR activity.

In order to understand prostate cancer progression and possibly to treat it more successfully, one of the most important questions addresses the development of its AI phase. Among human patients, androgen deprivation therapy is usually performed by administration of gonadotropin-releasing hormone analogs and/or surgical castration (orchiectomy), often in combination with anti-androgens, such as flutamide or bicalutamide to treat AS prostate cancer. Probably due to selective pressure, AI prostate cells have the capability to escape from the effect of castration and antiandrogens; exclusion of the AR activity by inhibition of dimerization or inhibition of DNA binding seems to be the next step [24]. Prostate cancer growth is stimulated initially by circulating testicular androgens [25]. Even when prostate cancer progresses to the castration resistant phase, AR activation and signaling remains sustained through a variety of mechanisms [26, 27]. Mechanisms for increased AR transactivation during prostate cancer progression to castration-resistant growth include AR gene amplification [28], somatic AR gene mutations that provide a gain of function by decreasing AR ligand specificity and increased AR interactions with coregulators, whose levels also increase during prostate cancer progression [29]. Prostate cancer tissue production of androgen develops during androgen deprivation therapy [30, 31] and increased mitogen signaling as well as AR phosphorylation influences AR transcriptional activity [32]. Mohler *et al.* [33], reported that the extent of AR transactivation in the presence of 5 α -androstane-3 α ,17 β -diol (androstane-3 α ,17 β -diol) in prostate-derived cell lines parallels the bioconversion of androstane-3 α ,17 β -diol to DHT. We were able to show that DHT increased proliferation in LNCaP prostate cancer AS cell line and the levels of AR and β -catenin expression. The results obtained confirm the participation of AR in the translocation of β -catenin, as liganded AR increased the nuclear level of β -catenin rather than inhibition as in the case of AR silencing. Other findings demonstrate that AR can regulate β -catenin signaling, that the AR- β -catenin interaction can enhance β -catenin/Tcf4 signaling and contribute to aggressive biological behavior of prostate cancer [34]. We observed that the β -catenin levels in the nucleus were higher when the levels of AR increased. Also Verras suggested a possible mechanism of cross talk between β -catenin and androgen signaling pathways [23, 35]. It seems that AR liganded by DHT has a more potent impact on the transport of β -catenin to the nucleus than AR liganded by testosterone. The subsequent observations indicated that DHT significantly influences the migration and MMPs activity in LNCaP cell line. Similar effects, namely enhanced cell migration, were observed after exposure of LNCaP T β RII cells to DHT [36].

Silencing of AR

We determined that treatment of LNCaP prostate cells with siRNA specific for AR significantly reduced: proliferation, expression of cell cycle proteins cyclin-D1, cyclin G1, c-Myc, cell migration and activity of MMPs. Furthermore, we observed that LNCaP cells transfected with siRNA specific for AR showed almost completely reduction of their migration ability. In contrast, similar transfection of siRNA specific for AR only reduced 15% of the ability of DU145 cells to migrate through Matrigel-coated Boyden chambers, while there were no changes in case of PC-3 cells. On the other hand, increased expression of E-cadherin, cell cycle inhibitors p21^{Cip-1} and p27^{Kip-1} were observed. Silencing of AR inhibited the nuclear translocation of β -catenin only in the AS LNCaP cell line. The inhibition of β -catenin nuclear translocation persisted for 72 hrs. A common feature shared by genes including: MMP-2, c-Myc, cyclin D1, IGFBP6, IGF-1R, IGF2 and PI3K [37] is that



B. Cell invasion assay through Matrigel coated Boyden chamber. Gelatinolytic activities of MMP2 and MMP-9 in prostate cells appears as clear zone in the gel

Factor \ Cell line	LNCaP		PC-3		DU145	
Medium (control)	~2-5		26-34		40-60	
siAR	72 hrs	120 hrs	72 hrs	120 hrs	72 hrs	120 hrs
	not detected	18-22	26-35	27-38	38-42	40-45

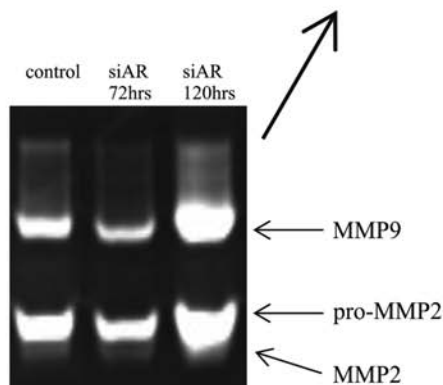
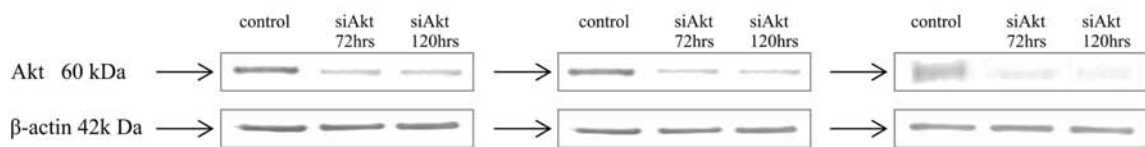
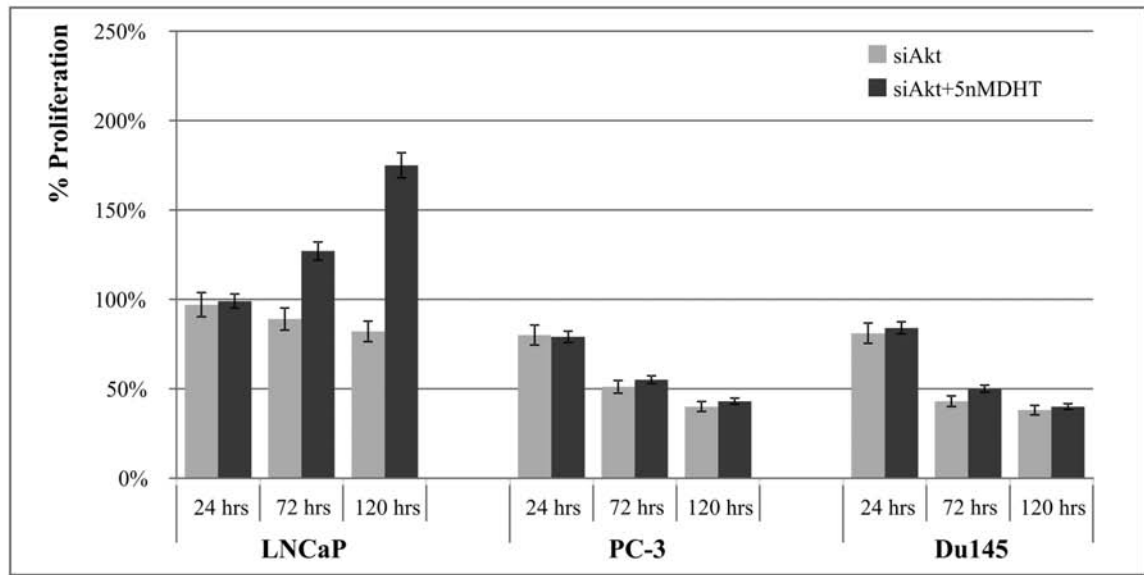


Fig. (4). The effects of prolonged AR silencing in LNCaP, PC-3 and DU145 prostate cancer cells on gene expression and migration. Panel A). β -catenin and β -catenin(pSer552) expression and their cellular localization. Also the levels total Akt and pAkt in the same lysates are presented. Panel B). migration through Matrigel coated Boyden chamber and activities of MMP-2 and MMP-9 (LNCaP cell line). Prostate cells were transfected with control siRNAs or three different 21 bp double-stranded siRNA targeting the AR. 72h and 120h after transfection, protein expression was analyzed by Western blot. Total protein loading was determined by probing the membranes for β -actin. Representative results of at least four independent experiments are presented.

A. PROLIFERATION



B. AR, Akt, β -catenin expression

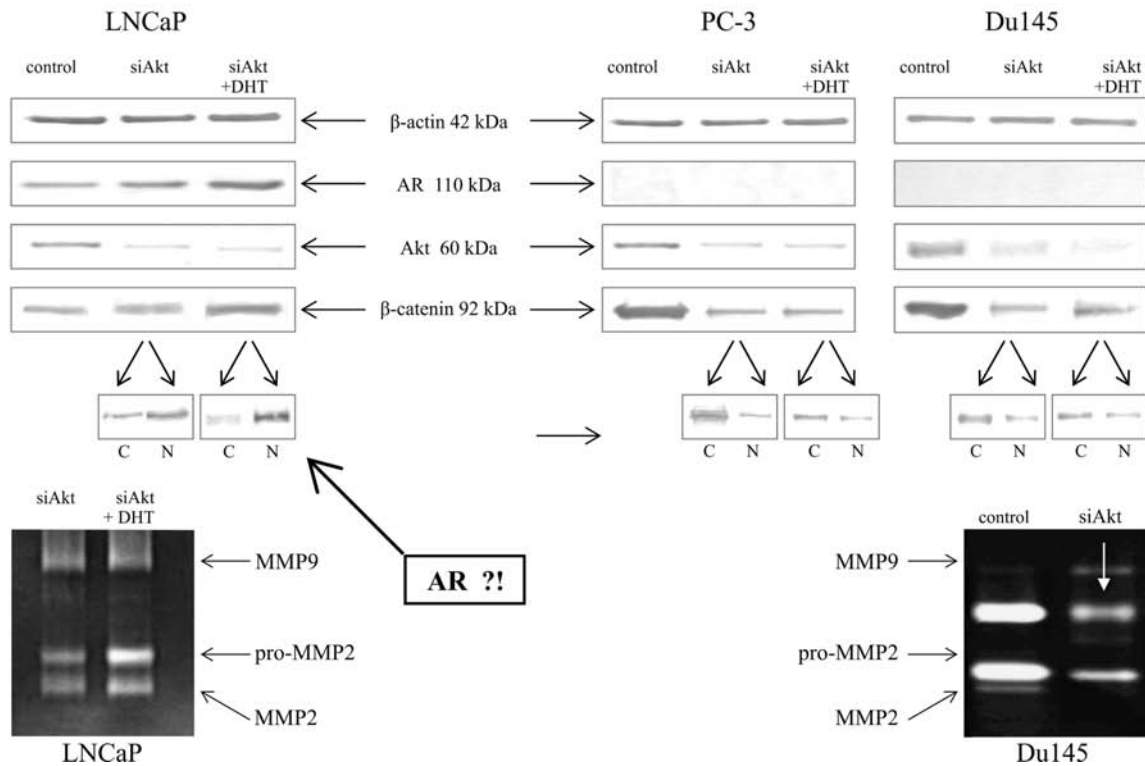
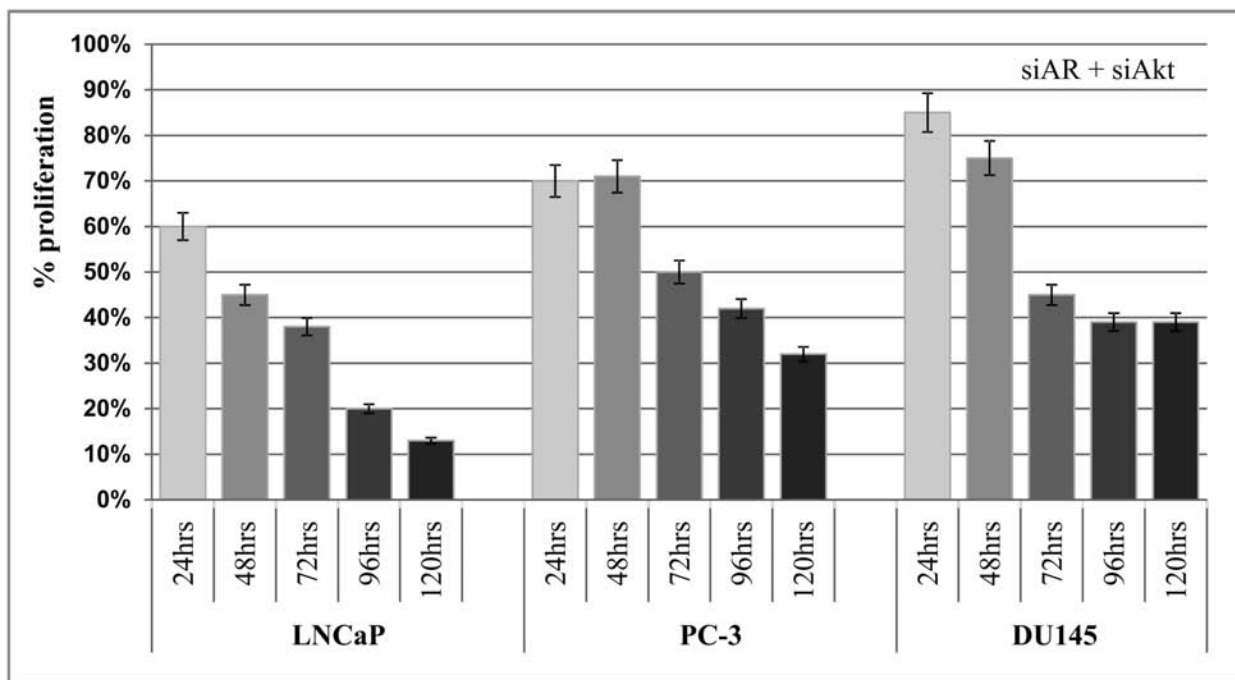


Fig. (5). Effects of Akt silencing in LNCaP, PC-3 and DU145 prostate cancer cells. Panel A). proliferation. Panel B). AR and nuclear β -catenin expression. Prostate cells were transfected with control siRNAs or three different 21 bp double-stranded siRNAs targeting the Akt. 72h after transfection, protein expression was analyzed by western blotting. Total protein loading was determined by probing the membranes for β -actin. Representative results of at least four independent experiments are presented.

A. Proliferation



B. Western blot Analysis

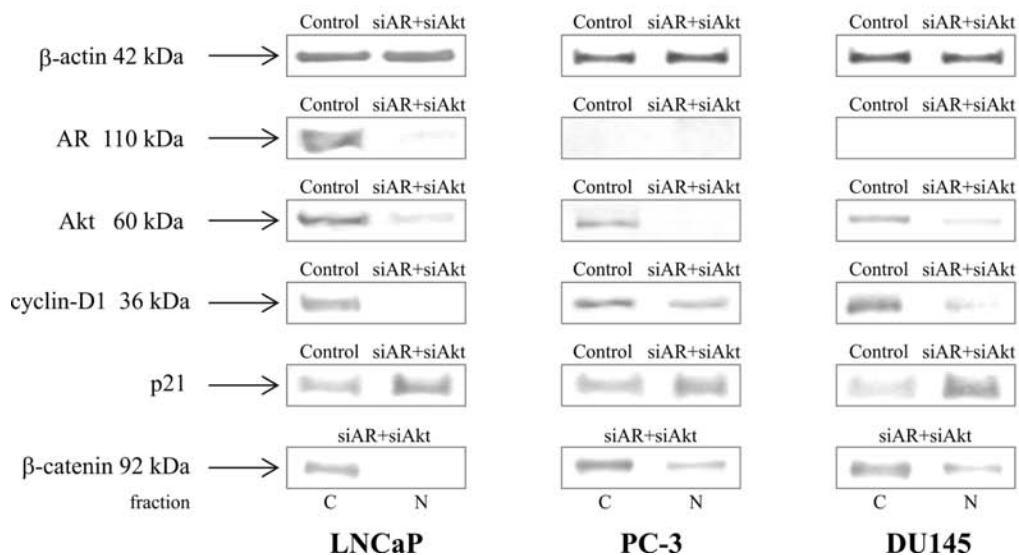
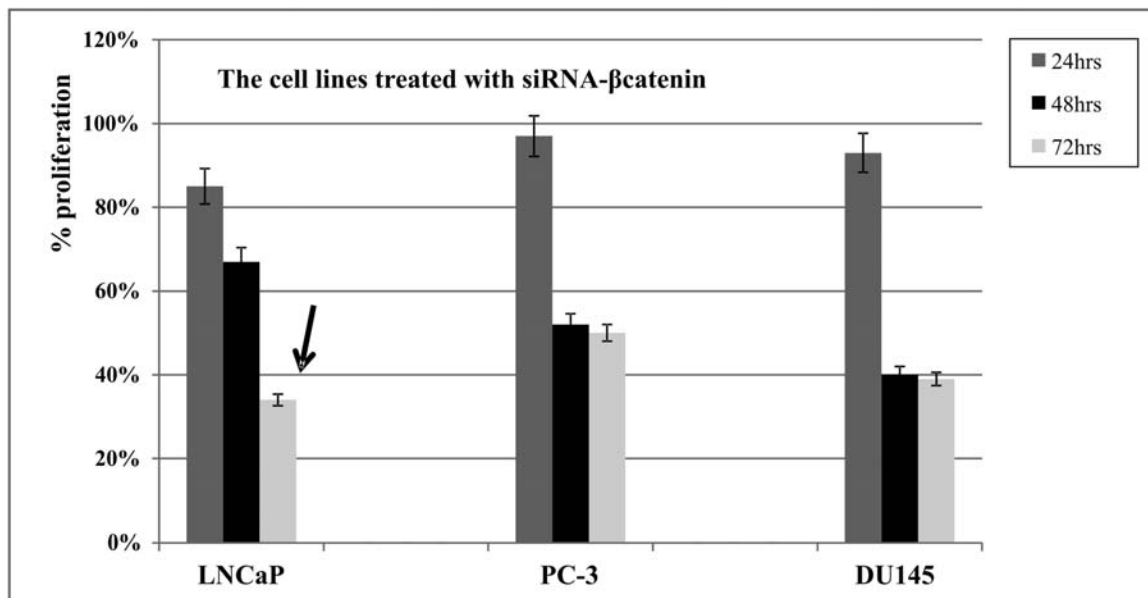


Fig. (6). Effects of co-targeting AR and Akt in LNCaP, PC-3 and DU145 prostate cancer cell lines. Panel A). Proliferation after 24-120hrs of treatment. Panel B). AR, Akt, cyclin D1, p21^{Cip-1} and β -catenin expression. Prostate cells were transfected with control siRNAs or three different 21 bp double-stranded siRNAs targeting the AR and Akt. 72h and 120h after transfection, protein expression was analyzed by western blotting. Total protein loading was determined by probing the membranes for β -actin. Representative results of at least four independent experiments are presented.

they are directly or indirectly associated with the β -catenin-signaling pathway [35]. Activation of the β -catenin pathway has been observed in prostate cancer patients. A recent study showed that 32% of patients with advanced disease carried mutations in the β -catenin gene [38]. Oncogenic activation of the β -catenin-signaling pathway has been reported to result in the abnormal accumulation of β -catenin. The translocation of β -catenin-TCF-4 complex to the nucleus leads to transcriptional activation of target

genes, such as c-Myc, MMP-2 and cyclin D1 [39, 40]. To determine the effects of AR silencing on proliferation, Akt activation and the expression of β -catenin-regulated genes in prostate cancer cells that are normally AS, we silenced AR in LNCaP cells for periods longer than 72hrs. To our surprise, we observed increased proliferation, increased cyclin D1, and c-Myc expression, increased MMP activity, elevation of p-Akt levels as well as p- β -catenin (pSer 552). The higher expression of p-Akt and p- β -catenin may

A. Proliferation



B. Western blot analysis

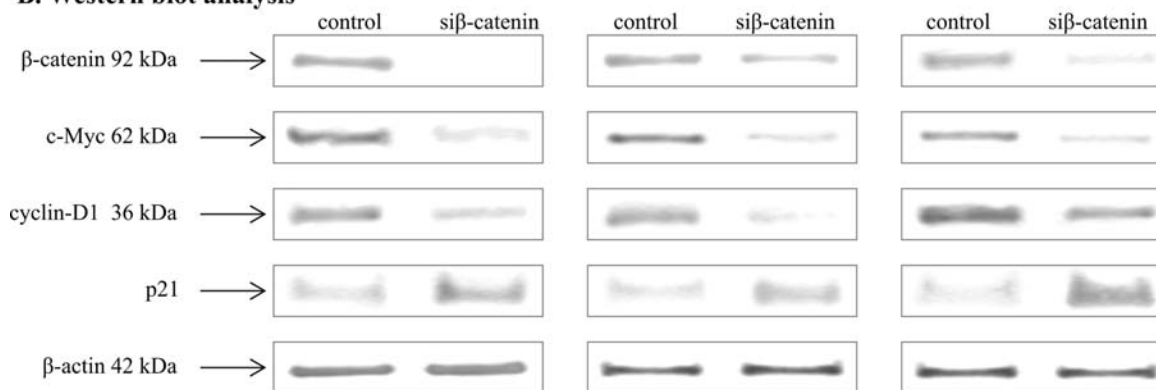


Fig. (7). The effects of β -catenin silencing in LNCaP, PC-3 and DU145 prostate cancer cell lines. Panel **A**). Proliferation, Panel **B**). Protein expression - β -catenin, c-Myc, cyclin D1, p21^{Cip-1}. Prostate cells were transfected with control siRNAs or three different 21 bp double-stranded siRNAs targeting β -catenin. 72h and 120h after transfection, protein expression was analyzed by western blotting. Total protein loading was determined by probing the membranes for β -actin. Representative results of at least five independent experiments are presented.

suggest the participation of Akt- β -catenin in the regulation of progression of prostate cancer in the case of acquired AI, which seems to be confirmed by their increased migration.

Akt Knock-Down

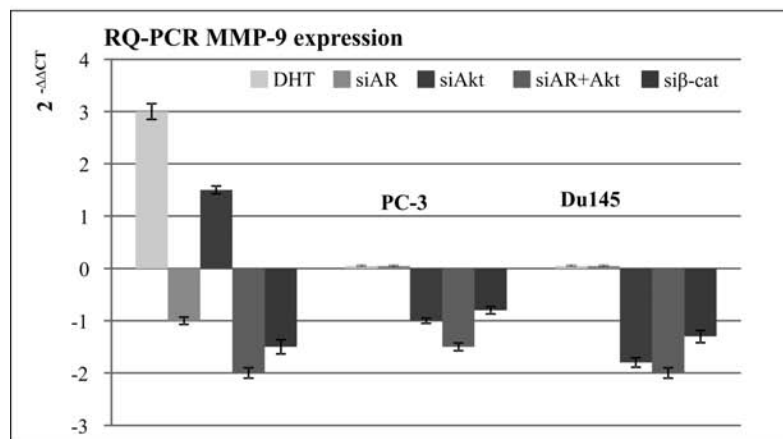
The effects of Akt knock-down on these biological processes were observed in AI PC-3 and DU145 cells and not in AS LNCaP cells. siRNA specific for Akt caused over 50% inhibition of proliferation in AI cells, but only 10-20% in AS cell line. Silencing of Akt gene caused inhibition both of Akt and β -catenin expression in AI cell lines PC-3 and DU145, without a significant change in β -catenin expression in AS LNCaP cell line. The exact mechanism by which the phosphorylation of β -catenin at Ser552 positively regulates the expression of its target genes remains unclear. One possibility exists that phosphorylation of Ser552 may modulate the translocation of β -catenin through nuclear pores, while a second possibility is that phosphorylation by Akt occurs within the nucleus and affects function rather than localization [41]. Further studies are

needed to determine whether the phosphorylation of β -catenin at Ser552 exerts the same effect as stabilized β -catenin on binding to the Tcf4/Lef1 complex and activating the transcription of Wnt target genes.

Function of β -Catenin in Cancer Progression

Up-regulation of β -catenin is a common feature of tumorigenesis that can be affected through mutations in β -catenin genes or other genes functioning in this pathway. Such genetic defects that result in up-regulation and stabilization of β -catenin play an important role in melanoma progression [42]. On the contrary, Fang *et al.* [43] showed that β -catenin accumulation in the nucleus is not sufficient for β -catenin/TCF transcriptional activity. They observed that cells stably transfected with β -catenin S552A had reduced transcriptional activity that has been closely related to tumor cell invasiveness. Daugherty and Gottardi [43] and Fang *et al.* [44] reported that phosphorylation of β -catenin at C-terminal residues Ser 552 by Akt was necessary for the promotion of β -catenin-regulated tran-

A. Quantitative PCR analysis of matrix metalloproteinase MMP-9 gene



B. Cell invasion assay through Matrigel coated Boyden chamber.

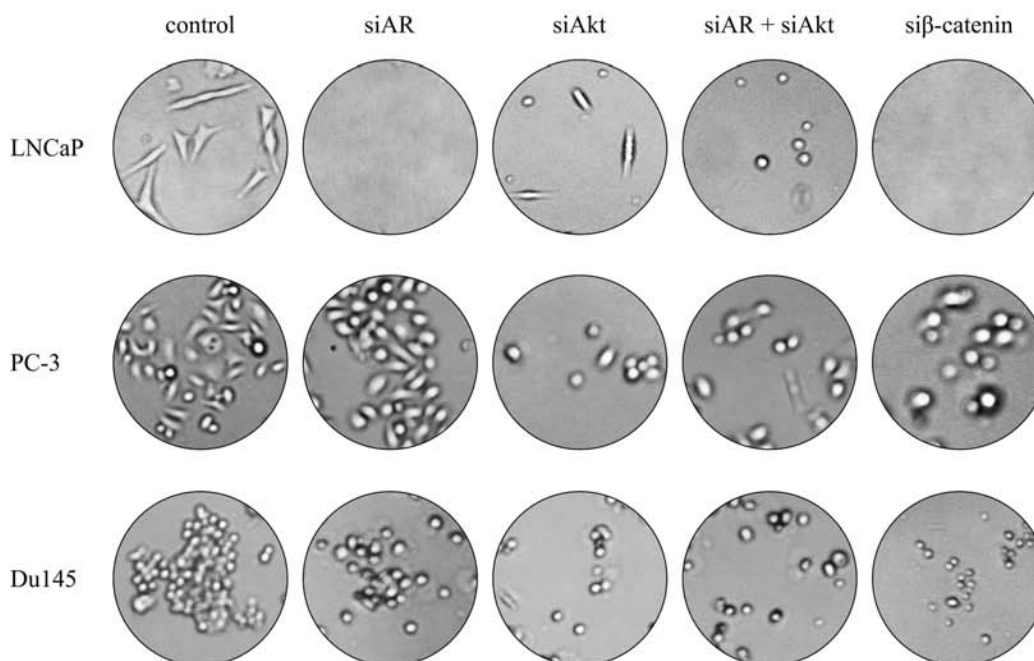


Fig. (8). Invasive potential of LNCaP, PC-3 and DU145 prostate cancer cells treated with siRNAs specific for AR, Akt and β -catenin. A. quantitative real-time PCR analysis of MMP-9 expression. Gene expression is presented as fold increase in $2^{-\Delta\Delta CT}$. B. Cell photographs that illustrate differences in the number of migrating cells - cell invasion assay through Matrigel coated Boyden chamber. Data were obtained from four experiments.

scriptional activity. In addition, Miyabayashi *et al.* [45] suggested that the particular co-activators recruited to β -catenin may dictate which target genes are activated and that this differential recruitment can be regulated by phosphorylation. Gil *et al.* [46] also suggested the central role of phosphorylation in the regulation of β -catenin signaling, since phosphorylation of β -catenin at Ser 552 is completely abolished upon ILK silencing in melanoma cell lines, also in the nuclear fraction from 1205Lu metastatic cell lines. Caspi *et al.* [47] and Gardner *et al.* [48] discovered GSK-3 β -dependent and independent mechanisms that regulate β -catenin activity. Gardner *et al.* [48] suggested that the phospho-inhibition of GSK-3 β at Ser 9 is not necessary for β -catenin/TCF signaling. In addition, Caspi *et al.* [47] using colorectal cell lines that express a mutant form of β -catenin, which cannot be phosphorylated by GSK-3 β and ectopically expressed mutant β -catenin, demonstrated that nuclear GSK-3 β functioned in a mechanism that did not involve β -

catenin phosphorylation. Our results indicated that translocation of β -catenin to nucleus in AS cells was to a higher degree dependent on AR than Akt activity. In contrast, in AI cells, the translocation of β -catenin depended mainly on Akt activity. Cells supplemented with DHT and additionally treated with siRNA specific for Akt increased proliferation only in case of AS LNCaP line. These results implicate both genes, AR and Akt, in prostate cancer progression, as well as in the regulation of important targets like β -catenin. Simultaneous silencing of AR and Akt resulted in inhibition of both Akt and β -catenin expression more in AS than AI cells.

β -catenin Knockdown

β -catenin knockdown caused significant inhibition of proliferation in all studied prostate cell line in both time and dose-dependent fashion and was sufficient to down-regulate the expression of cyclin

D1, c-Myc and to increase p21^{Cip-1} in all cell lines, but the greatest effect was again observed in LNCaP cell line. Similarly, the decrease in cell migration was observed in all lines when either siRNA specific for AR+, siRNA specific for Akt or siRNA specific for β -catenin were used. Li Xin [49] using cell-culture models has generated alternative views about the potential crosstalk between Akt and AR signaling by using reporter assays that measure AR transcriptional activity. They demonstrated a direct synergy between Akt and AR signaling that can transform prostatic epithelium into AI, but AR-dependent, carcinoma. Somewhat different cell response seem to depend on the presence of AR in LNCaP cells that expressed AR, DU145 with membrane AR [50] and PC-3 with no receptor.

CONCLUSION

Replacement of AR function by Akt in β -catenin signaling may directly contribute to cancer progression in the case of the loss of androgen sensitivity/dependence. Using the inducible system of Akt activation, we demonstrated that active Akt plays a functional role in AI progression of prostate cancer.

Akt signal transduction seems to be linked to AR modulation of basic cellular properties through as yet not fully understood mechanism(s). Our results may provide a novel target of nuclear receptor modulators and indicate their potential as therapeutic targets in the treatment of prostate cancer.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by MNiSzW grants: K/ZDS/001003 and K/PBW/ 000561 - UJCM Krakow, Poland. The authors' responsibilities were as follows; Joanna Dulińska-Litewka- conception and design of the study, provided essential reagents, materials, performed statistical analyses; Joanna Dulińska-Litewka, Piotr Laidler - data analysis and interpretation, Joanna Dulińska-Litewka, Piotr Laidler and James A. McCubrey - critical revision of the manuscript for content and approval of the final version of the manuscript. We are grateful to Aleksy Pawluczuk for preparing the final versions of the figures.

ABBREVIATIONS

AS	= Androgen-Sensitive
AI	= Androgen-Insensitive
AR	= Androgen Receptor
BPH-K	= Primary Benign Prostate Hyperplasia Cells
CA-K	= Primary Prostate Cancer Cells
DHT	= Dihydrotestosterone
MMPs	= Metalloproteinases
PPAR γ	= Peroxisome Proliferator-Activated Receptor Gamma
PSA	= Prostate-Specific Antigen
SF	= Steroid Free
T	= Testosterone

REFERENCES

- American Cancer Society. Atlanta, Georgia, Cancer Facts and Figures <http://www.cancer.org> (2010).
- Balk, S.P. Androgen receptor as a target in androgen-independent prostate cancer. *Urology*, **2002**; 60(3 suppl 1), 132-138.
- Culig, Z.; Georg, B. Androgen axis in prostate cancer. *JCB Journal of Cellular Biochemistry*, **2006**, 99(2), 373-381.
- Isaacs, J.T. Role of androgens in prostatic cancer. *Vitam. Horm. (N.Y.)*, **1994**, 49, 433-502.
- Shen, M.M.; Abate-Shen, C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & Development*, **2010**, 24, 1967-2000.
- Garnick, M. B. Prostate cancer : screening, diagnosis, and management. *Ann. Intern. Med.*, **1993**, 118, 804-818.
- Culig, Z.; Hobisch, A.; Hittmair, A.; Peterziel, H.; Cato, A.C.; Bartsch, G.; Klocker, H. Expression, structure, and function of androgen receptor in advanced prostatic carcinoma. *Prostate*, **1998**, 35, 63-70.
- van der Kwast, T. H.; Schalken, J.; Ruizeveld de Winter, J. A.; van Vroonhoven, C. C.; Mulder, E.; Boersma, W.; Trapman, J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int. J. Cancer*, **1991**, 48, 189-193.
- Laidler, P.; Dulińska, J.; Mroziki, S. Does the inhibition of c-myc expression mediate the anti-tumor activity of PPAR'S ligands in prostate cancer cell lines. *Arch. Biochem. Biophys.*, **2007**, 462, 1-12.
- Saxena, P.; Trerotola, M.; Wang, T.; Li, J.; Sayeed, A.; Vanoudenhove, J.; Adams, D.S.; Fitzgerald, T.; Altieri, D.C.; Languino, L.R. PSA regulates androgen receptor expression in prostate cancer cells. *Prostate*, **2012**, 72(7), 769-76.
- Ming, Z.; Zhu, C.; Sun, J.L.; Weis, W.I.; Sun, Z. The β -Catenin Binding Protein ICAT Modulates Androgen Receptor Activity Molecular. *Endocrinology*, **2011**, 25, 1677-1688.
- Alexander, N.R.; Tran, N.L.; Rekapally, H.; Summers, C.E.; Glackin, C.; Heimark, R.L. N-cadherin Gene Expression in Prostate Carcinoma Is Modulated by Integrin-Dependent Nuclear Translocation of Twist1. *Cancer Res.*, **2006**; 66, 3365-3369.
- Jennbacken, K.; Tešan, T.; Wang, W.; Gustavsson, H.; Damber, J.; Welén, K. N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer. *Endocrine-Related Cancer*, **2010**, 17 469-479.
- Vasioukhin, V.; Fuchs, E. Actin dynamics and cell-cell adhesion in epithelia. *Curr. Opin. Cell Biol.*, **2001**, 13, 76-84
- Nelson, W.J.; Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*, **2004**, 303, 1483-1487.
- Kochuparambil, S.T.; Al-Husein, B.; Goc, A.; Soliman, S.; Somanath, P. R. Anticancer Efficacy of Simvastatin on Prostate Cancer Cells and Tumor Xenografts Is Associated with Inhibition of Akt and Reduced Prostate-Specific Antigen Expression. *JPET*. **2011**, 336, 496-505.
- Dulińska, J.; Laidler, P.; Łabędź, M. Comparative analysis of prostate acid phosphatase and prostate specific-antigen mRNA levels in hyperplastic prostate stimulated with steroid hormones and growth factors. *Acta Biochim. Polon.*, **2002**, 49, 357-368.
- Dulińska, J.; Gil, D.; Zagajewski, J.; Hartwich, J.; Bodzioch, M.; Dembinska-Kiec, A.; Langmann, T.; Schmitz, G.; Laidler, P. Different effect of beta carotene on proliferation of prostate cancer cells. *BBA - Molecular Basis of Disease*, **2005**, 1740, 189-201.
- Jach, R.; Dulińska-Litewka J.; Laidler P., Szczudrawa A., Kopera K., Szczudlik L., Pawlik M., Zajac K., Mak M., Basta A. Expression of VEGF, VEGF-C and VEGFR-2 in insitu and invasive SCC of cervix. *Front. Biosci.*, **2010**, 2, 411-423.
- Sacha, T.; Zawada, M.; Dulinska-Litewka, J.; Lach, Z.; Szostek, M.; Bodzioch, M.; Laidler, P.; Dembińska-Kiec, A.; Florek, I.; Czekalska, S.; Skotnicki, A.B. Beta-carotene regulates the Expression of proapoptotic BAX and CAPN2 in HL-60, U-937 and TF-1 - human acute myeloid leukemia cell lines; microarray, RQ-PCR and Western Blot analysis. *Przegląd Lekarski*, **2011**, 68(4), 258-262.
- Macabeo-Ong, M.; Ginzinger, D.G.; Dekker, N.; McMillan, A.; Regezi, J.A.; Wong, D.T.W.; Jordan, R.C.K. Effect of Duration of Fixation on Quantitative Reverse Transcription Polymerase Chain Reaction Analyses. *Mod Pathol.*, **2001**, 15, 979- 987.
- Dudzik, P.; Dulinska-Litewka, J.; Wyszko, E.; Jedrychowska, P.; Barciszewski, J.; Laidler, P. Effects of kinetin riboside on proliferation and proapoptotic activities in human normal and cancer cell lines. *Journal of Cellular Biochemistry*, **2011**, 112, 2115-2124.
- Verras, M.; Sun, Z. Beta-catenin is involved in insulin-like growth factor 1-mediated transactivation of the androgen receptor. *Mol Endocrinol.*, **2005**, 19(2), 391-398.
- Schröder, F.H. Progress in Understanding Androgen-Independent Prostate Cancer (AIPC): A Review of Potential Endocrine-Mediated Mechanisms. *European Urology*, **2008**, 1129-1137.
- Cunha, G.R.; Alarid, E.T.; Turner, T.; Donjacour, A.A.; Boutin, E.L.; Foster, B.A. Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal- epithelial interactions, and growth factors. *J. Androl.*, **1992**, 13, 465-475.
- Attard, G.; Coope, C.S.; de Bono, J.S. Steroid hormone receptors in prostate cancer: A hard habit to break? *Cancer Cell*, **2009**, 16, 458-462.
- Bonkhoff, H.; Berges, R. From pathogenesis to prevention of castration resistant prostate cancer. *Prostate*, **2010**, 70, 100-112.
- Linja, M.J.; Savinainen, K.J.; Saramsaki, O.R.; Tammela, T.L.; Vessella, R.L.; Visakorpi, T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.*, **2001**; 61,3550-3555.

- [29] Karpf, A.R.; Bai, S.; James, S.R.; Mohler, J.L.; Wilson, E.M. Increased expression of androgen receptor coregulator *MAGE-11* in prostate cancer by DNA hypomethylation and cyclic AMP. *Mol Cancer Res.*, **2009**, *7*, 523–535.
- [30] Locke, J.A.; Guns, E.S.; Lubik, A.A.; Adomat, H.H.; Hendy, S.C.; Wood, C.A.; *Ettinger, S.L.*; *Gleave, M.E.*; *Nelson, C.C.* Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. *Cancer Res.*, **2008**, *68*, 6407–6415.
- [31] Lih, F.B.; Titus, M.A.; Mohler, J.L.; Tomer, K.B. Atmospheric pressure photoionization tandem mass spectrometry of androgens in prostate cancer. *Anal Chem.*, **2010**, *82*, 6000–6007.
- [32] Wang, G.; Sadar, M.D. Amino-terminus domain of the androgen receptor as a molecular target to prevent the hormonal progression of prostate cancer. *J Cell Biochem.*, **2006**, *98*, 36–53.
- [33] Mohler, J.L.; Titus, A.; Bai, S.; Kennerley, B.J.; Lih, F.B.; Tomer, K.B.; Wilson, E.M. Activation of the Androgen Receptor by Intratumoral Bioconversion of Androstenediol to Dihydrotestosterone in Prostate Cancer. *Cancer Res.*; **2011**, *71*(4), 1486–1496.
- [34] Shao-Yong, C.; Wulf, G.; Zhou, X.Z.; Rubin, M.a.; Lu, K.P.; Balk, S.P. Activation of β -Catenin Signaling in Prostate Cancer by Peptidyl-Prolyl Isomerase Pin1-Mediated Abrogation of the Androgen Receptor- β -Catenin Interaction. *Mol Cell Biol.*, **2006**, *26*(3), 929–939.
- [35] Verras, M.; Sum, Z. Roles and regulation of Wnt signaling and beta-catenin in prostate cancer. *Cancer Lett.*, **2006**, *237*, 22–32.
- [36] Meng-Lei, Z.; Kyprianou, N. Role of androgens and the androgen receptor in epithelial-mesenchymal transition and invasion of prostate cancer cells. *FASEB J.*, **2010**, *24*(3), 769–777.
- [37] Saleem, M.; Murtaza, I.; Tarapore, R.S.; Suh, Y.; Adhami, V.M.; Johnson, J.J.; Siddiqui, I.A.; Khan, N.; Asim, M.; Hafeez, B.B.; Shekhani, M.T.; Li, B.; Mukhtar, H. Lupeol inhibits proliferation of human prostate cancer cells by targeting β -catenin Signaling. *Carcinogenesis*, **2009**, *30*(5), 808–817.
- [38] Chesire, D.R.; Ewing, C.M.; Sauvageot, J.; Bova, G.S.; Isaacs, W.B. Detection and analysis of b-catenin mutations in prostate cancer. *Prostate*, **2000**, *45*, 323–334.
- [39] Yang, G.; Timme, T.L.; Frolov, A.; Wheeler, T.M.; Thompson, T.C. Combined c-Myc and caveolin-1 expression in human prostate carcinoma predicts prostate carcinoma progression. *Cancer*, **2005**, *103*, 1186–1194.
- [40] Wu, J.D.; Haugk, K.; Woodke, L.; Nelson, P.; Coleman, I.; Plymate, S.R. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *J. Cell. Biochem.*, **2006**, *99*, 392–401.
- [41] He, X.C.; Yin, T.; Grindley, J.C.; Tian, Q.; Sato, T.; Tao, W.A.; Dirisina, R.; Porter-Westpfahl, K.S.; Hembree, M.; Johnson, T.; Wiedemann, L.M.; Barrett, T.A.; Hood, L.; Wu, H.; Li, L. PTEN-deficient intestinal stem cells initiate intestinal polyposis. *Nat Genet.*, **2007**, *39*, 189–198.
- [42] Rubinfeld, B.; Robbins, P.; El-Gamil, M.; Albert, I.; Porfiri, E.; Polakis, P. Stabilization of β -catenin by genetic Defects in melanoma cell lines. *Science*, **1997**, *275*, 1790–1792.
- [43] Daugherty, R.L.; Gottardi, C.J. Phospho-regulation of β -catenin adhesion and signaling functions. *Physiology*, **2007**, *22*, 303–309.
- [44] Fang, D.; Hawke, D.; Zheng, Y.; Xia, Y.; Meisenhelder, J.; Nika, H.; Mills, G.B.; Kobayashi, R.; Hunter, T.; Lu, Z. Phosphorylation of β -catenin by AKT promotes β -catenin transcriptional Activity. *J Biol Chem.*, **2007**, *282*, 11221–11229.
- [45] Miyabayashi, T.; Teo, J.L.; Yamamoto, M.; McMillan, M.; Nguyen, C.; Kahn, M. Wnt/b-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. *Proc Natl Acad Sci USA*, **2007**, *104*, 5668–5673.
- [46] Gil, D.; Ciołczyk-Wierzbicka, D.; Dulińska-Litewka, J.; Żwawa, K.; McCubrey, J.A.; Laidler, P. The mechanism of contribution of integrin linked kinase (ILK) to epithelial mesenchymal transition (EMT). *Advances in Enzyme Regulation Advances in Enzyme Regulation*, **2011**, *51*, 195–207.
- [47] Caspi, M.; Zilberberg, A.; Eldar-Finkelman, H.; Rosin-Arbesfeld, R. Nuclear GSK-3 β inhibits the canonical Wnt signaling pathway in a β -catenin phosphorylation-independent manner. *Oncogene*, **2008**, *27*:3546–3555.
- [48] Gardner, S.; Maudsley, S.; Millar, R.P.; Pawson, A.J. Nuclear stabilization of β -catenin and inactivation of glycogen synthase kinase-3 β by gonadotropin-releasing hormone: targeting Wnt signaling in the pituitary gonadotrope. *Mol Endocrinology*, **2007**, *21*, 3028–3038.
- [49] Li, X.; Teitell, M.A.; Lawson, D.A.; Kwon, A.; Mellinshoff, I.K.; Witte, O.N. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. *PNAS*, **2006**, *103*(20), 7789–7794.
- [50] Kampa, M.; Kogia, C.; Theodoropoulos, P.A.; Anezinis, P.; Charalampopoulos, I.; Papakonstanti, E.A.; Stathopoulos, E.N.; Hatzoglou, A.; Stournaras, C.; Gravanis, A.; Castanas, E. Activation of membrane androgen receptors potentiates the antiproliferative effects of paclitaxel on human prostate cancer cells. *Mol Cancer Ther.*, **2006**, *5*, 1342–1351.