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Androgen Receptor Signaling Pathway in Prostate Cancer

ACADEMIC DISSERTATION
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CONTENTS

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I Waltering KK, Wallén MJ, Tammela TL, Vessella RL, Visakorpi T (2006): Mutation screening of the androgen receptor promoter and untranslated regions in prostate cancer. Prostate 66;15:1585-91.

II Waltering KK[1], Helenius MA[1], Sahu B, Manni V, Linja MJ, Jänne OA, Visakorpi T (2009): Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens. Cancer Res 69;20:8141-9.

III Waltering KK, Porkka KP, Jalava SE, Urbanucci A, Kohonen P, Latonen L, Kallioniemi O, Jenster G and Visakorpi T (2010): Androgen regulation of microRNAs in prostate cancer. Accepted to be published in Prostate.

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ABBREVIATIONS

ACPP	acid phosphatase, prostate; PAP
AF	activation function
AKT	v-akt murine thymoma viral oncogene homolog 1
APC	adenomatous polyposis coli
AR	androgen receptor
ARA	androgen receptor coactivator
ARE	AR responsive element
ARTIS	AR transcription initiation site
ATAD2	ATPase family, AAA domain containing 2, ANCCA
CAB	complete androgen blockade
cAMP	3'-5'-cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation
CDC20	cell division cycle 20 homolog
CDK1	cyclin-dependent kinase 1
CDK2	cyclin-dependent kinase 2
cDNA	complementary DNA
CP	3'-CCCUCCC poly(C)-binding protein
CRPC	castration resistant prostate cancer
DBD	DNA-binding domain
DHEA	dehydroepiandrosterone
DHPLC	denaturing high-performance liquid chromatography
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
EIF3S3	a subunit of translation factor eIF3
EP300	E1A binding protein p300, acetyltransferase p300
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ETS	avian erythroblastosis virus E26 homolog
ETV1	ets variant 1
EZH2	enhancer of zeste homolog 2
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOXA1	forkhead box A1
GTF2F	general transcription factor IIF, TFIIF
GTF2H3	general transcription factor IIH, TFIIH
GSTP1	glutathione S-transferase
HAT	histone acetylase
HDAC	histone deacetylase
ITGA6	integrin alpha 6
HSPA4	heat shock 70kDa protein 4
JUN	jun oncogene
KLK3	kallikrein-related peptidase 3
KXKK	Lys-x-Lys-Lys motif
LBD	ligand-binding domain
LOH	loss of heterozygosity
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone

MAK	male germ cell-associated kinase
MCM	minichromosome maintenance protein
miR-141	microRNA 141
miRNA	microRNA
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA1	nuclear receptor coactivator 1, SRC1
NCOR1	nuclear receptor co-repressor 1
NKX3-1	NK3 homeobox 1
NLS	nuclear localization signal
PAP	prostatic acid phosphatase
PC	prostate cancer
PCR	polymerase chain reaction
PIAS	protein inhibitor of activated STAT
PIN	prostatic intraepithelial neoplasia
POU2F1	POU class 2 homeobox 1, octamer-binding transcription factor 1, OCT1
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog
RAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog, HRAS
RNA	ribonucleic acid
qRT-PCR	quantitative real-time PCR
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SP1	Sp1 transcription factor
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
TAU	transcription activation unit
TGFB1I1	transforming growth factor beta 1 induced transcript 1, androgen receptor coactivator ARA55
TMPRSS2	transmembrane protease, serine 2
TP53	tumor protein 53, p53
TSS	transcription start site
TURP	transurethral resection of prostate
UBE2I	ubiquitin-conjugating enzyme E2I
UTR	untranslated region

ABSTRACT

The progression and growth of prostate cancer (PC) has been shown to be dependent on androgens. The standard treatment of advanced PC is androgen deprivation, which reduces the levels of testosterone in the body. Initially, the treatment inhibits tumor growth effectively, but it ultimately fails and leads to the emergence of castration-resistant PC (CRPC). Presently, no truly effective treatment for CRPC has been discovered. The androgen receptor gene (*AR*) is known to be altered in several ways during PC progression. Thus, *AR* is believed to be the one of the major contributors to the emergence of CRPC.

The objective of this thesis was to identify genetic alterations, other than gene amplification, which result in the overexpression of *AR* during the progression of PC. Furthermore, we investigated the effects of *AR* overexpression on the growth of PC cells and on the transcription of protein-coding and microRNA (miRNA) genes using cell line and xenograft models as well as clinical patient samples.

No novel genetic alterations were identified that could explain *AR* overexpression. Overexpression of *AR* was found to enhance the growth of PC cells and the expression of AR target genes under low androgen conditions. Overexpression of *AR* increased significantly the number of upregulated genes. Additionally, several novel AR target genes associated with regulation of the cell cycle and mitosis were identified. Thus, one effect of the overexpression of *AR* seems to be the enhancement of the cell cycle under low androgen conditions. Inhibition of these target genes significantly decreased the growth of *AR* overexpressing cells. Novel androgen-regulated and differentially expressed miRNAs, such as miR-18a, miR-141, miR-375 and miR-221, were also identified in the study. The exogenous overexpression of miR-141 was found to enhance the androgen-dependent growth of PC cells.

At present, androgen deprivation is the standard treatment for advanced PC, and it is known that the overexpression of *AR* is a common event in CRPC. Thus, this thesis provides important information, especially regarding AR target genes in PC cells expressing high levels of *AR*.

YHTEENVETO

Eturauhassyövän etenemisen ja kasvun on todettu olevan riippuvainen androgeenien eli miessukupuolihormonien toiminnasta. Edenneen eturauhasen syövän standardihoito, kastraatio, vähentää elimistön vapaan testosteronin määrää ja estääkin aluksi tehokkaasti syövän etenemisen. Hoitoa jatkettaessa vasteen tiedetään kuitenkin häviävän, ja eturauhassyöpä muuttuu kastraatioresistentiksi. Uusiutuneeseen kastraatioresistentiin eturauhassyöpään ei ole löydetty hyvää hoitomuotoa. Androgeenireseptorigeenin (*AR*) tiedetään muuttuvan eri tavoin eturauhassyövän edetessä ja *AR*:n oletetaankin olevan yksi tärkeimmistä tekijöistä kastraatioresistentin syövän kehittymisessä.

Tämän väitöskirjatutkimuksen tavoitteena oli määrittää muita kuin geenimonistumisesta aiheutuvia geneettisiä AR:n yli-ilmentymisen selittäviä muutoksia. Lisäksi tutkittiin eturauhassyövän etenemisen aikana yleisesti todettavan AR:n yli-ilmentymisen vaikutusta solukasvuun ja proteiineja koodaavien, sekä microRNA (miRNA) geenien ilmentymiseen hyödyntäen solulinja- ja kudossiirremalleja, sekä kliinisiä syöpänäytteitä.

Tutkimuksessa ei löydetty uusia *AR*:n yli-ilmentymistä selittäviä yleisiä vaikutusmekanismeja. Yli-ilmentyneen *AR*:n todettiin herkistävän eturauhassyöpäsolut matalille androgeeni-pitoisuuksille lisäten syöpäsolujen kasvua sekä kohdegeenien ilmentymistä. Yli-ilmentyneen AR:n todettiin lisäävän merkittävästi ylössäädeltyjen geenien lukumäärää. Tutkimuksessa tunnistettiin useita aikaisemmin julkaisemattomia suoria AR:n kohdegeenejä joiden tiedetään toimivan solusykliä ja mitoosia edistävinä tekijöinä. Kohonneen AR:n ilmentymisen yksi vaikutusmekanismi näyttääkin liittyvän solusyklin lisääntymiseen matalissa androgeenipitoisuuksissa. Näiden kohdegeenien toiminnan estäminen vaikutti lisäksi erityisesti *AR*:ia yli-ilmentävien solujen kasvun hidastumiseen. Työssä löydettiin uusia androgeenisäädeltyjä ja eturauhassyövässä ilmenemiseltään muuttuneita miRNA:ta, kuten miR-18a, miR-141, miR-375 ja miR-221. Keinotekoisesti yli-ilmennetyn miR-141:n todettiin lisäävän eturauhassyöpä-solujen androgeeniriippuvaista kasvua.

Koska androgeenien vaikutuksen estäminen on edenneen eturauhassyövän vallitseva hoitomuoto, jonka aikana tiedetään *AR*:n yli-ilmentyvän, antoi tämä väitöskirja merkittävää tutkimustietoa erityisesti AR:n kohdegeeneistä korkeasti *AR*:ia ilmentävissä eturauhassyöpäsoluissa.

1 INTRODUCTION

Prostate cancer (PC) is the most common malignancy in males and is the second highest cause of cancer-related mortality in developed countries (Curado *et al.* 2007, Coleman *et al.* 2008). The mean age at diagnosis is approximately 71 years. The rates of PC incidence have steadily increased in many developed countries over the last few decades. The age-adjusted incidence was 103.9 per 100,000 males in Finland during the period of 2002-2006. However, this trend seems to be reversed itself; in 2007 and 2008, the age-adjusted incidence was 85.6 and 83.1, respectively. In 2008, over 4200 new PC diagnoses were made in Finland, accounting for just over 30% of all new male cancers, with more than 800 men dying from the disease that year. In 2009, the prevalence of prostate cancer rose to over 35,000 in Finland. (Finnish Cancer Registry 2010, www.syoparekisteri.fi).

PC is a complex, multifactorial disease. Despite its high prevalence, the molecular mechanisms that induce PC progression are poorly understood. Tumorigenesis is generally shown to be driven by stepwise processes that involve the genetic alteration of critical genes, resulting in altered expression and function (Vogelstein and Kinzler 1993, Hanahan and Weinberg 2000). As the majority of prostate cancers arise from androgen-dependent secretory epithelial cells, androgen receptor (AR) signaling is one common element that affects both the development and progression of PC. The standard treatment for advanced PC is androgen deprivation, which has been used for over half a century (Huggins and Hodges 1941). Under normal conditions, local androgen metabolism maintains a balance between the proliferation and apoptotic cell death of prostatic epithelial cells. In PC, this balance is disturbed to drive proliferation and survival of the cancerous cells (Isaacs *et al.* 1994). The AR has also been shown to be altered in several ways during the progression of hormone-independent, castration-resistant PC (CRPC) (Visakorpi *et al.* 1995, Taplin *et al.* 1995, Dehm *et al.* 2008).

The first aim of this thesis was to identify novel genetic alterations that induce the increased expression of *AR* using PC cell lines, xenograft models and clinical PC samples. The other aim of this thesis was to investigate *AR* overexpression under conditions of varying androgen levels and the effect on the growth of PC cells using an *in vitro AR* overexpression model. An additional aim was to identify novel downstream candidate protein coding genes and microRNA genes that are involved in the emergence of CRPC.

2 REVIEW OF THE LITERATURE

2.1 Normal anatomy and function of the prostate gland

The prostate gland is a walnut-sized exocrine gland that belongs to the male reproductive system. It is located just below the bladder and surrounds the urethra. The function of the prostate is to store and secrete a slightly alkaline seminal fluid that usually constitutes 25-30% of the total volume of semen along with spermatozoa. Seminal fluid is generally composed of simple sugars, zinc and the proteolytic enzymes, prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA). The function of the seminal fluid is to protect the genetic material (DNA) of the spermatozoa by aiding in sperm motility and promoting survival within the acidic vaginal tract. Anatomically, the prostate can be divided into three different zones: the peripheral zone, the central zone and the transition zone. Each glandular zone has a specific architecture with varying composition of stromal and epithelial (both basal and differentiated secretory luminal epithelial) cells (reviewed by Cunha *et al.* 1987, Taplin and Ho 2001).

2.2 Androgen signaling in normal prostate

Androgens belong to the group of male steroid hormones that are produced by Leydig cells in the testicles. The hypothalamus initially regulates androgen production. It releases luteinizing hormone releasing hormone (LHRH) in short pulses when levels of blood testosterone are decreased. The activation of the LHRH receptors of the anterior pituitary gland leads to increased synthesis and release of luteinizing hormone (LH) into the circulation, which induces steroidogenesis in Leydig cells. Some androgens, like dehydroepiandrosterone (DHEA), are also produced in small amounts by the adrenal cortex. In the prostate, testosterone is transformed into a more active form, dihydrotestosterone (DHT), by 5-alpha-reductase enzymes. Testosterone (T) can be further metabolized into several different conjugates, such as androstenediol, which stimulates the hypothalamus, or androsterone, which is secreted into the urine (reviewed by Cunha *et al.* 1987, Taplin and Ho 2001).

To function correctly, the prostate gland requires androgens, especially testosterone and DHT. Prostate epithelial cells are androgen-dependent. The normal differentiation of prostatic basal epithelial cells into secretory luminal epithelial cells is androgen-regulated. Differentiation is the direct effect of androgens on prostatic epithelial cells. However, androgens also stimulate the proliferation of the epithelial cells via the paracrinal support of the stromal cells. These cells secrete andromedins, which are crucial for the survival of the epithelial cells. This survival support is mediated by androgens and AR signaling. Without androgens, *e.g.*, after castration, the stromal support of the epithelia is blocked, causing the rapid apoptosis of prostatic epithelial cells. The function of androgens is mediated by the androgen receptor (AR), which is a ligand-inducible transcription factor (reviewed by Leenders and Schalken 2003, Isaacs and Isaacs 2004, Vander Griend *et al.* 2010).

The androgen receptor (AR) modulates the expression of genes involved in proliferation and differentiation. The AR belongs to the steroid receptor family of the nuclear receptor superfamily. This family consists of the glucocorticoid, estrogene, progesterone and mineralocorticoid receptors. Androgens and AR are important not only in the prostate but also for the development and maintenance of the male sexual phenotype during embryogenesis and for male sexual maturation at puberty. In adulthood, androgens remain essential for the maintenance of reproductive function (prostate gland) and sexual drive. They are also important in a wide variety of non-reproductive tissues, including the skin, bone, muscle, and adipose tissues (Lubahn *et al.* 1988, Jenster *et al.* 1991, reviewed by Gelmann 2002, Heinlein and Chang 2002, Lee and Chang 2003).

2.2.1 Genetic structure of *AR*

Genes are composed of DNA located in specific and highly regulated regions. Regions that encode proteins are called exons, and the regions between exons are called introns. Every gene also contains untranslated expression regulation sites at the 5' and 3' ends of the gene called the 5' and 3' untranslated regions (UTRs), respectively. The transcription of the gene starts when certain transcription factors (TFs) bind to the open region of the promoter sites at the 5' end of a gene. Gene promotion sites are often highly conserved between species and consist of DNA sequences such as the TATA box (5'-TATAAA-3' sequence) and are often guanine and cytosine (GC) rich.

The human *AR* gene, located in the chromosome Xq11–12 region, is over 90 kb long and contains eight exons (Chang *et al.* 1988, Lubahn *et al.* 1988 and Trapman *et al.* 1988). The genetic structure of AR is illustrated in Figure 1, which has been adapted from Gelmann (2002). The first exon is approximately 1580 bp long and encodes the main portion of the activation function-1 (AF-1) domain (AR protein function will be discussed in the next paragraph). Exon 1 contains two highly polymorphic repeat regions (CAG and GGN) (Chamberlain *et al.* 1994, Choong *et al.* 1998). The length of the CAG (glutamine triplet) varies from 14 to 35 repeats, with an average of 21 ± 2 repeats (Irvine *et al.* 1995). The C-terminal polyglycine (GGN) repeat has an average of 16 repeats and shows a lesser degree of polymorphism than the CAG repeat (Macke *et al.* 1993, Irvine *et al.* 1995). Two transcription activation units (TAUs) have been identified in the N-terminal domain. The first (TAU-1) is responsible for AR transactivation capability (Jenster *et al.* 1995, Callewaert *et al.* 2006).

The second domain, which is a DNA-binding domain (DBD), is encoded by exon 2 and partially by exon 3. This domain contains a DNA-binding structure formed by two zinc fingers. A hinge region is located at the end of exon 3 and the beginning of exon 4, which contains the major nuclear localization signal (NLS). The hinge region is needed for intraprotein interaction between AF-1 and AF-2 domains (AR protein function is discussed in the next paragraph). The C-terminal domain is encoded by exons 4-8 and forms a ligand-binding domain (LBD), which includes the transcription activation function domains (AF-2) (Simental *et al.* 1991, Gelmann 2002).

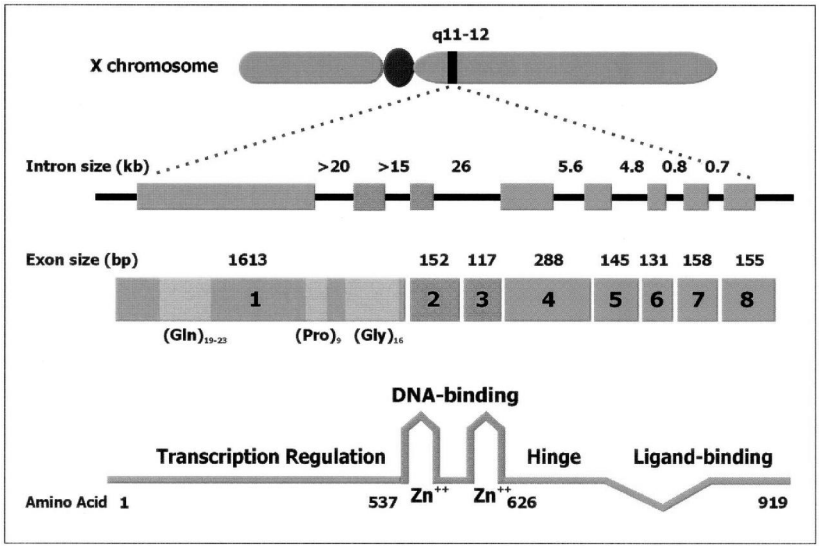

Figure 1. Genomic organization of the AR gene. *The genome spans more than 90 kb, which includes the exon organization shown in the second panel. The location of repeat regions in the first exon, which codes for the N-terminal domain, is shown in the third panel. The diagram of the protein structure demonstrates how the exon organization translates into discrete functional regions of the receptor. (Gelmann EP: J Clin Oncol. Molecular Biology of Androgen Receptor. 20. (13), 2002: 3001-15. Reprinted with permission from © 2002 American Society of Clinical Oncology. All rights reserved.)*

The *AR* gene has two transcription initiation sites (ARTIS I and ARTIS II) in a 13-base pair region (Faber et al 1991 and 1993). The core promoter (-74 to + 87) of *AR* lacks both a TATA and CAAT box but has an SP1-binding site (-52-57) and a palindromic homopurine (-129-70) repeat. ARTIS I and II have been demonstrated to function as independent overlapping pathways, in which SP1 binding induces the transcription of *AR* through ARTIS II but has no influence on ARTIS I (Faber *et al.* 1991 and 1993). However, the regulation and the roles of these two overlapping pathways are unclear. Several putative positively regulating *cis*-acting elements can be found upstream of *AR* (Mizokami *et al.* 1994a, Takane *et al.* 1996). Functional studies of the promoter have shown that the palindromic homopurine repeat is important for AR transcription and may facilitate transcription initiation from the GC-rich region (Chen *et al.* 1997, Takane *et al.* 1996). Mizokami *et al.* (1994) identified a cAMP-responsive element 518 bp upstream of the core promoter. They also found a putative suppression region from -540 to -150 bp from the core promoter and another *cis*-acting region(s) at -1390 to -940 bp. Other functional regulatory elements that may alter *AR* transcription have been found in helix-loop-helix-like motifs 1 and 2, -179 and -37 bp upstream from the core promoter (Takane et al 1996), and nuclear factor I/C (CCAAT-binding transcription factor, NFIC) in the distal part of the promoter (Song *et al.* 1999). AR also regulates itself via exonic androgen responsive elements (AREs) (Grad *et al.* 1999).

The untranslated regions (UTRs) of *AR* are very long. The 5'UTR is approximately 1.1 kb whilst this region usually spans a few hundred base pairs in most genes. The 5'UTR contains SP1 sites, which are binding sites that are essential for AR translation (Mizokami *et al.* 1994b). The 3'UTR is even longer at approximately 7 kb according to northern blot analysis (Lubahn *et al.* 1988, Trapman *et al.* 1988). AR has two differently spliced mRNAs in its 3'UTR region (the major forms are 10.6 kb and 7 kb)

(Faber *et al.* 1993 and 1991). The 3'UTR also contains highly conserved UC-rich motifs and 3'-CCCUCCC poly(C)-binding protein (CP) motifs that are 4036 and 4071 bp downstream of the ARTIS. The UC-rich region is a target of the Elav/Hu family of RNA binding proteins such as HuR, which is involved in the stabilization of several mRNAs containing AU-rich elements. The UC-rich region also simultaneously binds CP1 and 2, which both have a role in the control of mRNA turnover and the rate of translation. Thus, these proteins are suggested to have a cooperative role in controlling AR expression in prostate cancer (Wang *et al.* 2004). Many growth-related mRNAs are known to have atypical 5'UTRs, which are often long and GC-rich (Pickering *et al.* 2005). Interestingly, relatively recent findings have shown that short non-coding microRNAs (miRNAs) are known to downregulate growth related genes, in particular, by binding in a sequence-specific manner in their 3'UTR. miRNA controlled genes are also known to contain often AU-rich elements in their UTRs (Vasudevan *et al.* 2008).

2.2.2 Protein structure and function of AR

The most important domains of AR include the amino-terminal activation function-1 (AF-1) domain, the DNA-binding domain (in the middle) and the carboxy-terminal ligand-binding (LBD) activation function-2 (AF-2) domain. The LBD folds into 12 helices, which form a ligand-binding pocket also known to exist in other members of the steroid receptor family. Ligand (DHT or T) binding to AR induces the folding of helix 12 over the ligand pocket, enabling the interaction of AF-1 and -2 and the dimerization and activation of the protein (Matias *et al.* 2000, Gelmann 2002). Without its ligand, AR is located in the cytoplasm where it is bound with high affinity to a complex of chaperone proteins, which belong to the heat shock protein family. In the presence of ligand, the composition and conformation of the AR-chaperone complex is changed causing the release of AR. This release allows intramolecular interactions, activation and translocation of AR to the nucleus (Matias *et al.* 2000, Gelmann 2002, McEwan 2004). In the nucleus, the dimerized receptor complex binds to a palindromic AR response element (ARE) in the target genes, thus influencing their expression. Androgens are capable of regulating the expression of hundreds of target genes in the prostate gland including *prostate-specific antigen* (*PSA*) (Young *et al.* 1992), *prostate-acid phosphatase* (*PAP*), many growth factors, and genes involved in cell cycle control and apoptosis (Perry *et al.* 1996, Fasciana *et al.* 1996).

The interaction between the LBD and the N-terminal transactivation domain is needed for the full ligand-dependent transactivation and stabilization of AR (Ikonen *et al.* 1997, Schaufele *et al.* 2005). In total, ten phosphorylation, three acetylation, and two sumoylation sites of AR have been documented to influence and regulate the transcriptional activity, localization and stability of AR (Fig. 2, reviewed by McEwan 2004, Faus and Haendler 2006, AR coregulators to be discussed later). One site, Ser94, is constitutively phosphorylated, whereas S16, S81, S256, S308, S424 and S650 exhibit elevated phosphorylation following ligand binding in response to androgen (Zhou *et al.* 1995, Gioeli *et al.* 2002 and 2006). Phosphorylation of S650 by MAPK kinases in the hinge region is shown to increase the nuclear localization of AR (Gioeli *et al.* 2006). However, no single phosphorylation site seems to have a major impact on AR activity since no mutated phosphorylation site alone has been shown to dramatically affect the transcriptional activity of AR (Gioeli *et al.* 2006, Faus and Haendler 2006)

Three acetylation sites clustering within the hinge region, at positions 630, 632 and 633, of AR have been identified, forming a KXKK motif. They play a role in the modulation of the transcriptional activity of AR by favoring nuclear translocation and shifting the balance between coactivator and corepressor binding (Fu *et al.* 2000 and 2002, Thomas *et al.* 2004). Mutation of lysine residues to alanine in the acetylation motif dramatically impairs AR function, preventing the stimulation of coactivators and favoring the recruitment of corepressors (Fu *et al.* 2002, Gaughan *et al.* 2002).

AR has two sumoylation sites (K386 and K520) and was the first hormone receptor shown to be sumoylated (Poukka *et al.* 2000). AR sumoylation is hormone-dependent and involves the Ubc9 and E3 ligases of the PIAS family (Poukka 1999, Kotaja *et al.* 2002). The effects of sumoylation are mainly repressive but are also dependent on the cell context. Mutation of the SUMO acceptor sites has been found to stimulate AR activity. Furthermore, AR regulated promoters respond differently to expression changes of enzymes involved in sumoylation, thus modulating AR-dependent transcription activation in a gene-specific manner (Geserick *et al.* 2003).

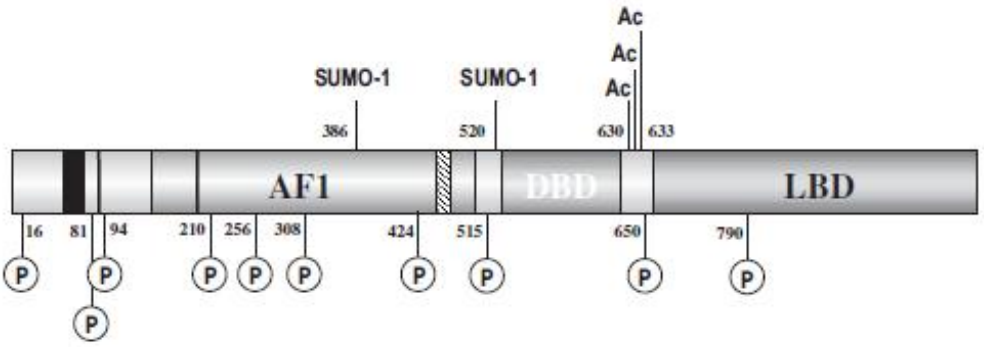

Figure 2. Post-translational modifications of the AR protein. *Sites of phosphorylation (P), sumoylation (SUMO-1) and acetylation (Ac) are shown. (McEwan IJ, Molecular mechanisms of androgen receptor-mediated gene regulation: structure–function analysis of the AF-1 domain Endocrine-Related Cancer (2004), 11, 281–293 Reprinted with permission from The Endocrine Society, Copyright 2004 .)*

Crosstalk between the phosphorylation, acetylation and sumoylation of AR has not yet been extensively studied. However, some initial studies provide evidence that AR acetylation mutants exhibit reduced phosphorylation and AR S94A phosphorylation mutants respond less well to p300-mediated acetylation (Fu *et al.* 2004).

2.2.3 AR coregulators

AR-mediated transactivation requires several auxiliary protein complexes. The transcriptional activity of AR is modulated by the interaction of AR with hundreds of coregulators and by posttranslational modifications of both AR and its coregulators (reviewed by Heemers and Tindall 2007). According to Heemers and Tindall (2007), AR coregulators may be divided into three general classes: 1) general transcription factors, 2) AR coregulators with diverse properties, and 3) specific transcription factors. The most commonly known and extensively studied coregulator is NCOA1 (SRC1), which was the first isolated nuclear receptor coactivator (Onate *et al.* 1995). Group 1 includes coregulators of the direct interaction of AR with the general transcription units GTF2F2 (TFIIF) and GTF2H3 (TFIIH), which facilitates AR transcription activity in a direct or indirect manner (McEwan *et al.* 1997, Lee *et al.* 2000). Group 2 can be further divided into several subclasses according to their main function in the nucleus. Especially interesting subclasses are those that include AR modifying properties such as phosphorylation, acetylation, sumoylation/ubiquitination, mentioned above, as well as those that interact directly with the chromatin. Several histone acetylases (HATs) have been shown to interact with the AR and modulate its transactivating properties, such as the coactivators NCOA1 (SRC1, AIB1), NCOA2 (TIF2, SRC2), NCOA3 (SRC3), EP300 (p300), KAT2B (P/CAF), KAT5 (Tip60) and the corepressors SIRT1, NCOR1 and the HDACs (Heemers and Tindall 2007). Interestingly, Tip60, p300 and P/CAF have also been shown to directly acetylate AR itself whilst AR activity is inhibited by the histone deacetylase activity of HDAC1 (Fu *et al.* 2000, Gaughan *et al.* 2002). Group 3 consists of multiple specific transcription factors including, *e.g.*, Foxa1, Oct1, ETS1, AP-1, and EGR (Heemers and Tindall 2007). Overall, several dynamic changes in covalent histone modification status have been associated with androgen/AR-stimulated transcription (Kang *et al.* 2004).

Coregulators that modify sumoylation and ubiquitination of AR are, *e.g.*, SUMO3, UBE2I and PIAS proteins (Zheng *et al.* 2006, Poukka *et al.* 1999, Kotaja *et al.* 2002). Several kinases, cell cycle regulators, chaperones and cytoskeletal proteins, as well as signal integrators and transducers such as MAK, CDK6, HSPA4 (Hsp70), TGFB1I1 (ARA55), ATAD2 (ANCCA) and STAT3, have been shown to directly interact with AR (Yeah *et al.* 1996, Zou *et al.* 2009). Classical transcription factors such as JUN, FOS, FOXA1 and POU2F1 (OCT1) have also been suggested to interact with AR functioning as coactivators or repressors (Sato *et al.* 1997, Yu *et al.* 2005, Wang *et al.* 2009b, reviewed by Heemers and Tindall 2007). The modulation and recruitment of AR and its coregulators in the transcriptome is a slow and very complex mechanism, as recently demonstrated by Wang *et al.* (2005 and 2009b).

2.3 Cancer of the prostate gland

Prostate cancer (PC) originates from glandular epithelial cells. Histological changes resembling *in situ* cancer are called prostatic intraepithelial neoplasias (PIN). The tumor normally grows very slowly, remaining confined to the organ and leaving the patient asymptomatic for decades. As the cancer advances, it first invades through the capsule and spreads locally to the surrounding tissues. It finally metastasizes further to lymph nodes and bones and to other organs such as the lungs and liver. Localized intracapsular prostate cancer can be cured by radical prostatectomy. However, 20-40% of cancers relapse (Van Poppel *et al.* 2009). Once the tumor has invaded the capsule, the rate of relapse increases significantly (Carver *et al.* 2006, Bill-Axelson *et al.* 2008). Locally advanced and metastasized PC is treated by androgen deprivation. Eventually, an androgen independent cell population arises during hormonal treatment and castration-resistant PC (CRPC) develops with an average expected survival period of 17 months (Fig. 3, Labrie *et al.* 2005, Isaacs and Isaacs 2004, Petrylak *et al.* 2004).

The most widespread method for PC screening is via the serum measurement of prostate-specific antigen (PSA). Increased levels of PSA may suggest the presence of PC, but PSA levels can be also increased by, *e.g.*, infection or benign prostatic hyperplasia (BPH). Thus, PSA is not a PC-specific marker (Gleason 1966, Papsidero *et al.* 1985, Stamey *et al.* 1987). TNM classification and Gleason scoring are more commonly used as prognostic tools for diagnosed cancer (Kattan *et al.* 1998, Epstein *et al.* 2006). TNM classification evaluates the size of the tumor (T), lymph node metastasis (N), and distal metastasis (M) (Chisholm *et al.* 1992). The Gleason score is the sum of the primary and secondary grades of the glandular differentiation. Gleason grades range from 1 (mild structural changes) to 5 (full disappearance of glandular structure). Thus, the sum of scores ranges from 2 to 10. A high Gleason score predicts a poorer prognosis for the patient (Epstein *et al.* 2006)

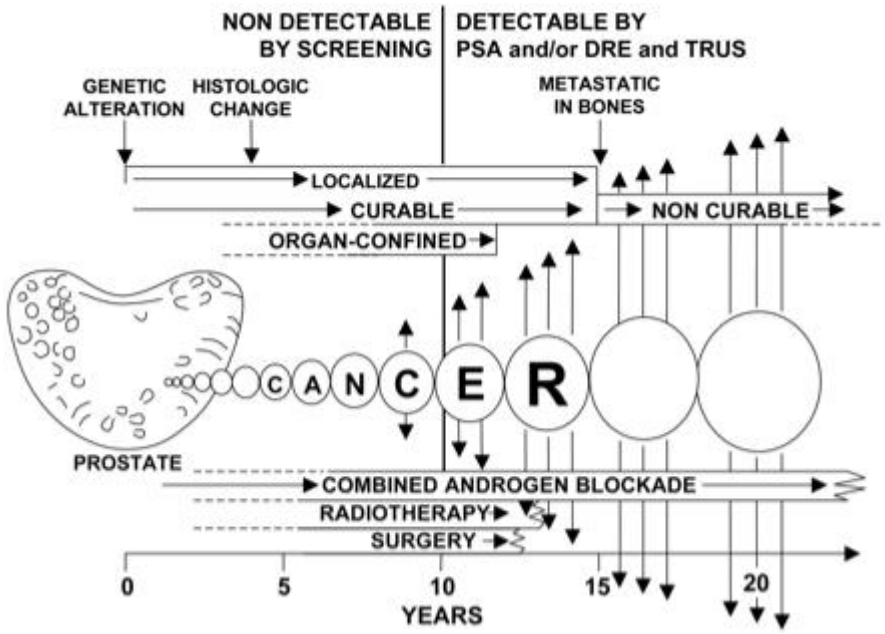

Figure 3. Schematic representation of the evolution of prostate cancer. *The represented scale is an estimated average. The diagnosis (PSA, digital rectal examination, and transrectal echography of the prostate) cannot be made until the tumor reaches a relatively large volume (0.3 cc or more). PC is only curable when the cancer is organ-confined. (Labrie F 2005, Gonadotropin-releasing hormone agonists in the treatment of prostate cancer. Endocrine Reviews. 26, 361-793 Reprinted with permission from The Endocrine Society, Copyright 2005)*

2.3.1 Genetic predisposition

Hereditary factors have been demonstrated to be important in the development of prostate cancer (reviewed by Grönberg 2000). Twin studies have estimated that up to 40% of the prostate cancer risk can be explained by heritable factors (Lichtenstein *et al.* 2000). The genetic predisposition may consist of both high- and low-penetrance genes. Mutations in high-penetrance susceptibility genes are generally very rare but may increase the risk of cancer several fold, whereas mutations or single nucleotide polymorphisms (SNPs) in the low-penetrance genes increase the risk of cancer only modestly (Lichtenstein *et al.* 2000). Thus far, only *elaC homolog 2* (*ELAC2*), *macrophage scavenger receptor 1* (*MSR1*), and *ribonuclease L* (*RNASEL*) have been suggested to be high-penetrance genes. ELAC2 is involved in processing endoribonuclease activity. MSR1 takes part in the regulation of scavenger receptor activity in macrophages, and RNASEL is a component of the antiviral and antiproliferative system of interferons. However, they explain only a small proportion of the genetic predisposition for PC. Several low-penetrance genes have been suggested, but their significance remains unclear (Seppälä *et al.* 2003, Gillanders *et al.* 2004, Rökman *et al.* 2004).

A recent genome-wide association study of approximately 3350 PCs as well as controls identified SNPs at seven highly significant susceptibility loci on chromosomes 2, 4, 8,

11 and 22 (Eeles *et al.* 2009). One SNP was located in 2q31 (in intron 1 of *ITGA6*, the gene encoding integrin alpha 6), and one was located in 8p21 (10 kb downstream of *NKX3-1*, which codes for an androgen-regulated homeobox protein, NKX3-1). The remaining SNPs were located at 2p21 (*THADA*), 4q22 (*PDLIM5*), 4q24 (*TET2*), 11p15 (*IGF2*) and 22q13 (*TTLL1*). Another recent genome-wide association study found four variants associated with the susceptibility to PC (Gudmundsson *et al.* 2009). The variants were one in 3q21.3, two in 8q24.21 and one in 11q13. Interestingly, *human prostatic acid phosphatase (ACPP)*, which has been used as a diagnostic marker for PC, is located at one of the locations identified in this study (Li and Sharief 1993, Sotelo *et al.* 2010). Another interesting finding was that two SNPs were located within the 8q24 region, a region containing enhancer elements that have been suggested to regulate the transcription of the *MYC* oncogene (Sotelo *et al.* 2010). The fourth SNP was located near the *CCND1* (*cyclin D1*) gene in the 11q13 region. This gene is also known to be amplified and functions as an oncogene in many cancers (Fu *et al.* 2004).

2.3.2 Somatic genetic changes in prostate cancer

Somatic genetic changes in PC can include mutations, copy number alterations, translocations or epigenetic changes. Aberrations may include gain- or loss-of-function changes depending on the change and the target gene (Vogelstein and Kinzler 1993, Hanahan and Weinberg 2000). In the next chapters, the genes that are commonly known to carry somatic alterations in prostate cancer are introduced, and they will be further sub-divided into both gain- and loss-of-function categories. Some of these genes are known to participate in AR signaling; however, gain-of-function alterations within the *AR* gene itself will be discussed later in their own individual chapters.

2.3.2.1 Loss-of-function alterations in prostate cancer

The most common regions for loss-of-function changes (and the putative target genes therein) are chromosome 5q (*APC*), 6q (not known), 8p (*NKX3-1*), 10q (*PTEN*), 13q (*RBI*), 16q, 17p, and 18q (Saramäki and Visakorpi 2007). Phosphatase and tensin homolog (*PTEN*), located at 10q23, negatively regulates intracellular levels of dephosphorylated phosphoinositide substrates and functions as a tumor suppressor by negatively regulating the AKT/PKB signaling pathway, which promotes cell survival and inhibits apoptosis. The *PTEN* locus has been shown to be deleted and/or mutated in roughly 40% of late stage prostate cancer cases (Li *et al.* 1997, Dong *et al.* 2006). APC acts as an antagonist of the Wnt signaling pathway. Mutations of *APC* are known to cause familial adenomatous polyposis (FAP) (Phelps *et al.* 2009). *NKX3-1* is a well-known AR target gene and will be discussed later.

Tumor protein p53 (TP53, 17p13.1) is a transcription factor that regulates cell cycle arrest, apoptosis and DNA repair. It is commonly known as “the guardian of the genome”, and the protein functions as a tumor suppressor. *TP53* is commonly deleted during the later stages of prostate cancer. Mutated TP53 protein has a prolonged half-life, leading to the nuclear accumulation of the abnormal protein, which fails to bind the consensus DNA binding site. Mutations of *TP53*, *PTEN* and *RB1* are rare in PC. (Visakorpi 1992, Isaacs WB 1995, Hollstein and Hainaut 2010, Taylor *et al.* 2010). Hypermethylation of the glutathione S-transferase gene (*GSTP1*) is the most

commonly reported epigenetic alteration in PC (reviewed by Meiers *et al.* 2007). In addition, hypermethylation of the well-known tumor suppressor, *APC*, has been reported to occur frequently in the early stages of PC, suggesting its potential use as a biomarker (Yegnasubramanian *et al.* 2004).

2.3.2.2 Gain-of-function alterations in prostate cancer

Somatic gain-of-function alterations, excluding mutations of the *AR* gene and *ETS* fusion genes, are largely unknown. The *AR* gene and *ETS* fusion genes will be discussed separately. Gain-of-function mutations in signature oncogenes, such as *RAS* and *EGFR*, have been found in several other cancers (reviewed by Dong 2005). However, only few have been identified in prostate cancer (Taylor *et al.* 2010). The most common chromosomal gains in PC are located in the 7p/q, 8q, 9p and Xq regions of the genome. In addition, a chromosomal rearrangement in 21q has been observed in over 50% of prostate cancers. The putative target genes include, 7q: *MCM7* and *EZH2*, 8q: *TCEB1*, *MYC*, and *EIF3S3*, Xq: *AR* (discussed later) and 21q: *TMPRSS2:ERG* fusion (discussed later) (Nupponen *et al.* 2000, Saramäki *et al.* 2001, Savinainen *et al.* 2004, Saramäki and Visakorpi 2007, Taylor *et al.* 2010).

MCM7 and *EZH2*, located in 7q, have both been suggested as potential prognostic markers in prostate cancer (Laitinen *et al.* 2008, Ren *et al.* 2006, Saramäki *et al.* 2006, Varambally *et al.* 2002). EZH2 functions in a multiprotein complex called polycomb repressive complex 2 (PRC2). The primary activity of the EZH2 protein complex is to trimethylate histone H3 lysine 27 (H3K27) at target gene promoters, leading to epigenetic silencing. Overexpression of *EZH2* promotes cell proliferation, colony formation and increased invasion of benign cells both *in vitro* and *in vivo* (Saramäki *et al.* 2006, reviewed by Simon and Lange 2008).

The most frequent high-level amplification in late stage prostate cancer is found at the 8q region. This region harbors *MYC*, a known oncogene; however, this region also contains *TCEB1* and *EIF3H*, which have been suggested to function as oncogenes (Savinainen *et al.* 2006, Jalava *et al.* 2009). *MYC* (8q24.21) encodes the v-myc myelocytomatosis viral oncogene homolog, which is a transcription factor involved in cell cycle progression, apoptosis and cellular transformation. Amplification of *MYC* has been shown to be a common event in late state PC. However, gene amplification is not necessarily correlated with overexpression at the protein level (Edwards *et al.* 2003, Savinainen *et al.* 2004, Li *et al.* 2008). Still, overexpression of *MYC* in transgenic mouse models results in prostatic intraepithelial neoplasia (PIN), and together with loss of *NKX3-1* it is associated in carcinogenesis (Zhang *et al.* 2000, Ellwood-Yen *et al.* 2003, Williams *et al.* 2005).

2.4 Androgen receptor (AR) signaling pathway in PC

The AR signaling pathway is dependent on androgen metabolism, ligand specificity, the expression level of AR, ligand-independent activation and cofactor interactions. Several alterations take place in the AR signaling pathway during the development and progression of PC. These changes include somatic mutations of *AR* that allow the usage of a wider spectrum of ligands, the amplification of *AR*, leading to higher expression levels and a shift from paracrine stromal growth support to an autocrine mode. In addition, changes in the balance of AR coregulators and AR splice variants allowing ligand-independent AR action have been suggested. It has also been shown that many of the androgen-regulated genes become up-regulated during the progression of the disease to CRPC (Holzbeierlein *et al.* 2004). Only a very few PCs and CRPCs are considered to have an inactive AR signaling pathway. In the following chapters, the most frequently studied alterations of *AR* and AR signaling in PC are introduced (reviewed by Feldman and Feldman 2001, Isaacs and Isaacs 2004). Changes in different parts of AR pathway signaling during PC progression are discussed individually.

2.4.1 Changes in androgen metabolism

There is abundant evidence that androgens influence the development of PC. In a large randomized PC prevention trial, over 18,800 men aged 55 years or older were treated with finasteride, an inhibitor of steroid 5α-reductase that converts testosterone to DHT. Finasteride treatment was found to reduce the risk of developing prostate cancer by 20% (Thompson *et al.* 2003). Similar results were shown in a prevention trial in which 6729 men aged 50 to 75 were treated with another 5α-reductase inhibitor, dutasteride. The relative risk reduction with dutasteride was 22.8% (Andriole *et al.* 2010). In advanced prostate cancer, even when androgen deprivation therapy is used, the intraprostatic DHT levels remain relatively high. Generally only a 50% reduction in DHT levels is observed after androgen depletion (reviewed by Labrie *et al.* 2005). Intracrine activity of the PC cells themselves has been suggested to be involved in increasing DHT levels (Gao *et al.* 2001, Vander Griend *et al.* 2010). It has also been reported that the expression of many enzymes involved in steroidogenesis are upregulated during CRPC progression (Holzbeierlein *et al.* 2004, Montgomery *et al.* 2008, Locke *et al.* 2010, Leon *et al.* 2010). However, in the recent study, Hofland *et al.* (2010) could only detect a low level of simultaneous expression of the enzymes *CYP17A1* and *HSD3B1*, which are essential for *de novo* synthesis of androgens, in 5 of 88 patients. *SRD5A1* and *AKR1C3* expression were shown to be increased during androgen deprivation, suggesting the importance of DHT synthesis of an adrenal origin androgens instead of from cholesterol (Fig. 4, adapted from Hofland *et al.* 2010). The importance of androgen metabolism was confirmed by recent clinical trials with abiraterone, a CYP17A1 inhibitor, which directly indicated that CRPC is still androgen-dependent (Attard *et al.* 2008, 2009a and b, Ryan *et al.* 2010).

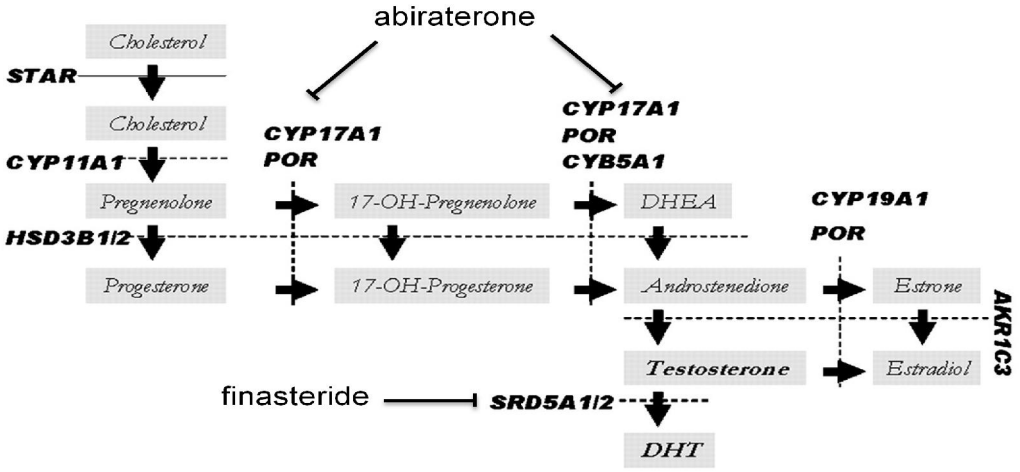

Figure 4. Scheme of the classic steroid biosynthetic pathway. *CYP, cytochrome P450; HSD, hydroxysteroid dehydrogenase; CYB5, cytochrome b_5; AKR, aldo-keto reductase; SRD, 5-alpha reductase. (Hofland J et al. Evidence of limited contributions for intratumoral steroidogenesis in prostate cancer. Cancer Research. 70(3):1256-64 Apated by permission from American Association for Cancer Research, Copyright 2010)*

2.4.2 Somatic mutations of *AR*

AR mutations seem to be rare in early stage, untreated PC (Newmarck *et al.* 1992, Culig *et al.* 1993, Taplin *et al.* 1995 & 2003, Wallen *et al.* 1999). However, the number of *AR* mutations increases with progression of the disease and after hormonal treatments. *AR* mutations can be identified in approximately 20-30% of late stage CRPC tumors. Collectively, only two reports identify point mutations within *AR* in a significant number of untreated tumor samples. Gaddipati and co-authors (1994) found the LNCaP mutation (T877A) in 25% of the transurethral resections of prostate (TURP) specimens from patients with untreated metastatic PC, whereas Tilley *et al.* (1996) reported that roughly 50% of hormone naïve PCs have a mutated *AR*. However, the tumors used in these studies were late stage and rare forms of PC. Generally, the highest frequency of mutations seems to be in PCs treated with antiandrogens (especially in patients treated with flutamide). Mutation frequencies of 10-30% have been reported in such cases (Suzuki *et al.* 1996, Taplin *et al.* 1999 and 2003, Buchanan *et al.* 2001).

The most significant known *AR* mutations are known to affect the ligand specificity of AR. The most frequently found point mutation of *AR* is the T877A mutation (threonine at position 877 is substituted to alanine). This mutation was the first *AR* mutation identified in PC and was originally characterized in the LNCaP cell line (Veldscholte *et al.* 1990). This amino acid is located on helix 11 at the ligand-binding pocket, which interacts directly with the ligand. It alters the stereochemistry of the binding pocket and broadens the ligand binding of AR (Sack *et al.* 2001). It allows other nuclear hormones (estrogen and progestin), corticosteroids (cortisol and cortisone) and antiandrogens (cyproterone and hydroxyflutamide) to activate AR (Culig *et al.* 1993 and 1999, Chang *et al.* 2001, Steketee *et al.* 2002).

In addition to the T877A, several other mutations at the AR-LBD, *e.g.*, L701H, V715M, V730M and H874Y, which enhance the transcriptional sensitivity of AR to other steroids including adrenal androgens and/or antiandrogens, have been identified (Suzuki *et al.* 1993, Culig *et al.* 1993, Newmark *et al.* 1992 and Taplin *et al.* 1995). L701H was originally found in CRPC (Suzuki *et al.* 1993, Watanabe *et al.* 1997) and in the MDA PCa 2a cell line, which also harbors the T877A mutation (Zhao *et al.* 1999). L701H mutated cells are highly responsive to glucocorticoids (cortisol and cortisone) at the concentrations found in humans (Zhao *et al.* 1999, van de Wijngaart *et al.* 2010).

H874Y was originally identified in CRPC patients treated with flutamide (Taplin *et al.* 1995). This mutation was also identified in the xenograft CWR22, which was derived from a patient suffering from primary PC who also had symptoms of bone metastasis. The original patient tumor was graded with a Gleason score 9 (Weinstain *et al.* 1994, Tan *et al.* 1997). DHEA, estradiol, progesterone, and hydroxyflutamide induced a greater transcriptional response from the H874Y mutant than the wild-type AR (Taplin *et al.* 1995, Tan *et al.* 1997, Steketee *et al.* 2002). This site is located distant from the ligand-binding pocket and affects the binding of coregulator proteins (enhancing *e.g.* p160 mediated AR transactivation). Thus, H874Y indirectly affects ligand specificity by causing a conformational change in the AR protein (Steketee *et al.* 2002, Duff *et al.* 2005). The 22Rv1 cell line, which is derived from the castration-resistant form of PC xenograft CWR22 (CWR22R), also carries an LBD deletion and a duplication of the DBD domain (exon 3). These mutations are not present in the androgen-sensitive CWR22Pc cell line (Dagvadorj *et al.* 2008, Dehm *et al.* 2008).

V715M and W741C are less frequently studied, as these mutations are rare within PC. However, they do result in functional changes in AR. V715M was originally found in CRPC patients and is reported to be activated by adrenal androgens and progesterone and is sensitive to low androgen concentrations (Culig *et al.* 1993, Thompson *et al.* 2001). The W741C mutation was found in bicalutamide treated patients (Haapala *et al.* 2001, Taplin *et al.* 2003). The growth of KUCaP xenografts carrying the W741C mutation is accelerated by treatment with bicalutamide and flutamide (Yoshida *et al.* 2005, Terada *et al.* 2010). Additionally, the LNCaP cell line has been shown to acquire the ability for bicalutamide-resistant growth via the W741C mutation when exposed to long-term treatment with bicalutamid (Hara *et al.* 2003).

A smaller number of missense mutations have been detected in other domains of AR. Missense mutations (K179R and C619Y) which affect the N-terminal and DBD regions of the AR protein have been identified in two patients with untreated primary prostate cancer (Tilley *et al.* 1996, Marcelli *et al.* 2000). K179R has been suggested to play a more potent role in AR deregulation (Callewaert et al 2006), whereas C619Y has been found to cause inactivation and mislocation of the receptor (Nazareth *et al.* 1999).

Buchanan *et al.* (2001b) found F671I at the boundary of the hinge and LBD regions in the TRAMP mouse model. This mutation broadens the range of AR ligand specificity and increases the transactivation capacity by 2- to 4-fold. The AR/E231G transgenic mouse model provided evidence that mutations within the N-terminal region of the AR protein may have an oncogenic effect (Han *et al.* 2005). Such mutations led to the development of PIN, which progressed further to the invasive, metastatic disease in

100% of the mouse models studied. Neither the F671I nor the E231G mutation have been found to occur in human PC. The locations of all *AR* mutations found in PC and have been shown to have a functional effect on AR action are shown in Figure 5.

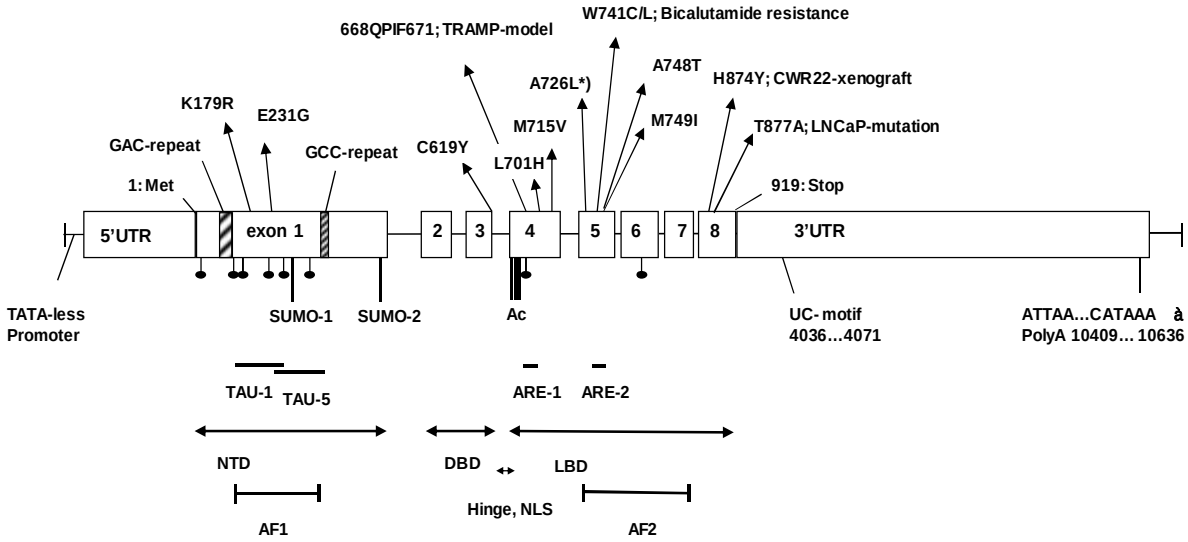

Figure 5. The genetic alterations of AR in PC. *Functionally active regions are illustrated under the structure and functionally active genetic changes involved in PC are noted above the structure. *) Germline mutation, phosphorylation sites (8) marked with pinheads. NTD = N-terminal Domain, DBD = DNA-binding Domain, NLS = Nuclear Localization Signal, LBD = Ligand-binding Domain, AF1 & 2 = Activation Function 1 & 2, TAU-1 & 5 = Transactivation Units 1 and 5, ARE1 & 2 = Androgen Responsive Elements 1 and 2, SUMO-1 & 2 = Sumoylation sites 1 and 2, Ac = Acetylation sites. (Current Clinical Oncology:Prostate Cancer: Signaling Networks, Genetics and New Treatment Strategies by R.G. Pestell and M.T. Nevalainen. 2008. Somatic Genetic Changes in Prostate Cancer: Androgen Receptor Alterations. pp 99-128. Reprinted by permission from ©2008 Humana Press).*

2.4.3 Amplification and overexpression of *AR*

Almost all PCs, except rare small cell carcinomas of the prostate, express *AR* at both the mRNA and protein level. Expression of *AR* is maintained and often elevated during prostate carcinogenesis from androgen-dependent PC to hormone-refractory CRPC, especially during long-term androgen ablation (Ruizeveld de Winter *et al.* 1994, Kokontis *et al.* 1994, Visakorpi *et al.* 1995, Hobisch *et al.* 1995, Culig *et al.* 1999, Latil *et al.* 2001, Linja *et al.* 2001, Edwards *et al.* 2003, Hofland *et al.* 2010). High *AR* expression has been suggested to be associated with short recurrent-free survival (Henshall *et al.* 2001, Lee *et al.* 2003, Donavan *et al.* 2009). Interestingly, AR always localizes to the nucleus in clinical tumors, irrespective of circulating androgen levels and androgen-dependence status, *i.e.*, hormone-naïve, recently castrated or castration-resistant. This finding indicates that AR is continuously activated during PC progression (Laitinen *et al.* 2007). The expression of *AR* is abolished in only a very rare fraction of CRPCs, possibly through hypermethylation of the *AR* promoter (Kinoshita *et al.* 2000).

Gain and amplification of the *AR* gene is one of the most frequent chromosomal gains in CRPC (reviewed by Nupponen & Visakorpi 1999). Nearly 80% of CRPCs have been reported to carry an elevated *AR* gene copy number (Edwards *et al.* 2003), with 20-30% showing a high level of *AR* gene amplification. In contrast, untreated primary PCs very rarely contain an *AR* gene amplification (Visakorpi *et al.* 1995, Koivisto *et al.* 1997, Bubendorf *et al.* 1999, Kaltz-Wittmer 2000, Edwards *et al.* 2003). Amplification was detected in the untreated samples of only 2 cases out of 205 primary PCs, indicating that the amplification is selected during the emergence of CRPC (Bubendorf *et al.* 1999). Recently, two studies reported that *AR* gene amplification was found in >50% of circulating tumor cells (CTC) from metastasized CRPC cases (Leversha *et al.* 2009, Attard *et al.* 2009b). However, AR gene amplification only partially explains the overexpression of AR. The other still unknown mechanisms leading to *AR* overexpression could include, *e.g.*, genetic aberrations in the regulatory regions of *AR* and miRNA deregulation.

The significance of the overexpression of *AR* in CRPC was first demonstrated by Chen and co-authors (2004), who showed that the common nominator in gene expression profiles of CRPC xenograft models as compared to androgen-dependent counterparts was the increased *AR* level. They also showed that overexpression of *AR* alone was necessary and sufficient to cause androgen-sensitive xenografts to become castration- and bicalutamide-resistant.

2.4.4 AR splice variants

Three novel AR isoforms lacking the ligand-binding domain (designated as AR3, AR4, and AR5, according to Guo *et al.* 2009, Fig. 6) have been reported in CRPC. AR3, one of the major splice variants expressed in human prostate tissues, has been suggested to be constitutively active (Guo *et al.* 2009, Hu *et al.* 2009). The molecular mechanisms that lead to differential AR splicing are not known. Immunohistochemical analysis of 429 PC tissues showed that AR3 is significantly up-regulated during CRPC progression and AR3 expression levels are correlated with the risk of tumor recurrence after radical prostatectomy. Unlike wildtype AR, AR3 was shown to directly increase AKT1 expression (Dehm *et al.* 2008, Guo *et al.* 2009, Hu *et al.* 2009).

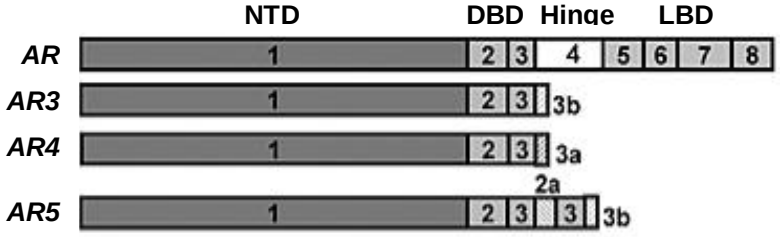

Figure 6. *Schematic structure of the human AR splice variants. (Guo Z et al. 2009. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Research. 69(6):2305-13 Reprinted by permission from American Association for Cancer Research, Copyright 2009)*

2.4.5 Changes in AR cofactors

AR cofactor imbalances have been studied in PC progression. Thus far, no AR cofactor has been shown to influence a great number of cancers. However, some non-recurrent cofactor changes have been reported in a small number of PC cases. More studies are required to clearly define the changes of all the cofactors implicated in PC (reviewed by Xu *et al.* 2009a, Fujimoto *et al.* 2001, Linja *et al.* 2004, Mäki *et al.* 2006 and 2007, Zou *et al.* 2009).

High expression levels of some AR cofactors have been reported in PC. The increased expression of *NCOA1* (*SRC1*), *NCOA2* (*TIF2*, *SRC2*), *KDM1A* (*LSD1*), *KDM4C* (*JMJD2C*), *RNF6*, *TRIM68* and *TGFB1I1* (*ARA55*) has been linked with increased activation of AR in PC (Fujimoto *et al.* 2001, Agoulnik *et al.* 2006, Mäki *et al.* 2006 and 2007, Miyajima *et al.* 2008, Xu *et al.* 2009b, Taylor *et al.* 2010). Increased levels of NCOA2, BAG1 and ATAD2 (ANCCA) protein have also been associated with the progression of CRPC or higher grade PCs (Fujimoto *et al.* 2001, Agoulnik *et al.* 2006, Mäki *et al.* 2007, Zou *et al.* 2009). Interestingly, *ATAD2* was also shown to be upregulated by androgens (Zou *et al.* 2009).

Genetic alterations of AR co-regulators have not been intensively studied. Only a few studies have reported somatic DNA copy number alterations or nonsense mutations of known AR coregulators. Two cases of *NCOA1* gene amplification and one case of a *NCOA1* missense mutation in PC have been reported thus far (Linja *et al.* 2004, Mäki *et al.* 2006). The low frequency of such aberrations suggests that genetic alterations in *NCOA1* are not commonly involved in the progression of PC (Linja *et al.* 2001, Mäki *et al.* 2006, Xu *et al.* 2009). Another AR coregulator reported to be amplified in PC is *BAG1*, which has been shown to activate AR by interacting with the N-terminal region of the receptor. *BAG1* was found to be amplified in 7% of CRPC samples, with significantly higher protein expression compared to primary PC samples (Shatkina *et al.* 2003, Mäki *et al.* 2007). Recently *NCOA2* was also shown to be amplified and overexpressed in PC (Taylor et al. 2010).

2.4.6 Well known AR target genes

Microarray studies of androgen-regulated genes suggest that approximately 2-4% of all transcripts could be directly or indirectly regulated by androgens (Amler *et al.* 2000, Nelson *et al.* 2002). AR regulates the expression of androgen-responsive genes by binding to the androgen response elements (AREs) of target genes. Sequence analysis and new chromatin immunoprecipitation (ChIP) technologies have identified, in addition to classical AREs, several new noncanonical AR binding sites, which have been shown to function even up to 300 kb up- or downstream from the target gene (Wang *et al.* 2007a). The most commonly known and studied AR target genes include *KLK2*, *KLK3*(*PSA*) and *TMPRSS2*, which produce prostate gland enzymes including phosphatases and several serine proteases secreted by the epithelial cells into seminal plasma (Young *et al.* 1992, Perry *et al.* 1996). The following paragraphs introduce the most well known AR target genes.

2.4.6.1 *ACPP* (*PAP*)

The *acid phosphatase prostate* (*ACPP*) gene, also known as *prostate acidic phosphatase* (*PAP*), is located in chromosome 3q21-q23. *ACPP* encodes a 100 kDa tyrosine and lipid phosphatase which is synthesized in the prostatic epithelial cells and secreted into prostatic fluid. There are two forms of PAP, a cellular and secreted form, which have different biochemical properties. *PAP* has been shown to be directly regulated by androgens in a biphasic manner and is highly expressed in both the normal prostate and in PC (Gutman and Gutman 1938, Vihko 1979, Li and Sharief 1993, Henttu *et al.* 1992, Lin *et al.* 1993, Ulrix *et al.* 1998). PAP was used as early as the 1930s as a biomarker for PC (Gutman and Gutman 1938, Huggins and Hodges 1941). Cellular PAP levels are decreased in advanced PC. *PAP* expression correlates negatively with cell growth and cancer progression. It has been suggested that PAP dephosphorylates HER-2, which in turn activates ERK/MAPK signaling (Sharma *et al.* 2005). Decreased PAP levels and increased tyrosine phosphorylation of HER-2 correlate with Gleason score and PC progression. The molecular mechanisms that cause decreased PAP levels in PC are not known (Veeramani *et al.* 2005).

2.4.6.2 *KLK3* (*PSA*)

Kallikrein-related peptidase 3 (*KLK3*), better known as *prostate-specific antigen* (*PSA*), is located in chromosome 19q13.41. *KLK3* encodes a single chain glycoprotein with a molecular mass of 33 kDa and functions as a serine protease. It belongs to the family of the fifteen *kallikrein* members located in a cluster in the same chromosomal region. All *kallikrein* genes encode five exons of similar size and have high sequence homology with other family members. Many of these peptidases also have several alternative splice variants and are known to be regulated by androgens (reviewed by Lawrence *et al.* 2010). KLK3 was cloned in 1987 (Lundwall and Linja). *KLK3* expression has been shown to be elevated in BPH and in highly differentiated PCs, but it is decreased during PC progression (Abrahamsson *et al.* 1988, Hakalahti *et al.* 1993). The use of KLK3 as a PC biomarker (the so-called PSA test) began in the mid 1980s (Stamey *et al.* 1987). In a recent European study, which included more than 160,000 men aged 55 to 69, it was found that PSA-based screening reduced PC mortality by 20%. However, there was a high risk of overdiagnosis (Schröder *et al.* 2009). Androgen regulation of *KLK3* includes both the proximal promoter and the enhancer ARE located 4 kb upstream from the TSS. Recruitment of AR and its coregulators create a chromosomal loop from the enhancer to the core promoter (Young *et al.* 1992, Riegman *et al.* 1991, Wang *et al.* 2005). Kallikrein family members have also been suggested to play a putative role in PC progression. For example, KLK3 has been suggested to directly degrade extracellular matrix glycoproteins and facilitate cell migration (reviewed by Lilja 2003, Hollenberg *et al.* 2008).

2.4.6.3 *TMPRSS2*:*ERG* and other fusion ETS

The fusion of *E-twenty-six* family (*ETS*) genes with a hormone-dependent promoter region occurs in 30-70% of therapy-naîve prostate cancers (Tomlins *et al.* 2005 and 2007, Saramäki *et al.* 2008, reviewed by Kumar-Sinha *et al.* 2008, Tomlins *et al.* 2009). Thus far, rearrangements of the *ERG*, *ETV1*, *ETV4* and *ETV5* gene loci have

been reported (Tomlins *et al.* 2005, 2006 and 2007, Helgeson *et al.* 2008). The most common variants involve *transmembrane protease, serine 2* (*TMPRSS2*) exon 1 or 2 fused to *v-ets erythroblastosis virus E26 oncogene homolog* (*ERG*) exon 2, 3, 4 or 5 (Tomlins *et al.* 2005 and 2007). These two genes are located at the same chromosomal region, 21q22.3. *TMPRSS2* is an androgen-regulated gene which is highly expressed in the normal prostate and in PC (Lin *et al.* 1999, Vaarala *et al.* 2001). The gene encodes a serine protease that contains a predicted protein of 492 amino acids in length. Relatively recently, androgen regulation by *cis*-regulation of noncanonical AREs in *TMPRSS2* gene was discovered (Wang *et al.* 2007a).

Additional 5' partners for *ETV1, ETV4, ETV5* and *ELK4* have also been identified. These 5' partners include *SLC45A3, HERV-K_22q11.23, CANT1* and *KLK2*, which are prostate-specific and androgen-inducible (reviewed by Kumar-Sinha *et al.* 2008, Tomlins *et al.* 2009). It is not clear how these gene fusions participate in carcinogenesis in the prostate. However, *in vitro* studies of overexpressed *ETV1* or *ERG* have suggested their role in invasion via the urokinase plasminogen activator (UPA, or PLAU) pathway. It has also been shown that transgenic mice that overexpress *ETV1* or *ERG* develop mouse prostatic intraepithelial neoplasia (mPIN), but not tumors. (Tomlins *et al.* 2007, Cai *et al.* 2007, Klezovitch *et al.* 2008). These fusion genes are early events that are most commonly associated with localized PC. They are found in similar frequency in CRPC, suggesting their role in driving the transformation but probably having less significance in the CRPC progression (Perner *et al.* 2007, Saramäki *et al.* 2008, reviewed by Tomlins *et al.* 2009). Recently, two studies with transgenic *ERG* overexpression in *Pten* heterozygous background mice showed significant progression of high-grade PIN and PC by inducing downstream checkpoint genes that would usually be blocked by AKT (Carver *et al.* 2009, King *et al.* 2009, Squire 2009).

2.4.6.4 *NKX3-1*

The *NK3 homeobox 1* (*NKX3-1*) gene encodes a transcription factor with tumor suppressor functions. This gene is localized in the chromosomal region 8p21, which is commonly deleted in PC. The expression of *NKX3-1* is androgen-regulated and stimulates the differentiation of prostatic epithelial cells. *NKX3*-1 expression is often lost during the progression of PC (He *et al.* 1997, Asatiani *et al.* 2005, Bethel *et al.* 2007). However, some examples of *NKX3-1* overexpression have been reported (Xu *et al.* 2000). NKX3-1 has also been shown to regulate *AR* expression in LNCaP cells (Possner *et al.* 2008). Interestingly, Wang *et al.* (2009a) recently suggested that in mouse, in rare castration resistant Ar- and Nkx3-1 positive luminal epithelial PC stem cells, Nkx3-1 is required for stem cell maintenance *in vivo* during hormonal treatment. This finding may indicate that NKX3-1 is not a classical tumor suppressor gene but could have a role in PC stem cell survival and differentiation in androgen-deprived conditions.

2.5 Endocrine treatments of prostate cancer

PC treatments that target AR signaling have been investigated heavily for over half a century. The revolutionary finding was made in 1941 by Huggins and Hodges, who showed that castration or estrogen treatment inhibits the growth of PC and that the growth of PC was activated by androgen injections (Huggins and Hodges 1941). They also noticed the association between hormonal manipulations and the serum level of PAP. Currently, a wide spectrum of AR-targeted therapies is available. These therapies focus mainly on either preventing androgen production from the testes and/or blocking the function of AR with antiandrogens.

2.5.1 Targeting androgen synthesis

Androgen synthesis can be prevented by classical orchiectomy or by continuous stimulation of the pituitary with high concentrations of luteinizing hormone releasing hormone (LHRH) agonist or antagonist. Continuous agonist stimulation results in receptor desensitization and inhibition of LH release, which further inhibits the production of testosterone by the testes. Several agonists, *e.g.*, goserelin and leupropelin, are used in the clinic with well-proven equivalence to orchiectomy. LHRH antagonists directly affect the LHRH receptor regulatory system, causing a rapid decrease in serum androgen levels (reviewed by Tammela 2004, Weckermann and Harzmann 2004, Labrié *et al.* 2005). Since residual serum androgens, as well as upregulated intracrine androgen synthesis, may be sufficient to promote CRPC growth in patients receiving androgen-deprivation therapy, strategies to further lower androgen levels have been suggested. One of the promising new drugs that targets both adrenal and tumor intracrine androgen synthesis is abiraterone. It is a selective irreversible inhibitor of the p450 enzyme, 17α-hydroxylase/C17,20-lyase (CYP17), which catalyzes the biosynthesis of androgens from pregnane precursors. Recent phase I/II clinical trials of patients with CRPC have shown significant antitumor activity and up to 70% of PSA response (Attard *et al.* 2008 and 2009a, Ryan *et al.* 2010).

2.5.2 AR targeting inhibitors

Direct AR inhibitors can be divided into steroidal or non-steroidal antiandrogens. They specifically block T and DHT from binding the ligand-biding pocket of AR. Nonsteroidal antiandrogens such as bicalutamide, flutamide and nilutamide inhibit the activity of androgens by their competitive interaction with AR. Steroidal antiandrogens (cyproterone acetate) also function in the pituitary axis and inhibit LH release. However, with the use of steroidal antiandrogens, more complications have been reported compared to LHRH analogs and non-steroidal antiandrogens (reviewed by Tammela 2004, Loblaw *et al.* 2007). The binding of flutamide or bicalutamide to AR prevents androgen binding and modulates AR-protein structure, allowing the binding of AR corepressors (Yoon & Wong 2006). However, somatic *AR* mutations (introduced above) that occur after the use of antiandrogens have been reported to release the transcriptional inhibition (Chen *et al.* 2004, Steketee *et al.* 2002, Culig *et al.* 1999). To improve the inhibition of the androgen signaling axis, complete androgen

blockade (CAB) therapies have been investigated. In CAB, castration is combined with antiandrogen therapy. CAB has been shown to prolong life in CRPC patients, but the significance is only marginal (Palmberg *et al.* 2000, Labrie *et al.* 2002 and Scher *et al.* 2004). Recently, new antiandrogens for CRPC have been developed. For example, MDV-3100 binds to AR with 10-fold higher affinity than bicalutamide and inhibits PSA secretion at 10-fold lower concentrations. Unlike bicalutamide, MDV-3100 impairs AR nuclear translocation and blocks DNA binding (Tran *et al.* 2009, Jung *et al.* 2010). MDV-3100 is currently in a phase III clinical trial for use in CRPC (reviewed by Chen *et al.* 2008, Tran *et al.* 2009, Jung *et al.* 2010).

3. AIMS OF THE STUDY

The purpose of the study was to investigate the molecular mechanisms leading to *AR* overexpression and to establish an *AR* overexpression model that can be used to identify and investigate the downstream AR target genes involved in PC progression.

The specific aims were the following:

1) To investigate whether mutations in the *AR* gene regulatory regions could underlie its overexpression;
2) To establish an *in vitro* model of *AR* overexpression;
3) To identify AR downstream protein-coding genes involved in PC progression;
4) To identify AR downstream miRNAs involved in PC progression.

4. MATERIALS AND METHODS

4.1 Cell lines and xenografts

DU145, LNCaP, PC3 and 22Rv1 PC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). LAPC4 cells were kindly given by Prof. C. Sawyers (University of California at Los Angeles, Los Angeles, CA, USA). VCaP and DuCaP were provided by Prof. J. Schalken (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands). Cells were cultured under the recommended conditions (original communications I-III).

Nineteen LuCaP series xenografts (LuCap 23.1, 23.8, 23.12, 35, 35V, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 105, 115) were obtained from Prof. R. Vessella (University of Washington, Seattle, WA, USA), and 13 PC series xenografts (PC-82, 133, 135, 295, 310, 324, 339, 346, 346B, 346I, 346BI, 374 and 374F) were obtained from Prof. G. Jenster (University of Rotterdam, The Netherlands). Tumor samples were collected either from intact male (LuCaP series) mice or both intact mice and 7 to 14 days after castration (PC xenografts) and were freshly frozen at -80°C (original communications I and III).

4.2 Clinical samples

All together, 52 freshly frozen clinical samples (6 BPH, 30 primary prostatectomy PC and 14 CRPC samples described in original communication I, and 7 BPH and 15 CRPC samples described in original communication III) were used in the study. Of those, the BPH and CRPC samples were transurethal resections (TURP). The endocrine therapy included either orchiectomy, LHRH analog, estrogens, orchiectomy and estrogen, bicalutamide or unspecified hormone therapy. Samples were snap frozen in liquid nitrogen. Tumor samples contained at least 60% cancer cells. Thirty normal samples for general SNP analysis (60 chromosome X) were obtained from healthy Finnish female blood donors. The use of the clinical material was approved by the ethical committee of the Tampere University Hospital (original communications I and III).

4.3 DNA and RNA extractions, DNA amplification and PCR

DNA and RNA were purified using routine phenol-chloroform and Trizol™ techniques. For mutation analysis, DNA samples were first amplified with a Genome Phi™ genome amplification kit (Invitrogen) according to the manufacturer's instructions. For PCR reactions, Accutype (Stratagene) or Platinum Taq (Invitrogen) added with 1:4 ratio Pfu (Fermentas) polymerases. The enzymes were used in detergent-free buffer according to the manufacturer's instructions. Primers and annealing temperatures are listed in the original publications. All primers were designed with the publicly available Primer3 program (http://frodo.wi.mit.edu /primer3/) (original communications I-III).

4.4 DHPLC and sequencing

For promoter and 5'UTR mutation analyses, sample PCR fragments were each mixed at a 1:1 ratio with the corresponding normal PCR fragment, denatured (95°C for 3 min) and renatured (65°C for 30 min). DHPLC (denaturing high-performance liquid chromatography) was performed with an Agilent 1100 LC machine and a Varian CP28353 Helix DNA column (50 x 3.0 mm) at a 12% diluent gradient over 8 min at fragment-specific temperatures. Primers and DHPLC driving conditions were designed with the freely available DHPLC Melt program (http://insertion.stanford.edu/melt. html). Sequencing was performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and run in the ABI3100 Genetic Analyzer according to the manufacturer's instructions (original communication I).

4.5 mRNA and miRNA qRT-PCR reactions

First strand cDNA synthesis was carried out from total RNA using either AMV reverse transcriptase (Finnzymes) or SuperScriptIII (Invitrogen) for miRNA-specific targets according to the manufacturer's instructions. To measure mRNA expression, a SYBR GreenII Fast Start kit (Roche Diagnostics) was used according to the manufacturer's instructions using *TBP* (TATA-box binding protein) mRNA as a reference gene. For measuring mature miRNA expression, TaqMan® MicroRNA Assays (Applied Biosystems) and a Probe Fast Start kit (Roche Diagnostics) were used. A Light Cycler apparatus (Roche Diagnostics) was used. The RT reactions and the qPCR reactions were performed separately for each miRNA using RNU6b as a small reference RNA (original communications II and III).

4.6 Western blot

Protein extracts (12.5 µg) were separated by 12% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore Corp.) using the standard semidry transfer technique (BIORAD Transblot, Bio-Rad Lab.). Mouse anti-AR 441 was used as the primary antibody (NeoMarkers), and anti-mouse IgG-HRP conjugate (DAKO A/S) was used as the secondary antibody (original communications II and III).

4.7 Transfection methods

The parental LNCaP cells were stably transfected either with the pcDNA3.1(+) empty expression vector (Invitrogen Inc., Carlsbad, CA, USA) or with the pcDNA3.1-AR (Acc#_M23263) into the NotI/BamHI site in the pcDNA3.1 vector) with Lipofectamine Plus transfection reagent (Invitrogen, Inc.), according to the manufacturer's instructions. Transfected clones were selected under 400 µg/ml geneticin (G418, Invitrogen, Inc.). Two clones expressing moderate and high levels of AR protein (LNCaP-ARmo and LNCaP-ARhi) were selected for further studies and maintained in 200 µg/ml geneticin-containing medium (original communication II).

The LNCaP-pcDNA3.1 cells were transiently transfected with 20 nM Pre-miR™ miR-141 precursor (#PM10860, Ambion, Inc., Austin, TX, USA), and the LNCaP-ARhi cells with 100 nM Anti-miR™ miR-141 inhibitor (#AM10860, Ambion, Inc.) using INTERFERINin™ siRNA transfection reagent (POLYPLUS-TRANSFECTION Inc.,

NY, USA) under various DHT concentrations, according to manufacturer's protocol. The scrambled Anti- or Pre-miR™ negative controls (#AM17010, #AM17110, Ambion, Inc.) were used as corresponding reference treatments (original communication III).

4.8 DHT and roscovitine treatments and cell proliferation assays

Before each hormone exposure experiment, cells were grown in 5% charcoal/dextran-treated serum (CSS, HyClone, Inc.) in medium without phenol red for three or four days. The medium was subsequently replaced with various concentrations of DHT (Steraloids, Inc., USA). Roscovitine (Calbiochem®, EMD Chemicals Inc., Germany) was used for CDK1/2 growth inhibition treatments. The relative number of cells at each time point was analyzed either by using the Beckman Coulter® Z2-series particle counter (Beckman Coulter, Inc., USA) or AlamarBlue reagent (AbD Serotec, UK) and luminometric detection using a fluorometer (Wallac 1420 Victor, PerkinElmer, USA) (original communications II and III).

4.9 Microarray hybridizations

AR and androgen regulation of different protein coding genes were studied with an Illumina Microarray platform on a HumanRef-8 v2 chip and performed in the Finnish DNA Microarray Center at the Turku Centre for Biotechnology. Total RNA (300 ng) from each sample was transcribed *in vitro*, biotinylated and amplified with an Illumina RNA TotalPrep Amplification kit (Ambion, #IL1791, USA), and hybridized to an Illumina Sentrix® HumanRef-8_V2 Expression Bead Chip (cat. no. BD-25-213). The probes (>22,000) of the Illumina HumanRef-8 v2 chip are based on the well annotated protein coding genes of the Reference Sequence (RefSeq) database 1, release 17 (original communication II).

miRNA expression was analyzed with Agilent human miRNA microarray v2 chips (Agilent Technologies, Santa Clara, CA, USA) containing 723 human and 76 human viral miRNAs (Sanger Cambridge, Database v10.1, http://microrna.sanger.ac.uk) according to the manufacturer's instructions. Total RNA (100 ng) was labeled with pCp-Cy3 and hybridized according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). miR-141 target gene expression was studied with Whole Genome Human 4x44K microarray chips (Agilent Technologies) according to the manufacturer's instructions (original communication III).

4.10 Data analysis

The data analysis was performed with the GeneSpring Analysis Platform, version GX 7.3.1 (Agilent Technologies, CA, USA) using standard normalization methods such as median normalization and Lowess smoothing. The average linkage method was used for unsupervised hierarchical clustering, and the similarities were estimated with Pearson's correlation. For ontology classifications, all gene ontology (GO) lists containing at least ten genes with p-values <0.001 (hypergeometric p-value without multiple testing correction) were filtered and organized with the GeneSpring Ontology browser (original communications II and III).

4.11 ChIP-on-chip assays

ChIP-on-chip assays were performed with custom anti-AR antibody (BJ14-AR3) using LNCaP cells that were treated with 100 nM DHT or vehicle for 2 h. Three micrograms of the immunoprecipitated DNA samples were hybridized to Affymetrix whole genome tiling arrays (GeneChip Human Tiling 2.OR Array Set, Affymetrix Inc., USA). The regions enriched for AR-binding sites (ARBs) were identified using the MAT algorithm, scored at a p-value of 10^{-4} and mapped to the differentially expressed genes in the AR overexpression model (original communication II).

4.12 Statistical methods

The unpaired, two-tailed Student's t-test was used to examine the statistical significance of the differences in growth and gene expression between the DHT-treated and vehicle-treated cells (original communication II and III). The significance of the number of genes in different gene ontology classes between DHT-treated and untreated cells, as well as between different cell lines, was calculated by using the chi-square test (original communication II). To evaluate the significance of the gene expression levels between the two cancer groups (PC and CRPC) and the non-cancer group (BPH), the two-tailed Mann-Whitney U-test was used (original communication III).

5. RESULTS AND DISCUSSION

5.1 Mutations in the regulatory regions of the *AR* gene are rare in PC

To study the possible mutations that may cause AR overexpression, the wide promoter region, the entire 5'UTR and a 4.6 kb region of the putative 3'UTR were screened for unknown mutations in PC cell lines, PC xenografts and in clinical samples. In the promoter and the >2 kb upstream regulatory region, four single base changes were found in the cell lines and xenografts. Single substitutions C-1863T, G-1474A, T-640G and G-572T were found in LuCaP35, LuCaP69, LAPC4 and DU145, respectively. The alteration found in LuCaP35 was also present in the CRPC sub-line of the xenograft (LuCaP35V). In the 5'-UTR, only C+456G, was found in the LAPC4 cell line. In the 4.6 kb region of the putative 3'UTR, three substitutions were found: G4550C, T5729A and C7696A were found in DU145, LAPC4 and LuCaP 73, respectively. In addition, a single base deletion (4037delT) was found in DU145. None of these alterations were recurrent nor were they present in the 30 PC, 6 BPH, 14 CRPC specimens or in the 60 normal female germ line DNA samples used in the study, indicating that the found variants are rare mutations (original communication I).

Of the mutations found, only the 4037delT mutation in DU145 is located in a known functionally active motif (Faber *et al.* 1993, Mizokami *et al.* 1994a, Takane *et al.* 1996, Chen *et al.* 1997, Yeap *et al.* 2002, Wang *et al.* 2004). However, the functional analysis of the $C(U)_9C$ motif has shown that a single base substitution has no effect on the stability of the *AR* mRNA (Yeap *et al.* 2002). On the other hand, DU145 cells are completely AR negative, indicating that the mutation cannot be involved in AR overexpression.

Single substitutions C-1863T and G-1474A were found in LuCaP35 and LuCaP69, which both contain an amplification of the *AR* gene and express high levels of *AR* (Linja *et al.* 2001). The amplification of the *AR* by itself is already a mechanism for high *AR* levels. Both DU145 and LAPC4 had three single base mutations, indicating that these cell lines are genetically unstable. DU145 has already been shown to lack functional MLH1, leading to a loss of mismatch repair (Chen *et al.* 2001, Martin *et al.* 2009). These mutations were found only in the most genetically unstable cancer samples with no recurrence; none were found in the clinical samples. Therefore, it is unlikely that they contribute to the overexpression of *AR* in CRPC.

It is, however, possible that mutations existing outside the studied regions affect the expression of *AR*. An interesting and still poorly characterized example of this possibility is the full length of the 3'UTR. The databases, such as GenBank, recognize only a 4313 bp mRNA, whereas the size of the 3'UTR is 7 kb long according to northern analysis (Lubahn *et al.* 1988, Trapman *et al.* 1988). Due to the heterogenic distribution of AT-repetitive sequences in the 3'UTR, only 4.6 kb of the 3'UTR region could be sequenced. The function of the AT-repetitive sequence in the putative 3'UTR is unknown. This type of repetition belongs to a non-random pattern of repeated elements that are typically 60-80 nucleotides long. These repeats are also known as

pyknons and are found more frequently in the 3'UTR regions of the human genome (Rigoutsos *et al.* 2006). The pyknons include approximately 40% of the known miRNA sequences, suggesting a novel putative regulatory mechanism possibly linked with posttranscriptional regulation and RNA interference (Meynert & Birney 2006, Glinsky 2009).

Other mechanisms of protein overexpression might include several posttranslational modification of the AR. Increased stability and the nuclear localization of AR in CRPC cells has been associated with an increased sensitivity to the growth-promoting effects of dihydrotestosterone (Gregory *et al.* 2001, Chen *et al.* 2004). Akt has been shown to both stimulate and inhibit AR activity in LNCaP cells at high and low passage numbers, respectively (Lin *et al.* 2003, Ghosh *et al.* 2005). Akt has been shown to directly phosphorylate AR at Ser-210 and Ser-790. However, this phosphorylation leads to the inhibition of AR-regulated p21-mediated apoptosis and has not been shown to increase or stabilize AR levels (Lu *et al.* 1999, Lin *et al.* 2001). Cyclin dependent kinase 1 (CDK1) has been shown to both phosphorylate and stabilize AR (Chen *et al.* 2006). CDK1 protein levels also correlate with a high Gleason grade (Kallakury *et al.* 1997). However, these mechanisms could only explain the observed overexpression of the protein product, but not the changes in mRNA level.

miRNAs are a relatively recently discovered group of small non-coding RNAs involved in the post-transcrptional regulation of mRNA levels. miRNAs most often target AU-rich 3'UTRs (Jing *et al.* 2005). Since the *AR* has a relatively AU-rich and a very long 3'UTR, several miRNAs might regulate *AR* mRNA. Deletion or hypermethylation of AR-targeting miRNAs may increase the expression of *AR* mRNA. On the other hand, a shortened *AR* 3'UTR might induce higher *AR* expression even in the presence AR-targeting miRNAs. However, no AR targeting miRNAs have yet been reported. One challenge in this field of research is the complexity of the putative 3'UTR of AR.

5.2 Increased AR levels sensitize PC cells to low androgen levels

5.2.1 The growth of the AR-overexpressing LNCaP cells

To study the consequences of overexpressed *AR*, three different levels of *AR* expressing LNCaP clones were constructed and then selected for detailed studies and further experiments. These constructs were the empty vector control-carrying LNCaP-pcDNA3.1, the LNCaP-ARmo and the LNCaP-ARhi cells. LNCaP-ARmo cells expressed approximately four times more AR mRNA and two to four times more AR protein; whereas, LNCaP-ARhi cells expressed 13 times more mRNA and 5-6 times more protein than the LNCaP-pcDNA3.1 cells. The most intense AR nuclear staining was seen in the LNCaP-ARhi cells. The proliferation rates of all three cell lines were highest under 10 nM DHT conditions, with no differences between the cell lines. However, in the presence of 1 nM DHT or lower, LNCaP-ARhi cells grew significantly faster than the LNCaP-ARmo or LNCaP-pcDNA3.1 cells. The cells overexpressing *AR* were also capable of growing longer in charcoal-stripped fetal bovine serum (CSS) medium without any DHT supply (original communication II).

The results coincide with earlier studies of Kokontis *et al.* (1994 and 1998), Gregory *et al.* (2001) and Chen *et al.* (2004), indicating that high levels of AR sensitizes the growth of the cells under low androgen levels. In low androgen levels, the growth is enhanced by higher AR levels, and in higher DHT concentrations, the proliferation effect is decreased in high AR-expressing cells, whereas the growth of low AR-expressing cells is still enhanced. In the original studies of Kokonties *et al.* (1994), LNCaP cells adapted to the low androgen levels. Chen *et al.* (2004) showed that the common denominator for the PC cells adapted to growth in castrated mice was the increased AR level. We showed that exogenously enhanced AR directly increases the proliferation of PC cells in low androgen medium. The adaptation of the PC cells to low androgen levels is also supported in CRPC cells *in vivo*. It has been shown that intraprostatic DHT levels remain relatively high and, in general, only 50% reductions have been observed, despite androgen deprivation therapy (Labrie *et al.* 2005).

5.2.2 The effect of increased AR levels on the transcription of target genes

To study the effect of overexpressed *AR* on transcription, microarrays were performed at different time points and DHT concentration for all of the different AR-overexpressing cells. The number of androgen-responsive genes was clearly associated with the *AR* expression level. The number of androgen-regulated genes was highest in the LNCaP-ARhi and VCaP cells expressing the highest amount of *AR*. However, the number of differentially expressed genes was not significantly higher in the VCaP cells compared to the LNCaP-ARhi cells. LNCaP-ARmo cells had concordantly more androgen-regulated genes than the LNCaP-pcDNA3.1 cells, but less than the LNCaP-ARhi or VCaP cells (original publication II). Interestingly, in unsupervised hierarchical clustering which was based on differentially expressed genes, the VCaP and the

LNCaP-ARhi cells clustered together despite their different genetic backgrounds. This result indicates a very strong influence of AR on the genome-wide expression of PC cells. The well-known androgen-regulated genes, such as *PSA*, *ACPP*, *TMPRSS2* and *NKX3-1*, were found to be sensitized up to 10-fold in the LNCaP-ARhi cells compared to the LNCaP-pcDNA3.1 cells. Approximately, a 10-fold higher DHT concentration was required for the LNCaP-pcDNA3.1 cells than for the LNCaP-ARhi or LNCaP-ARmo cells for an equivalent level of transcription. Thus, the higher AR level sensitizes both the number of androgen-regulated genes as well as the level of transcription of the target genes (original publication II).

Several microarray studies have addressed androgen regulation of gene expression (Amler *et al.* 2000, Nelson *et al.* 2002, Velasco *et al.* 2004, York *et al.* 2005, Hendriksen *et al.* 2006, Steele *et al.* 2006), the effects of castration on gene expression (Holzbeierlein *et al.* 2004, Hendriksen *et al.* 2006, Wang et al 2007b, Mostaghel *et al.* 2007, Ma *et al.* 2009) and expression profiles comparing different stages of PC (Sirotnak *et al.* 2004, Chen *et al.* 2006, Murillo *et al.* 2006, Morgenbesser *et al.* 2007, Kawada *et al.* 2007, Tamura *et al.* 2007). However, the data from these studies are only partially concordant. Reasons for the discrepant findings could be the use of different ligands in the different studies (T, DHT or synthetic androgen R1881), different concentrations, different time points and heterogeneity of the PC cells in different laboratories. Importantly, in all other studies, only one androgen concentration was used. The expression profiles are also time-dependent. In our data, *e.g.*, several genes including *tumor necrosis factor receptor superfamily member 10b* (*TNFRSF10B*), *forkhead box O1* (*FOXO1*), *tumor necrosis factor alpha-induced protein3* (*TNFAIP3*), and *serum/glucocorticoid regulated kinase* (*SGK*) were upregulated strongly at the 4 h time point, but showed less or very little upregulation at the 24 h time point (original communication II).

LNCaP and VCaP cells contain the known genetic rearrangements of *TMPRSS2*:*ETV1* and *TMPRSS2*:*ERG*, respectively (Tomlins *et al.* 2005, Saramäki *et al.* 2008, Kumar-Sinha *et al.* 2008). Over two-fold upregulation of *ERG* and *ETV1* expression was detected in the VCaP cells in 1 nM DHT and in LNCaP-pcDNA3.1 in 10 nM DHT compared to untreated cells. In LNCaP-ARhi cells, *ETV1* expression was higher under lower androgen level conditions compared to LNCaP-pcDNA3.1 cells (original communication II). On the other hand, the *ETV1* promoter has also been shown to be directly AR-regulated (Cai *et al.* 2007). Of the other *Ets* family members, *ELK4* and *EHF* mRNA levels were also increased with androgens in LNCaP-ARhi cells (original communication II). The *ELK4* gene is known to be an AR target in human prostate cancer cells with an association with cell growth *in vitro* (Makkonen *et al.* 2007). *ELK4* has also been reported to be fused with the *SLC45A3* gene, which is strongly regulated by androgens. This fusion is possibly due to an over trans-splicing mechanism (Rickman *et al.* 2009). *EHF* is methylated and has been suggested to function as a candidate tumor suppressor in AR-negative prostate cancer cells (Cangemi *et al.* 2008).

5.2.3 Gene ontology classes associated with androgen and AR levels

To identify the pathways that are differentially regulated, gene ontology analyses were performed for both up- and downregulated genes. LNCaP-ARhi cells had more upregulated genes related to the mitotic cell cycle, regulation of cell cycle, organelle organization and biogenesis, cellular protein metabolism and DNA metabolism when compared to both LNCaP-pcDNA3.1 and LNCaP-ARmo cells at 24 h. At the 4 h time point, intracellular signaling cascades, including phosphoinositide-mediated signaling and the androgen receptor signaling pathway, cell cycle, and lipid metabolism (including cholesterol biosynthesis) were also upregulated significantly. Only apoptosis was significantly upregulated at the 4 h time point, but was no longer upregulated at the 24 h time point (original communication II).

DNA metabolism (DNA replication), cell cycle, cell organization and biogenesis (spindle organization and biogenesis), cell division and intracellular signaling (phosphoinositide-mediated signaling) were found to be significantly upregulated in LNCaP-ARhi cells treated with 1 nM DHT, but not in LNCaP-pcDNA3.1 or LNCaP-ARmo cells. At higher DHT concentrations, the same ontology classes were upregulated in all cells. The number of upregulated genes was, however, always highest in LNCaP-ARhi cells. The most significantly upregulated gene classes in LNCaP-ARmo cells were lipid metabolism (cholesterol biosynthesis) and secretory pathway genes (ER-to-Golgi transport). No significantly downregulated ontology classes were found (original communication II).

The gene expression profiles and ontology analysis are well in concordance with the growth analysis data. DHT treatments showed the most significant increased growth in 1 nM DHT and the most significant upregulation of genes associated with proliferation- and mitotic spindle-associated genes in LNCaP-ARhi cells at the same concentration (original communication II). The upregulation of cell cycle genes and enhanced steroidogenesis, DNA, RNA and protein metabolism have also been reported by others (Gregory *et al.* 1998 and 2001, Sirotnak *et al.* 2004, Zimmerman *et al.* 2004, Swinnen *et al.* 2004, Ma *et al.* 2009, Mostaghel *et al.* 2009, Leon *et al.* 2010, Locke *et al.* 2010, Vander Griend *et al.* 2010).

5.2.4 Direct AR target genes involved in growth during PC progression

To see what genes are concordantly upregulated by androgens and AR, and are specific for AR overexpression as well as PC progression, Venn diagrams were constructed from the up- and downregulated genes and were studied in the clinical PC datasets. All together, 55 genes showed a greater than two-fold higher expression in LNCaP-ARhi cells compared to LNCaP-pcDNA3.1 or LNCaP-ARmo cells in 1 nM DHT, were upregulated at least >1.5 fold and >2 fold by 1 nM DHT stimulation after 4 h and 24 h, respectively, and were expressed at similar or higher levels in the VCaP as the LNCaP-ARhi cells at any DHT concentration (original communication II). Of those genes, 27 were expressed significantly higher in CRPC or metastatic PC and were found to have an AR binding site (ARB) <200 kb from the transcription start site (TSS), indicating direct AR regulation. The list of genes consists of cell cycle genes, *e.g.*, *CDK1*, *CDK2*,

cyclin A2, *cyclin B2*, and *CDC20*. All of them are involved in cell cycle, cell cycle control, the mitotic spindle or DNA replication (original communication II, Fig. 7). Since several cell cycle genes were upregulated, we tested the effect of a small molecule CDK1/2 and cyclin inhibitor, roscovitine, on the growth of the LNCaP-AR cells. The growth of the LNCaP-ARhi cells was significantly more sensitive to the roscovitine in the presence of androgens than the growth of the LNCaP-pcDNA3.1 and LNCaP-ARmo cells (original communication II).

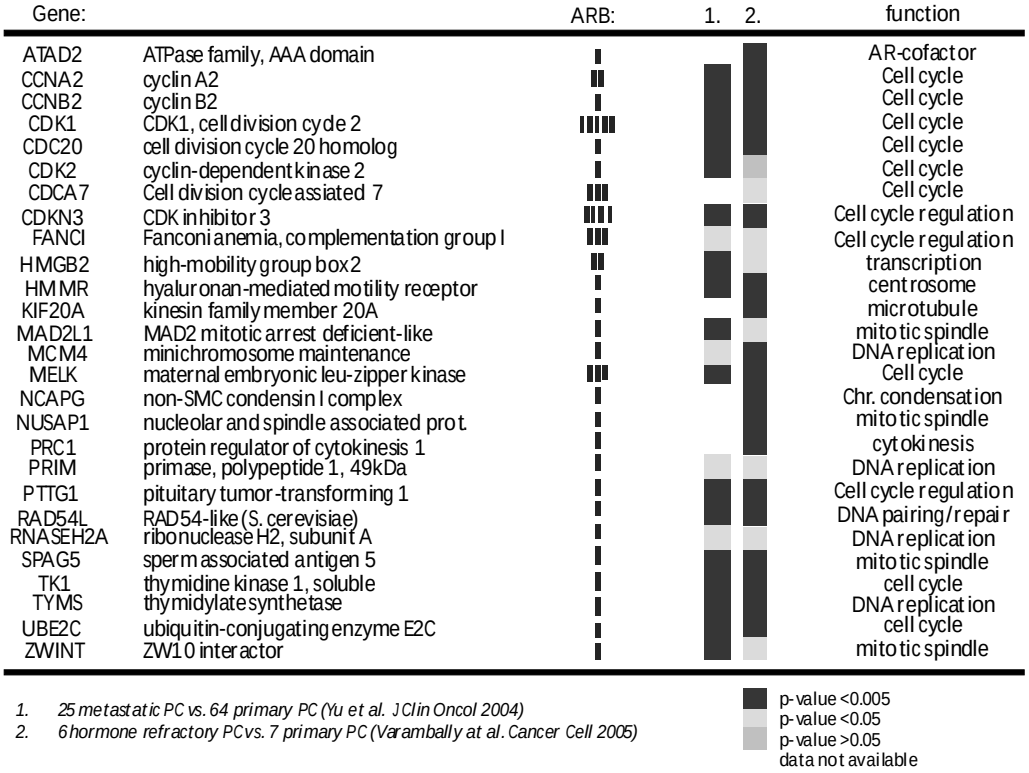

Figure 7. Direct AR target genes upregulated in CRPC or metastatic PC. *Direct AR target genes overexpressed in CRPC. ARB (AR binding sites) are indicated as thick lines with the number of observed bindings varying from 1 to 5. Simplified figure from original communication II.*

Cyclin A1, *B1* and *CDK1*, *CDK2*, *CDK4* have previously been reported to be increased by androgens in PC cells and decreased after castration in CWR22 xenografts (Lu *et al.* 1997, Gregory *et al.* 1998 and 2001, Wang *et al.* 2009b). Wang *et al.* (2009b) also showed that greater AR binding to the enhancer elements of the M-phase genes *CDK1*, *cyclin A2*, *CDC20*, *UBE2C*, *NUSAP1* and *PTTG1* correlated with increased expression in LNCaP-abl cells. On the contrary, the rest of the 27 mitotic phase-associated genes such as *cyclin B2*, *CDCA7*, *FANCI*, *KIF20A*, *MCM4*, *MELK* and *NCAPG* have not previously been reported to be directly androgen-regulated (Fig. 7). According to the results of Wang *et al.* (2009b), AR selectively upregulates M-phase cell cycle genes in CRPC cells by histone H3K4 methylation and by binding the FoxA1 transcription factor. In a study of 140 radical prostatectomy specimens, CDK1 protein expression was correlated with a high Gleason score, advanced pathologic stage, nondiploid DNA content, and metastases. On multivariate analysis, a high Gleason grade and CDK1 immunoreactivity predicted disease recurrence independently of the pathologic stage

(Kallakury *et al.* 1997, 1999). Interestingly, CDK1 has previously been shown to phosphorylate and stabilize AR (Chen *et al.* 2006). Thus, there may also be a positive feedback mechanism between AR and CDK1. These results, together with the higher sensitivity to roscovitine inhibition in LNCaP-ARhi cells, indicate the high significance of M-phase genes for causing a growth advantage in the CRPC cells.

Approximately 40% of the 55 upregulated genes had no ARB within 200 kb of the TSS. These genes also included several M-phase-associated genes known to be upregulated in CRPC, such as *aurora kinase A* and *B, topoisomerase (DNA) II alpha, cell division cycle 25 homolog A* and *cell division cycle associated 3,4* and *5*. It has been demonstrated that the chromosomal binding sites of steroid hormone receptors are often distal and can even be located as far as 1 Mb away from the TSS (Fullwood *et al.* 2009, Paakinaho *et al.* 2010). Thus, these genes might still be directly regulated by AR.

Biphasic androgen regulation of the *MYC* oncogene was detected in the LNCaP-AR model (original communication II), which is concordant with earlier studies (Katz *et al.* 1989, Wolf *et al.* 1992, Kokontis *et al.* 1994, Bièche *et al.* 2001). Similar to our studies, the *AR* overexpressing cell line showed higher expression of *MYC* in low levels of androgens than the parental cell line. In addition, the expression was decreased under higher concentrations of DHT (Kokontis *et al.* 1994, original communication II). MYC regulates the cell cycle and apoptosis in a complex manner (reviewed by Herold *et al.* 2009). MYC has been suggested, for example, to inhibit cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) and enhance the transcription of *E2F transcription factors* (*E2F1* and *E2F2*) in cancer cells via a mechanism that also includes miRNAs (Wang *et al.* 2008, Leone *et al.* 2001, O'Donnell *et al.* 2005). On the other hand, putative MYC regulation of *AR* expression has also been suggested (Grad *et al.* 1999). Interestingly, ATAD2 has been suggested to act as coactivator of both MYC and AR, and according to our results, it is androgen-regulated (Ciró *et al.* 2009, Zou *et al.* 2009, original communication II).

MYC- and *PI3K* (PTEN-Akt pathway)-overexpressing, immortalized normal human prostate epithelial cells (PrECs) have been shown to be very aggressive, closely resembling human PC, when also transduced with *AR* and injected into the mouse prostate. Tumor size correlated directly with blood androgen levels (Berger *et al.* 2004). A simplified schematic presentation of AR-induced gene expression in PC with possible feedback interactions is illustrated in Figure 8.

5.3 Androgen regulation of microRNAs in prostate cancer

5.3.1 Androgen-regulated miRNAs

To study putative androgen- and AR-regulated miRNAs, AR-overexpressing cell lines and castrated xenograft sample pairs were used for miRNA microarray studies. Seventeen miRNAs were found to be >1.5-fold up- or downregulated in LNCaP or VCaP cell lines 24 h after DHT treatment. Only two miRNAs, miR-29a and miR-29b, were >2-fold upregulated by 100 nM DHT. No miRNA was up- or down regulated >1.5-fold after 4 h of DHT stimulation. A total of 103 miRNAs were >1.5-fold up- or downregulated after castration in all of the xenografts. A total of 42 miRNAs were up- or downregulated in AR-positive xenografts (n=9), whereas 49 were found in both AR-positive and AR-negative xenografts; 12 miRNAs were only affected in the AR-negative xenografts. Altogether, 34 miRNAs showed similar DHT regulation according to standardized RANK-based analysis of the combined data for both the cell lines and the xenografts (original communication III).

Three studies were previously published using R1881-stimulated LNCaP and/or LAPC4 cells (Shi *et al.* 2007, Ambs *et al.* 2008 and Ribas et al 2009). Compared to those studies, similar androgen regulation was seen our study for miR-21, 29a, 29b and 221 in cell lines and for miR-17, 18b, 19b, 20a, 20b, 93 and 148a in xenografts. As seen in mRNA studies, differences may result from variations in the ligands used, the time points assessed and the cell lines used in the different studies. It is noteworthy that in the Ribas *et al.* (2009) study, a 72 h time point was used, which means that the cells were exposed to the synthetic androgen for three times longer than in our study. Similar to our study, Ambs *et al.* (2008) used a 24 h time point and detected only one upregulated miRNA in the parental LNCaP cells. The need for longer time points in order to detect miRNA regulation may indicate the slow progression of miRNA maturation via the drosha and dicer enzymes (reviewed by Bartel 2009), or that the regulation involves a very complex system that requires the assembly of several *cis*-acting co-regulators. It may also indicate that androgen regulation of these miRNAs is not directly AR induced.

While 1.6% (349/22,177) of protein-coding genes showed >2-fold upregulation in LNCaP-ARhi cells after 24 h at 100 nM DHT, only 2 of the 723 (0.3%) miRNAs showed >2-fold upregulation in the same cells at the same time point and DHT concentration (original communication III). This finding indicates that there are probably less directly androgen-regulated miRNAs than mRNAs. In light of the recent documentation which shows that many miRNAs specifically downregulate cell cycle and proliferation genes (reviewed by Bueno *et al.* 2008), the reduction of androgen regulated miRNA expression during CRPC progression seems reasonable. In fact, in the PC xenograft panel, the number of differentially expressed miRNAs after castration was highest in androgen-dependent compared to androgen-independent xenografts and was lowest in the totally AR-negative and androgen-independent xenografts (original communication III).

DHT-regulated miRNAs in the PC cell lines were also very different from the miRNAs induced or suppressed by castration in the xenografts. The expression level changes

were also more moderate in the PC cell lines. One reason for this finding could be, as discussed above, that DHT stimulation time was 24 h, whereas the time of castration was 7-14 days before sample collection. Only four miRNAs were regulated with similar intensity (>1.5 fold) in cell lines upon DHT stimulation and in xenografts after castration. Strikingly, some of the miRNAs, which were upregulated in LNCaP and VCaP cells, were also upregulated after castration (*e.g.*, miR-29a and miR-29b) in both AR-positive and -negative xenografts (original communication III). Differential regulation may also reflect the ongoing apoptosis after castration. In particular, the miRNAs altered in AR-negative xenografts cannot be directly regulated by the classical AR-dependent pathway. The differential expression could be due to the non-AR-mediated action of androgens (Heinlein and Chang 2002).

5.3.2 Differentially expressed miRNAs in clinical samples

To study the behavior of androgen-regulated miRNAs in clinical PC samples, both untreated and CRPC samples were assessed for miRNA expression. Of the androgen-regulated miRNAs, the expression levels of miR-18a, 18b, 19a, 20b, 21, 32, 126, 141, 148a, 203 and 375 were significantly upregulated in CRPC, and miR-100, 125b, 199a-5p, 214 and 221 were consistently downregulated in CRPC compared to BPH. miR-18a, -141, and -375 were also significantly upregulated and miR-221 was downregulated in untreated PC samples (original communication III).

Consistent with our expression profiles of androgen-regulated miRNAs in clinical samples, miR-21, miR-100, miR-125b, miR-141 and miR-221 were differentially expressed in other studies as well. miR-21, miR-100, miR-141, miR-375 and miR-221 show concordant results with other studies both in terms of androgen-regulation and in the expression profiles of clinical PC samples (Leite *et al.* 2009, Schaefer *et al.* 2009, Ribas *et al.* 2009 and 2010, Spahn *et al.* 2010, Szczyrba *et al.* 2010). Oncogenic miR-21 has been shown to be androgen-upregulated. It has also been shown to target programmed cell death 4 (PDGF4) and is upregulated in PC (Ribas *et al.* 2009 and 2010, Lu *et al.* 2008). miR-125b, the first reported androgen-regulated miRNA, has been shown to be both up- and downregulated in clinical samples and dysregulated by androgens (Shi *et al.* 2007, Schaefer *et al.* 2010).

5.3.3 Effect of miR-141 on the growth of PC cells

Since miR-141 was concordantly androgen-upregulated and overexpressed during PC progression, the biological effect of overexpressed miR-141 was studied *in vitro*. The forced overexpression of miR-141 by transient transfection enhanced the growth of LNCaP-pcDNA3.1 cells in the presence of low levels of DHT. Similarly, inhibition of miR-141 by transient transfection (anti-miR-141) reduced the growth of LNCaP-ARhi cells in low or depleted androgen medium. Ten putative miR-141 mRNA targets were affected by transfections with miR-141 and anti-miR-141 over 1.5 fold. Of those, *diaphanous homolog 3 (Drosophila) (DIAPH3), clock homolog* (mouse) *(CLOCK), zinc finger protein 800 (ZNF800)* and *OTU domain containing 4 (OTUD4)* contained predicted miR-141 binding sites (original communication III).

miR-141 is an epithelial-specific microRNA belonging to an evolutionarily conserved family of miRNAs including *miR-200a, miR-200b, miR-200c,* and *miR-429*. miR-141 has been shown to inhibit the differentiation of pre-osteoblasts to mature osteoblasts by targeting the bone-generating transcription factor *Dlx5* and can also induce embryonic stem cell differentiation in mice, which is regulated by Myc (Itoh *et al.* 2009, Lin *et al.* 2009). High expression of miR-141 has been reported in a wide range of common epithelial cancers including breast, lung, nasopharyngeal and ovarian cancer (Iorio *et al.* 2007, Nam *et al.* 2008, Zhang *et al.* 2010, Szczyrba *et al.* 2010). Higher serum levels of miR-141 have also been suggested as a novel biomarker for PC with high sensitivity (Mitchell *et al.* 2008). The downregulation of miR-141 has been reported in renal cancers and pair-matched gastric, colon, lung and breast cancers (Nakada *et al.* 2008, Du *et al.* 2009, Baffa *et al.* 2009). Interestingly, the putative miR-141 target gene *diaphanous homolog 3* (*DIAPH3*) has been reported to be deleted, controlling an oncosomic secretion formation in PC cells (Di Vizio *et al.* 2009). The SNPs in the other putative miR-141 target gene, *clock homolog* (*CLOCK*), have also been linked to PC (Zhu *et al.* 2009). *CLOCK* genes are responsible for the circadian rhythms important for hormone balance and have been suggested as possible tumor suppressor genes (Fu *et al.* 2003). However, we were not able to confirm the reduction of the putative target genes at the protein level. A summary and simplified schematic presentation of AR-induced miRNAs and the protein coding genes putatively involved in the emergence of PC and CRPC, according to our results and the literature, with a possible feedback interaction is illustrated in Figure 8.

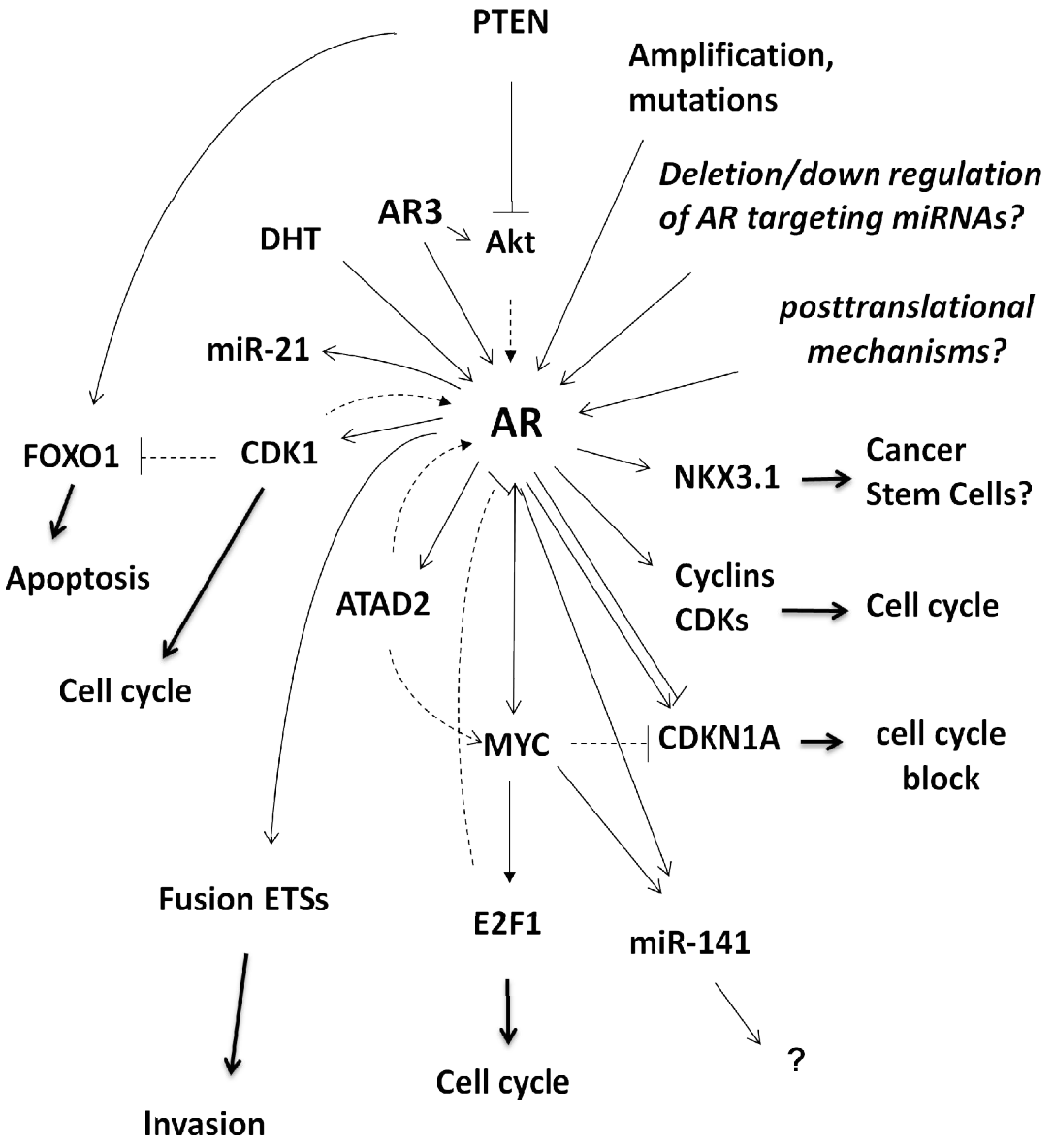

Figure 8. Simplified schematic presentation of the AR signaling pathway in prostate cancer. *This figure shows the main activities associated with AR which promote PC progression, CRPC, cell growth and invasion. Solid lines indicate transcriptional activation (arrowhead) or inhibition (blunt end), according to our stated results and the previous literature. A dashed line indicates post-translational regulation according to the literature referred to in the text. Bold lines show the downstream biological events.*

6. CONCLUSIONS

The main findings and conclusions of this thesis were:

Altogether, nine novel mutations were found in the promoter and UTR regions in genetically unstable cell lines and xenografts. None of these alterations were recurrent, nor were they present in the clinical prostate cancer specimens or normal controls. Six single base changes were found in two geneticly unstable cell lines. Results indicate that the found variants are rare mutations which are unlikely to be found in the majority of PC or CRPC cases.

In the presence of 1 nM DHT or lower, high *AR*-expressing cells grew significantly faster than lower level *AR*-expressing LNCaP cells. The cells overexpressing *AR* were also capable of growing longer in CSS medium without any DHT supplementation. The results indicate that *AR* overexpression increases the proliferation of PC cells in low androgen medium and the growth is balanced by the level of both the ligand and the receptor.

The number of androgen-responsive genes was clearly associated with *AR* expression level. The number of androgen-regulated genes was highest in LNCaP-ARhi and VCaP cells expressing the highest amount of *AR*. VCaP and the LNCaP-ARhi cells clustering together in unsupervised hierarchical clustering indicates a very strong influence of AR on the genome-wide gene expression in PC cells. Well-known androgen-regulated genes, such as *PSA*, *ACPP*, *TMPRSS2* and *NKX3-1*, were found to be sensitized up to 10-fold in the LNCaP-ARhi cells compared to the LNCaP-pcDNA3.1 cells.

Altogether, 55 genes were >2-fold induced in low androgen levels and were more highly expressed in high AR-containing cells. Of those, 27 had an AR binding site <200 kb from the TSS and were expressed at significantly higher levels in CRPC. All of these genes were mitosis- or cell cycle-associated genes. The growth of the LNCaP-ARhi cells was consistently more sensitive to the inhibition of CDK1 and CDK2 by the small molecule inhibitor, roscovitine, than the growth of LNCaP-pcDNA3.1 and LNCaP-ARmo cells. Results indicate that at least one of the main mechanisms of CRPC growth is the direct ability of AR to enhance the transcription of cell cycle- and mitosis-associated genes.

Only two miRNAs were >2-fold upregulated by 100 nM DHT and no miRNAs were >1.5-fold up- or downregulated at lower, physiological DHT concentrations. One to two weeks after castration, more androgen-regulated miRNAs were found. The highest number of castration-affected miRNAs was found in androgen-dependent xenografts and the lowest was found in androgen-independent and AR-negative xenografts. Of the androgen- or castration-regulated miRNAs, the expression levels of miR-18a, -141, and -375 were significantly upregulated and miR-221 was downregulated in untreated PC and CRPC. The transient transfection studies with miR-141 suggest that miR-141 might have a role in supporting androgen-dependent and independent-growth, but overexpression of miR-141 alone was not sufficient to induce androgen-independent growth in PC cells.

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Kati Waltering

REFERENCES

Abrahamsson PA, Lilja H, Falkmer S, Wadström LB (1988). Immunohistochemical distribution of the three predominant secretory proteins in the parenchyma of hyperplastic and neoplastic prostate glands. Prostate.12(1):39-46.

Agoulnik IU, Vaid A, Nakka M, Alvarado M, Bingman WE 3rd, Erdem H, Frolov A, Smith CL, Ayala GE, Ittmann MM, Weigel NL. (2006) Androgens modulate expression of transcription intermediary factor 2, an androgen receptor coactivator whose expression level correlates with early biochemical recurrence in prostate cancer. Cancer Res. 66(21):10594-602.

Andriole GL, Bostwick DG, Brawley OW, Gomella LG, Marberger M, Montorsi F, Pettaway CA, Tammela TL, Teloken C, Tindall DJ, Somerville MC, Wilson TH, Fowler IL, Rittmaster RS; REDUCE Study Group. (2010) Effect of dutasteride on the risk of prostate cancer. N Engl J Med. 362(13):1192-202.

Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, Yfantis HG, Stephens RM, Croce CM. (2008) Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res. 68(15):6162-70.

Amler LC, Agus DB, LeDuc C, Sapinoso ML, Fox WD, Kern S, Lee D, Wang V, Leysens M, Higgins B, Martin J, Gerald W, Dracopoli N, Cordon-Cardo C, Scher HI, Hampton GM. (2000) Dysregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R1. Cancer Res. 60(21):6134-41.

Asatiani E, Huang WX, Wang A, Rodriguez Ortner E, Cavalli LR, Haddad BR, Gelmann EP.(2005) Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res. 65(4):1164-73.

Attard G, Reid AH, Yap TA, Raynaud F, Dowsett M, Settatree S, Barrett M, Parker C, Martins V, Folkerd E, Clark J, Cooper CS, Kaye SB, Dearnaley D, Lee G, de Bono JS. (2008) Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J Clin Oncol. 26(28):4563-71.

Attard G, Reid AH, A'Hern R, Parker C, Oommen NB, Folkerd E, Messiou C, Molife LR, Maier G, Thompson E, Olmos D, Sinha R, Lee G, Dowsett M, Kaye SB, Dearnaley D, Kheoh T, Molina A, de Bono JS. (2009a) Selective inhibition of CYP17 with abiraterone acetate is highly active in the treatment of castration-resistant prostate cancer. J Clin Oncol. 27(23):3742-8.

Attard G, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, Levink R, Coumans F, Moreira J, Riisnaes R, Oommen NB, Hawche G, Jameson C, Thompson E, Sipkema R, Carden CP, Parker C, Dearnaley D, Kaye SB, Cooper CS, Molina A, Cox ME, Terstappen LW, de Bono JS. (2009b) Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. Cancer Res. 69(7):2912-8.

Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM, Rosenberg A. (2009) MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. J Pathol.219(2):214-21.

Bartel DP. (2009) MicroRNAs: target recognition and regulatory functions. Cell.136:215-33.

Berger R, Febbo PG, Majumder PK, Zhao JJ, Mukherjee S, Signoretti S, Campbell KT, Sellers WR, Roberts TM, Loda M, Golub TR, Hahn WC. (2004) Androgen-

induced differentiation and tumorigenicity of human prostate epithelial cells. Cancer Res. 64(24):8867-75.

Bethel CR, Faith D, Li X, Guan B, Hicks JL, Lan F, Jenkins RB, Bieberich CJ, De Marzo AM. (2006) Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. Cancer Res. 66(22):10683-90.

Bièche I, Parfait B, Tozlu S, Lidereau R, Vidaud M. (2001) Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene. Carcinogenesis. 22(9):1521-6.

Bill-Axelson A, Holmberg L, Filén F, Ruutu M, Garmo H, Busch C, Nordling S, Häggman M, Andersson SO, Bratell S, Spångberg A, Palmgren J, Adami HO, Johansson JE; Scandinavian Prostate Cancer Group Study Number 4. (2008) Radical prostatectomy versus watchful waiting in localized prostate cancer: the Scandinavian prostate cancer group-4 randomized trial. J Natl Cancer Inst. 100(16):1144-54.

Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP. (1999) Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. Cancer Res. 59(4):803-6.

Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD. (2001a) Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res. 7(5):1273-81.

Buchanan G, Yang M, Harris JM, Nahm HS, Han G, Moore N, Bentel JM, Matusik RJ, Horsfall DJ, Marshall VR, Greenberg NM, Tilley WD. (2001b) Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. Mol Endocrinol. 15(1), 46-56.

Bueno MJ, de Castro IP, Malumbres M. (2008) Control of cell proliferation pathways by microRNAs. Cell Cycle. 7(20):3143-8.

Cai C, Hsieh CL, Omwancha J, Zheng Z, Chen SY, Baert JL, Shemshedini L. ETV1 is a novel androgen receptor-regulated gene that mediates prostate cancer cell invasion. Mol Endocrinol. 2007;21(8):1835-46.

Callewaert L, Van Tilborgh N, Claessens F. (2006) Interplay between two hormone-independent activation domains in the androgen receptor. Cancer Res. 66(1), 543-53.

Cangemi R, Mensah A, Albertini V, Jain A, Mello-Grand M, Chiorino G, Catapano CV, Carbone GM.(2008) Reduced expression and tumor suppressor function of the ETS transcription factor ESE-3 in prostate cancer. Oncogene. 27(20):2877-85.

Carver BS, Bianco FJ Jr, Scardino PT, Eastham JA. (2006) Long-term outcome following radical prostatectomy in men with clinical stage T3 prostate cancer. J Urol. 176(2):564-8.

Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A, Alimonti A, Nardella C, Varmeh S, Scardino PT, Cordon-Cardo C, Gerald W, Pandolfi PP. (2009) Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. Nat Genet. 41(5):619-24.

Chamberlain NL, Driver ED, Miesfeld RL. (1994) The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res. 22: 3181–6.

Chang C, Kokontis J, Liao S. (1988) Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science. 240: 324-326.

Chang CY, Walther PJ, McDonnell DP. (2001) Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer.Cancer Res. 61(24):8712-7.

Chen S, Supakar PC, Vellanoweth RL, Song CS, Chatterjee B and Roy AK. (1997): Functional Role of a Conformationally Flexible Homopurine/ Homopyrimidine Domain of the Androgen Receptor Gene Promoter Interacting with SP1 and a Pyrimidine Single Strand DNA-Binding Protein.Mol Endocrinol. 11:3-15.

Chen Y, Wang J, Fraig MM, Metcalf J, Turner WR, Bissada NK, Watson DK, Schweinfest CW. (2001) Defects of DNA mismatch repair in human prostate cancer. Cancer Res. 61:4112-4121.

Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. (2004) Molecular determinants of resistance to antiandrogen therapy. Nat Med. 10(1):33-9.

Chen S, Xu Y, Yuan X, Bubley GJ, Balk SP. (2006a) Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. Proc Natl Acad Sci U S A. 103(43):15969-74.

Chen Q, Watson JT, Marengo SR, Decker KS, Coleman I, Nelson PS, Sikes RA. (2006b)Gene expression in the LNCaP human prostate cancer progression model: progression associated expression in vitro corresponds to expression changes associated with prostate cancer progression in vivo. Cancer Lett. 244(2):274-88.

Chen Y, Sawyers CL, Scher HI. (2008) Targeting the androgen receptor pathway in prostate cancer. Curr Opin Pharmacol. 8(4):440-8.

Chisholm GD, Hedlund PO, Adolfsson J, Denis LJ, Friberg S, Johansson JE, Peeling B. The TNM system of 1992. Comments from the TNM working group. Consensus Conference on Diagnosis and Prognostic Parameters in Localized Prostate Cancer. Stockholm, Sweden (1994). Scand J Urol Nephrol Suppl. 162:107-14;

Choong CS, Wilson EM. (1998): Trinucleotide repeats in the human androgen receptor: a molecular basis for disease. J Mol Endocrinol. 21: 235-257.

Ciró M, Prosperini E, Quarto M, Grazini U, Walfridsson J, McBlane F, Nucifero P, Pacchiana G, Capra M, Christensen J, Helin K. (2009) ATAD2 is a novel cofactor for MYC, overexpressed and amplified in aggressive tumors. Cancer Res. 69(21):8491-8.

Coleman MP, Quaresma M, Berrino F, Lutz JM, De Angelis R, Capocaccia R, Baili P, Rachet B, Gatta G, Hakulinen T, Micheli A, Sant M, Weir HK, Elwood JM, Tsukuma H, Koifman S, E Silva GA, Francisci S, Santaquilani M, Verdecchia A, Storm HH, Young JL; CONCORD Working Group. (2008) Cancer survival in five continents: a worldwide population-based study (CONCORD). Lancet Oncol 9:730-56.

Culig Z, Hobisch A, Cronauer MV, Cato AC, Hittmair A, Radmayr C, Eberle J, Bartsch G, Klocker H. (1993) Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol. 7(12):1541-50

Culig Z, Hoffmann J, Erdel M, Eder IE, Hobisch A, Hittmair A, Bartsch G, Utermann G, Schneider MR, Parczyk K, Klocker H. (1999) Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system. Br J Cancer. 81(2), 242-51

Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y. (1987) The endocrinology and developmental biology of the prostate. Endocr Rev. 8(3):338-62.

Curado. MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M and Boyle P, eds Cancer Incidence in Five Continents (2007) Vol. IX IARC Scientific Publications No. 160, Lyon, IARC.

Dagvadorj A, Tan SH, Liao Z, Cavalli LR, Haddad BR, Nevalainen MT. (2008) Androgen-regulated and highly tumorigenic human prostate cancer cell line established from a transplantable primary CWR22 tumor. Clin Cancer Res. 14(19):6062-72.

Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. (2008) Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. Cancer Res. 68(13):5469-77.

Dong JT. (2006) Prevalent mutations in prostate cancer. J Cell Biochem. 97(3):433-47.

Di Vizio D, Kim J, Hager MH, Morello M, Yang W, Lafargue CJ, True LD, Rubin MA, Adam RM, Beroukhim R, Demichelis F, Freeman MR. (2009) Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. Cancer Res. 69(13):5601-9.

Donovan MJ, Osman I, Khan FM, Vengrenyuk Y, Capodieci P, Koscuiszka M, Anand A, Cordon-Cardo C, Costa J, Scher HI. (2009) Androgen receptor expression is associated with prostate cancer-specific survival in castrate patients with metastatic disease. BJU Int. Jul 9. [Epub ahead of print]

Du Y, Xu Y, Ding L, Yao H, Yu H, Zhou T, Si J. (2009) Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. J Gastroenterol. 44(6):556-61.

Duff J, McEwan IJ. (2005) Mutation of histidine 874 in the androgen receptor ligand-binding domain leads to promiscuous ligand activation and altered p160 coactivator interactions.Mol Endocrinol. 19(12):2943-54.

Edwards J, Krishna NS, Witton CJ, Bartlett JM. (2003a) Gene amplifications associated with the development of hormone-resistant prostate cancer. Clin Cancer Res. 9(14):5271-81.

Edwards J, Krishna NS, Grigor KM, Bartlett JM. (2003) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer. 89(3), 552-6.

Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, Sawyers CL. (2003) Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell. 4:223–38

Eeles RA, Kote-Jarai Z, Al Olama AA, Giles GG, Guy M, Severi G, Muir K, Hopper JL, Henderson BE, Haiman CA, Schleutker J, Hamdy FC, Neal DE, Donovan JL, Stanford JL, Ostrander EA, Ingles SA, John EM, Thibodeau SN, Schaid D, Park JY, Spurdle A, Clements J, Dickinson JL, Maier C, Vogel W, Dörk T, Rebbeck TR, Cooney KA, Cannon-Albright L, Chappuis PO, Hutter P, Zeegers M, Kaneva R, Zhang HW, Lu YJ, Foulkes WD, English DR, Leongamornlert DA, Tymrakiewicz M, Morrison J, Ardern-Jones AT, Hall AL, O'Brien LT, Wilkinson RA, Saunders EJ, Page EC, Sawyer EJ, Edwards SM, Dearnaley DP, Horwich A, Huddart RA, Khoo VS, Parker CC, Van As N, Woodhouse CJ, Thompson A, Christmas T, Ogden C, Cooper CS, Southey MC, Lophatananon A, Liu JF, Kolonel LN, Le Marchand L, Wahlfors T, Tammela TL, Auvinen A, Lewis SJ, Cox A, FitzGerald LM, Koopmeiners JS, Karyadi DM, Kwon EM, Stern MC, Corral R, Joshi AD, Shahabi A, McDonnell SK, Sellers TA, Pow-

Sang J, Chambers S, Aitken J, Gardiner RA, Batra J, Kedda MA, Lose F, Polanowski A, Patterson B, Serth J, Meyer A, Luedeke M, Stefflova K, Ray AM, Lange EM, Farnham J, Khan H, Slavov C, Mitkova A, Cao G; UK Genetic Prostate Cancer Study Collaborators/British Association of Urological Surgeons' Section of Oncology; UK ProtecT Study Collaborators; PRACTICAL Consortium, Easton DF. (2009) Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. Nat Genet. 41(10):1116-21.

Epstein JI, Allsbrook WC Jr, Amin MB, Egevad LL. (2006) Update on the Gleason grading system for prostate cancer: results of an international consensus conference of urologic pathologists.Adv Anat Pathol. 13(1):57-9.

Faber PW, van Rooij HC, van der Korput HA, Baarends WM, Brinkmann AO, Grootegoed JA, Trapman J. (1991): Characterization of the human androgen receptor transcription unit. J Biol Chem 266: 10743-10749.

Faber PW, van Rooij HC, Schipper HJ, Brinkmann AO, Trapman J. (1993): Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. The role of Sp1. J Biol Chem 268: 9296-9301.

Fasciana C, van der Made AC, Faber PW, Trapman J. (1996) Androgen regulation of the rat keratinocyte growth factor (KGF/FGF7) promoter. Biochem Biophys Res Commun. 220(3):858-63.

Faus H, Haendler B. (2006) Post-translational modifications of steroid receptors. Biomed Pharmacother. 60(9):520-8.

Feldman BJ, Feldman D. (2001) The development of androgen-independent prostate cancer. Nat Rev Cancer. 1(1):34-45.

Fu M, Wang C, Reutens AT, Wang J, Angeletti RH, Siconolfi-Baez L, Ogryzko V, Avantaggiati ML, Pestell RG. (2000) p300 and p300/cAMP-response element-binding proteinassociated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 275(27):20853–60.

Fu M, Wang C, Wang J, Zhang X, Sakamaki T, Yeung YG, Chang C, Hopp T, Fuqua SA, Jaffray E, Hay RT, Palvimo JJ, Jänne OA, Pestell RG. (2002) Androgen receptor acetylation governs trans activation and MEKK1-induced apoptosis without affecting in vitro sumoylation and trans-repression function. Mol Cell Biol. 22(10):3373-88.

Fu L, Lee CC. (2003) The circadian clock: pacemaker and tumour suppressor. Nat Rev Cancer. 3(5):350-61.

Fu M, Rao M, Wu K, Wang C, Zhang X, Hessien M, Yeung YG, Gioeli D, Weber MJ, Pestell RG. (2004a) The androgen receptor acetylation site regulates cAMP and AKT but not ERK-induced activity. J Biol Chem. 279(28):29436-49.

Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. (2004b) Minireview: Cyclin D1: normal and abnormal functions. Endocrinology. 145(12):5439-47.

Fu YF, Liu J, Xu H, Mohamed YB, Orlov YL, Velkov S, Ho A, Mei PH, Chew EG, Huang PY, Welboren WJ, Han Y, Ooi HS, Ariyaratne PN, Vega VB, Luo Y, Tan PY, Choy PY, Wansa KD, Zhao B, Lim KS, Leow SC, Yow JS, Joseph R, Li H, Desai KV, Thomsen JS, Lee YK, Karuturi RK, Herve T, Bourque G, Stunnenberg HG, Ruan X, Cacheux-Rataboul V, Sung WK, Liu ET, Wei CL, Cheung E, Ruan Y. (2009) An oestrogen-receptor-alpha-bound human chromatin interactome. Nature. 462(7269):58-64.

Fujimoto N, Mizokami A, Harada S, Matsumoto T. (2001) Different expression of androgen receptor coactivators in human prostate. Urology. 58(2):289-94.

Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, Orlov YL, Velkov S, Ho A, Mei PH, Chew EG, Huang PY, Welboren WJ, Han Y, Ooi HS, Ariyaratne PN, Vega VB, Luo Y, Tan PY, Choy PY, Wansa KD, Zhao B, Lim KS, Leow SC, Yow JS, Joseph R, Li H, Desai KV, Thomsen JS, Lee YK, Karuturi RK, Herve T, Bourque G, Stunnenberg HG, Ruan X, Cacheux-Rataboul V, Sung WK, Liu ET, Wei CL, Cheung E, Ruan Y. (2009) An oestrogen-receptor-alpha-bound human chromatin interactome. Nature. 462(7269):58-64.

Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, Srivastava S. (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res. 54(11):2861-4.

Gao J, Arnold JT, Isaacs JT. (2001) Conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth during malignant transformation of prostatic epithelial cells. Cancer Res. 61(13), 5038-44.

Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. (2002) Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. J Biol Chem. 277(29):25904-13

Gelmann EP. (2002): Molecular biology of the androgen receptor. J Clin Oncol 20: 3001-3015.

Geserick C, Meyer HA, Barbulescu K, Haendler B. (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements. Mol Endocrinol. 17(9):1738–50.

Ghosh PM, Malik SN, Bedolla RG, Wang Y, Mikhailova M, Prihoda TJ, Troyer DA, Kreisberg JI. (2005) Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. Endocr Relat Cancer. 12(1):119-34.

Gillanders EM, Xu J, Chang BL, Lange EM, Wiklund F, Bailey-Wilson JE, Baffoe-Bonnie A, Jones M, Gildea D, Riedesel E, Albertus J, Isaacs SD, Wiley KE, Mohai CE, Matikainen MP, Tammela TL, Zheng SL, Brown WM, Rökman A, Carpten JD, Meyers DA, Walsh PC, Schleutker J, Gronberg H, Cooney KA, Isaacs WB, Trent JM. (2004) Combined genome-wide scan for prostate cancer susceptibility genes. J Natl Cancer Inst. 96(16):1240-7.

Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlage RE, Shabanowitz J, Hunt DF, Weber MJ. (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem. 277(32):29304-14.

Gioeli D, Black BE, Gordon V, Spencer A, Kesler CT, Eblen ST, Paschal BM, Weber MJ. (2006) Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization. Mol Endocrinol. 20(3):503-15.

Gleason D. Classification of prostate carcinomas. Cancer Chemother Rep. (1966) 50:125-128.

Glinsky GV. Human genome connectivity code links disease-associated SNPs, microRNAs and pyknons. Cell Cycle. 2009;8(6):925-30.

Grad JM, Dai JL, Wu S, Burnstein KL. (1999) Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. Mol Endocrinol. 13(11):1896-911.

Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, French FS.(1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res. 58(24):5718-24.

Gregory CW, Johnson RT Jr, Mohler JL, French FS, Wilson EM. (2001) Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res. 61(7):2892-8.

Grönberg H. Prostate cancer epidemiology. Lancet 361 (2003), 859–864.

Gutman AB, Gutman EB. (1938) An "acid " phosphatase occurring in the serum of patients with metastasizing carcinoma of the prostate gland. J Clin Invest. 17(4):473-8.

Gudmundsson J, Sulem P, Gudbjartsson DF, Blondal T, Gylfason A, Agnarsson BA, Benediktsdottir KR, Magnusdottir DN, Orlygsdottir G, Jakobsdottir M, Stacey SN, Sigurdsson A, Wahlfors T, Tammela T, Breyer JP, McReynolds KM, Bradley KM, Saez B, Godino J, Navarrete S, Fuertes F, Murillo L, Polo E, Aben KK, van Oort IM, Suarez BK, Helfand BT, Kan D, Zanon C, Frigge ML, Kristjansson K, Gulcher JR, Einarsson GV, Jonsson E, Catalona WJ, Mayordomo JI, Kiemeney LA, Smith JR, Schleutker J, Barkardottir RB, Kong A, Thorsteinsdottir U, Rafnar T, Stefansson K. (2009) Genome-wide association and replication studies identify four variants associated with prostate cancer susceptibility. Nat Genet. 41(10):1122-6.

Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, Chen H, Kong X, Melamed J, Tepper CG, Kung HJ, Brodie AM, Edwards J, Qiu Y. (2009) A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Res. 69(6):2305-13.

Haapala K, Hyytinen ER, Roiha M, Laurila M, Rantala I, Helin HJ, Koivisto PA. (2001) Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. Lab Invest. 81(12):1647-51.

Hakalahti L, Vihko P, Henttu P, Autio-Harmainen H, Soini Y, Vihko R. (1993) Evaluation of PAP and PSA gene expression in prostatic hyperplasia and prostatic carcinoma using northern-blot analyses, in situ hybridization and immunohistochemical stainings with monoclonal and bispecific antibodies. Int J Cancer. 55(4):590-7.

Han G, Buchanan G, Ittmann M, Harris JM, Yu X, Demayo FJ, Tilley W, Greenberg NM. (2005) Mutation of the androgen receptor causes oncogenic transformation of the prostate. Proc Natl Acad Sci U S A. 102(4), 1151-6.

Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. Cell. 100(1):57-70.

Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M. (2003) Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res. 63(1):149-53.

He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, Carter KC. (1997) A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics. 43(1):69-77.

Heemers HV, Tindall DJ. (2007) Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev. 28(7):778-808.

Heinlein CA, Chang C. (2002) The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol. 16:2181-7.

Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, Mehra R, Chinnaiyan AM. (2008) Characterization of

TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res. 68(1):73-80.

Helenius MA, Waltering KK and Visakorpi T. (2008) Somatic Genetic Changes in Prostate Cancer: Current Clinical Oncology Ser. Prostate Cancer: Signaling Networks, Genetics, and New Treatment. Richard G. Pestell, Marja T. Nevalainen. Springer-Verlag New York, LLC

Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, Jenster G. (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012-20.

Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, Delprado W, Stricker PD, Grygiel JJ & Sutherland RL (2001) Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer Res. 61;423–7.

Henttu P, Liao SS, Vihko P. (1992) Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. Endocrinology. 130(2):766-72.

Herold S, Herkert B, Eilers M. (2009) Facilitating replication under stress: an oncogenic function of MYC? Nat Rev Cancer. 9(6):441-4.

Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein, Cancer Res. 55 (1995) 3068–3072.

Hofland J, van Weerden WM, Dits NF, Steenbergen J, van Leenders GJ, Jenster G, Schröder FH, de Jong FH. (2010) Evidence of limited contributions for intratumoral steroidogenesis in prostate cancer. Cancer Res. 70(3):1256-64.

Hollenberg MD, Oikonomopoulou K, Hansen KK, Saifeddine M, Ramachandran R, Diamandis EP. (2008) Kallikreins and proteinase-mediated signaling: proteinase-activated receptors (PARs) and the pathophysiology of inflammatory diseases and cancer. Biol Chem. 389(6):643-51.

Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, Ryan C, Smith S, Scher H, Scardino P, Reuter V, Gerald WL. (2004) Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol. 164(1):217-27.

Hollstein M, Hainaut P. (2010) Massively regulated genes: the example of TP53. J Pathol. 220(2):164-73.

Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, Han M, Partin AW, Vessella RL, Isaacs WB, Bova GS, Luo J. (2009) Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res. 69(1):16-22.

Huggins CB, Hodges CV. (1941) Studies on prostate cancer: 1. The effects of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res. 1:293-7.

Ikonen T, Palvimo JJ, Janne OA. (1997): Interaction between the amino- and carboxylterminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem 272: 29821-29828.

Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. (2007) MicroRNA signatures in human ovarian cancer.Cancer Res. 67:8699-707.

Irvine RA, Yu MC, Ross RK, Coetzee GA. (1995): The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer.Cancer Res 55: 1937–40.

Isaacs WB, Bova GS, Morton RA, Bussemakers MJ, Brooks JD, Ewing CM. Molecular biology of prostate cancer. (1994) Semin Oncol. 21(5):514-21.

Isaacs JT, Isaacs WB. (2004) Androgen receptor outwits prostate cancer drugs. Nat Med. 10(1):26-7.

Isaacs JT, Furuya Y, Berges R. (1994) The role of androgen in the regulation of programmed cell death/apoptosis in normal and malignant prostatic tissue. Semin Cancer Biol. 5(5):391-400.

Itoh T, Nozawa Y, Akao Y. (2009) MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. J Biol Chem. 284:19272-9.

Jalava SE, Porkka KP, Rauhala HE, Isotalo J, Tammela TL, Visakorpi T. (2009) TCEB1 promotes invasion of prostate cancer cells. Int J Cancer. 124(1):95-102.

Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. (1991): Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol 5: 1396–1404.

Jenster G, van der Korput HA, Trapman J, Brinkmann AO. (1995) Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270:7341–6.

Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H, Han J. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell. 120(5):623-34.

Jung ME, Ouk S, Yoo D, Sawyers CL, Chen C, Tran C, Wongvipat J. (2010) Structure-activity relationship for thiohydantoin androgen receptor antagonists for castration-resistant prostate cancer (CRPC). J Med Chem. 53(7):2779-96.

Kallakury BV, Sheehan CE, Ambros RA, Fisher HA, Kaufman RP Jr, Ross JS. (1997) The prognostic significance of p34cdc2 and cyclin D1 protein expression in prostate adenocarcinoma. Cancer. 80(4):753-63.

Kallakury BV, Sheehan CE, Rhee SJ, Fisher HA, Kaufman RP Jr, Rifkin MD, Ross JS. (1999) The prognostic significance of proliferation-associated nucleolar protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen, and p34cdc2. Cancer. 85(7):1569-76.

Kaltz-Wittmer C, Klenk U, Glaessgen A, Aust DE, Diebold J, Löhrs U, Baretton GB. (2000) FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostatic carcinomas before and after androgen deprivation therapy. Lab Invest. 80(9):1455-64.

Kang Z, Jänne OA, Palvimo JJ (2004) Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. Mol Endocrinol. 18:2633-48

Kattan MW, Eastham JA, Stapleton AM, Wheeler TM, Scardino PT. (1998) A preoperative nomogram for disease recurrence following radical prostatectomy for prostate cancer. J Natl Cancer Inst. 90(10):766-71.

Katz AE, Benson MC, Wise GJ, Olsson CA, Bandyk MG, Sawczuk IS, Tomashefsky P, Buttyan R. (1989) Gene activity during the early phase of androgen-stimulated rat prostate regrowth. Cancer Res. 49(21):5889-94.

Kawada M, Inoue H, Arakawa M, Takamoto K, Masuda T, Ikeda D. (2007) Highly tumorigenic human androgen receptor-positive prostate cancer cells overexpress angiogenin. Cancer Sci. 98(3):350-6.

King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, Taylor BS, Sander C, Cardiff RD, Couto SS, Gerald WL, Sawyers CL. (2009) Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. Nat Genet. 41(5):524-6.

Kinoshita H, Shi Y, Sandefur C, Meisner LF, Chang C, Choon A, Reznikoff KA, Bova GS, Friedl A, Jarrard DF. (2000) Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer, Cancer Res. 60: 3623-30.

Klezovitch O, Risk M, Coleman I, Lucas JM, Null M, True LD, Nelson PS, Vasioukhin V. (2008) A causal role for ERG in neoplastic transformation of prostate epithelium. Proc Natl Acad Sci U S A. 105(6):2105-10.

Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP. (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res. 57(2), 314-9.

Kokontis J, Takakura K, Hay N, Liao S. (1994) Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. Cancer Res. 54(6), 1566-73.

Kokontis JM, Hay N, Liao S. (1998) Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. Mol Endocrinol. 12(7):941-53.

Kotaja N, Karvonen U, Jänne OA, Palvimo JJ. (2002) PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol. 22(14):5222–34.

Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. (2008) Recurrent gene fusions in prostate cancer. Nat Rev Cancer. 8(7):497-511.

Labrie F, Candas B, Gomez JL, Cusan L. (2002) Can combined androgen blockade provide long-term control or possible cure of localized prostate cancer? Urology. 60(1):115-9.

Labrie F, Bélanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez J, Candas B. (2005) Gonadotropin-releasing hormone agonists in the treatment of prostate cancer. Endocr Rev. 26(3):361-79.

Laitinen S, Martikainen PM, Tammela TL, Visakorpi T. (2007) Cellular changes in prostate cancer cells induced by intermittent androgen suppression. Eur Urol. 52(3):725-32.

Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TL, Visakorpi T. (2008) EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients. Int J Cancer. 122(3):595-602.

Latil A, Bieche I, Vidaud D, Lidereau R, Berthon P, Cussenot O, Vidaud M. (2001) Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res. 61(5), 1919-26.

Lawrence MG, Lai J, Clements JA. (2010) Kallikreins on Steroids: Structure, Function, and Hormonal Regulation of Prostate-Specific Antigen and the Extended Kallikrein Locus. Endocr Rev. Jan 26 [Epub ahead of print].

Lee DK, Duan HO, Chang C (2000) From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem 275:9308–9313

Lee DK, Chang C. (2003) Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication. J Clin Endocrinol Metab. 88(9), 4043-54.

Leite KR, Sousa-Canavez JM, Reis ST, Tomiyama AH, Camara-Lopes LH, Sañudo A, Antunes AA, Srougi M. (2009) Change in expression of miR-let7c, miR-100, and miR-218 from high grade localized prostate cancer to metastasis. Urol Oncol. Apr 15. [Epub ahead of print]

Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, Nelson CC, Guns ES, Wasan KM. (2010) Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. Prostate. 70(4):390-400.

Leone G, Sears R, Huang E, Rempel R, Nuckolls F, Park CH, Giangrande P, Wu L, Saavedra HI, Field SJ, Thompson MA, Yang H, Fujiwara Y, Greenberg ME, Orkin S, Smith C, Nevins JR. (2001) Myc requires distinct E2F activities to induce S phase and apoptosis. Mol Cell. 8(1):105-13.

Leversha MA, Han J, Asgari Z, Danila DC, Lin O, Gonzalez-Espinoza R, Anand A, Lilja H, Heller G, Fleisher M, Scher HI. (2009) Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. Clin Cancer Res. 15(6):2091-7.

Li SS, Sharief FS. (1993) The prostatic acid phosphatase (ACPP) gene is localized to human chromosome 3q21-q23. Genomics. 17(3):765-6.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997 275: 1943-1947.

Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. (2000) Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med. 343(2):78-85.

Lilja H. (2003) Biology of prostate-specific antigen. Urology. 62(5 Suppl 1):27-33.

Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, Nelson PS. (1999) Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res. 59(17):4180-4.

Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, Chang C. (2003) Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J Biol Chem. 278:50902–7.

Lin HK, Yeh S, Kang HY, Chang C. (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A. 98(13):7200-5.

Lin CH, Jackson AL, Guo J, Linsley PS, Eisenman RN. (2009) Myc-regulated microRNAs attenuate embryonic stem cell differentiation. EMBO J. 28:3157-70.

Linja M, Savinainen K, Saramäki O, Tammela T, Vessella R, Visakorpi T. (2001) Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res. 61:3550-3555.

Linja MJ, Porkka KP, Kang Z, Savinainen KJ, Jänne OA, Tammela TL, Vessella RL, Palvimo JJ, Visakorpi T. (2004) Expression of androgen receptor coregulators in prostate cancer. Clin Cancer Res. 10(3):1032-40.

Liu W, Xie CC, Zhu Y, Li T, Sun J, Cheng Y, Ewing CM, Dalrymple S, Turner AR, Sun J, Isaacs JT, Chang BL, Zheng SL, Isaacs WB, Xu J. (2008) Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. Neoplasia. 10(8):897-907.

Loblaw DA, Virgo KS, Nam R, Somerfield MR, Ben-Josef E, Mendelson DS, Middleton R, Sharp SA, Smith TJ, Talcott J, Taplin M, Vogelzang NJ, Wade JL 3rd, Bennett CL, Scher HI; American Society of Clinical Oncology. (2007) Initial hormonal management of androgen-sensitive metastatic, recurrent, or progressive prostate cancer: 2006 update of an American Society of Clinical Oncology practice guideline. J Clin Oncol. 25(12):1596-605.

Locke JA, Guns ES, Lehman ML, Ettinger S, Zoubeidi A, Lubik A, Margiotti K, Fazli L, Adomat H, Wasan KM, Gleave ME, Nelson CC. (2010) Arachidonic acid activation of intratumoral steroid synthesis during prostate cancer progression to castration resistance. Prostate. 70(3):239-51.

Lu S, Tsai SY, Tsai MJ. (1997) Regulation of androgen-dependent prostatic cancer cell growth: androgen regulation of CDK2, CDK4, and CKI p16 genes. Cancer Res. 57(20):4511-6.

Lu S, Liu M, Epner DE, Tsai SY, Tsai MJ. (1999) Androgen regulation of the cyclin-dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. Mol Endocrinol. 13(3):376-84.

Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, Li Y. (2008) MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene. 27(31):4373-9.

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. (1988): Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240: 327-330.

Lundwall A, Lilja H. (1987) Molecular cloning of human prostate specific antigen cDNA. FEBS Lett. 214(2):317-22.

Lundwall A, Brattsand M. (2008) Kallikrein-related peptidases. Cell Mol Life Sci. 65(13):2019-38.

Ma C, Yoshioka M, Boivin A, Gan L, Takase Y, Labrie F, St-Amand J. (2009) Atlas of dihydrotestosterone actions on the transcriptome of prostate in vivo. Prostate. 69(3):293-316.

Macke JP, Hu N, Hu S, Bailey M, King VL, Brown T, Hamer D, Nathans J. (1993) Sequence variation in the androgen receptor gene is not a common determinant of male sexual orientation. Am J Hum Genet. 53(4):844-52.

Makkonen H, Jääskeläinen T, Pitkänen-Arsiola T, Rytinki M, Waltering KK, Mättö M, Visakorpi T, Palvimo JJ. (2008) Identification of ETS-like transcription factor 4 as a novel androgen receptor target in prostate cancer cells. Oncogene. 27(36):4865-76.

Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ. (2000) Androgen receptor mutations in prostate cancer. Cancer Res. 60(4):944-9.

Martin L, Coffey M, Lawler M, Hollywood D, Marignol L. (2009) DNA mismatch repair and the transition to hormone independence in breast and prostate cancer. Cancer Lett. [Epub ahead of print]

Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Bäsler S, Schäfer M, Egner U, Carrondo MA. (2000) Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem. 275(34):26164-71.

McEwan IJ, Gustafsson J. (1997) Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc Natl Acad Sci USA. 94:8485–8490

McEwan IJ. (2004) Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr Relat Cancer. 11: 281-93.

Meiers I, Shanks JH, Bostwick DG. (2007) Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. Pathology. 39(3):299-304.

Meynert A, Birney E. (2006) Picking pyknons out of the human genome. Cell. 125(5):836-8.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 105(30):10513-8.

Miyajima N, Maruyama S, Bohgaki M, Kano S, Shigemura M, Shinohara N, Nonomura K, Hatakeyama S. (2008) TRIM68 regulates ligand-dependent transcription of androgen receptor in prostate cancer cells. Cancer Res. 68(9):3486-94.

Mizokami A, Yeh SY and Chang C. (1994a) Identification of 3',5'-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. Mol Endocrinol. 8:77-88.

Mizokami A and Chang C. (1994b) Induction of Translation by the 5'Untranslated Region of Human Androgen Receptor mRNA. J Biol Chem.(269:25655-25659.

Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, True LD, Nelson PS. (2008) Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res. 68:4447-54.

Morgenbesser SD, McLaren RP, Richards B, Zhang M, Akmaev VR, Winter SF, Mineva ND, Kaplan-Lefko PJ, Foster BA, Cook BP, Dufault MR, Cao X, Wang CJ, Teicher BA, Klinger KW, Greenberg NM, Madden SL. (2007) Identification of genes potentially involved in the acquisition of androgen-independent and metastatic tumor growth in an autochthonous genetically engineered mouse prostate cancer model. Prostate. 67(1):83-106.

Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS. (2007) Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res. 67(10):5033-41.

Mostaghel EA, Montgomery B, Nelson PS. (2009) Castration-resistant prostate cancer: targeting androgen metabolic pathways in recurrent disease. Urol Oncol. 27(3):251-7.

Murillo H, Schmidt LJ, Karter M, Hafner KA, Kondo Y, Ballman KV, Vasmatzis G, Jenkins RB, Tindall DJ. (2006) Prostate cancer cells use genetic and epigenetic

mechanisms for progression to androgen independence. Genes Chromosomes Cancer. 45(7):702-16.
Mäki HE, Waltering KK, Wallén MJ, Martikainen PM, Tammela TL, van Weerden WM, Vessella RL, Visakorpi T. (2006) Screening of genetic and expression alterations of SRC1 gene in prostate cancer. Prostate. 66(13):1391-8.
Mäki HE, Saramäki OR, Shatkina L, Martikainen PM, Tammela TL, van Weerden WM, Vessella RL, Cato AC, Visakorpi T. (2007) Overexpression and gene amplification of BAG-1L in hormone-refractory prostate cancer. J Pathol. 212(4):395-401.
Nakada C, Matsuura K, Tsukamoto Y, Tanigawa M, Yoshimoto T, Narimatsu T, Nguyen LT, Hijiya N, Uchida T, Sato F, Mimata H, Seto M, Moriyama M. (2008) Genome-wide microRNA expression profiling in renal cell carcinoma: significant down-regulation of miR-141 and miR-200c. J Pathol. 216(4):418-27.
Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S. (2008) MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res. 14(9):2690-5.
Nazareth LV, Stenoien DL, Bingman WE 3rd, James AJ, Wu C, Zhang Y, Edwards DP, Mancini M, Marcelli M, Lamb DJ, Weigel NL. (1999) A C619Y mutation in the human androgen receptor causes inactivation and mislocalization of the receptor with concomitant sequestration of SRC-1 (steroid receptor coactivator 1) Mol Endocrinol. 13(12):2065-75.
Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B. (2002) The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A. 99(18):11890-5.
Newmark JR, Hardy DO, Tonb DC, Carter BS, Epstein JI, Isaacs WB, Brown TR, Barrack ER. (1992) Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci U S A. 89(14):6319-23.
Nupponen N, Visakorpi T. (1999) Molecular biology of progression of prostate cancer. Eur Urol. 35(5-6):351-4.
Nupponen NN, Isola J, Visakorpi T. (2000) Mapping the amplification of EIF3S3 in breast and prostate cancer. Genes Chromosomes Cancer. 28(2):203-10.
O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. Nature. 435(7043):839-43.
Onate SA, Tsai SY, Tsai MJ, O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science. 270:1354–57
Papsidero LD, Croghan GA, Asirwatham J, Gaeta J, Abenoza P, Englander L, Valenzuela L.(1985) Immunohistochemical demonstration of prostate-specific antigen in metastases with the use of monoclonal antibody F5. Am J Pathol. 121(3):451-4.
Perry JE, Grossmann ME, Tindall DJ. (1996) Androgen regulation of gene expression. Prostate Suppl. 6, 79-81.
Paakinaho V, Makkonen H, Jääskeläinen T, Palvimo JJ. (2010) Glucocorticoid receptor activates poised FKBP51 locus through long-distance interactions. Mol Endocrinol.;24(3):511-25.
Palmberg C, Koivisto P, Kakkola L, Tammela TL, Kallioniemi OP, Visakorpi T. (2000) Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. J Urol. 164(6):1992-5.
Perner S, Mosquera JM, Demichelis F, Hofer MD, Paris PL, Simko J, Collins C, Bismar TA, Chinnaiyan AM, De Marzo AM, Rubin MA. (2007) TMPRSS2-

ERG fusion prostate cancer: an early molecular event associated with invasion. Am J Surg Pathol. 31(6):882-8.

Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med. 351(15):1513-20.

Phelps RA, Broadbent TJ, Stafforini DM, Jones DA. (2009) New perspectives on APC control of cell fate and proliferation in colorectal cancer. Cell Cycle. 8(16):2549-56.

Pickering BM, Willis AE. (2005) The implications of structured 5' untranslated regions on translation and disease. Semin Cell Dev Biol. 16(1):39-47.

Possner M, Heuser M, Kaulfuss S, Scharf JG, Schulz W, Hermann-Ringert R, Thelen P. (2008) Functional analysis of NKX3.1 in LNCaP prostate cancer cells by RNA interference. Int J Oncol. 32(4):877-84.

Poukka H, Aarnisalo P, Karvonen U, Palvimo JJ, Jänne OA. (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. J Biol Chem.274(27):19441–6.

Poukka H, Karvonen U, Jänne OA, Palvimo JJ. (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci USA 97(26):14145-50.

Ryan CJ, Smith MR, Fong L, Rosenberg JE, Kantoff P, Raynaud F, Martins V, Lee G, Kheoh T, Kim J, Molina A, Small EJ. (2010) Phase I clinical trial of the CYP17 inhibitor abiraterone acetate demonstrating clinical activity in patients with castration-resistant prostate cancer who received prior ketoconazole therapy. J Clin Oncol. 28(9):1481-8.

Ren B, Yu G, Tseng GC, Cieply K, Gavel T, Nelson J, Michalopoulos G, Yu YP, Luo JH. (2006) MCM7 amplification and overexpression are associated with prostate cancer progression. Oncogene. 25(7):1090-8.

Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, Kudrolli TA, Yegnasubramanian S, Luo J, Rodriguez R, Mendell JT, Lupold SE. (2009) miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res. 69(18):7165-9.

Ribas J, Lupold SE. (2010) The transcriptional regulation of miR-21, its multiple transcripts, and their implication in prostate cancer. Cell Cycle. 9(5):923-9.

Rickman DS, Pflueger D, Moss B, VanDoren VE, Chen CX, de la Taille A, Kuefer R, Tewari AK, Setlur SR, Demichelis F, Rubin MA. (2009) SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. Cancer Res. 69(7):2734-8.

Rigoutsos I, Huynh T, Miranda K, Tsirigos A, McHardy A, Platt D. (2006) Short blocks from the noncoding parts of the human genome have instances within nearly all known genes and relate to biological processes. Proc Natl Acad Sci U S A. 103(17):6605-10.

Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, Santerse AB, Schroder FH, van der Kwast TH. (1994) Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol. 144(4), 735-46.

Russo A, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N; TP53-CRC Collaborative Study Group. (2005) The TP53 colorectal cancer international collaborative

study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol. 23(30):7518-28.

Rökman A, Ikonen T, Seppälä EH, Nupponen N, Autio V, Mononen N, Bailey-Wilson J, Trent J, Carpten J, Matikainen MP, Koivisto PA, Tammela TL, Kallioniemi OP, Schleutker J. (2002) Germline alterations of the RNASEL gene, a candidate HPC1 gene at 1q25, in patients and families with prostate cancer. Am J Hum Genet. 70(5):1299-304.

Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR Jr, Weinmann R, Einspahr HM. (2001) Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A. 98(9):4904-9.

Saramäki O, Willi N, Bratt O, Gasser TC, Koivisto P, Nupponen NN, Bubendorf L, Visakorpi T. (2001a) Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. Am J Pathol. 159(6):2089-94.

Saramäki OR, Tammela TL, Martikainen PM, Vessella RL, Visakorpi T. (2006) The gene for polycomb group protein enhancer of zeste homolog 2 (EZH2) is amplified in late-stage prostate cancer. Genes Chromosomes Cancer. 45(7):639-45.

Saramaki O, Visakorpi T. (2007) Chromosomal aberrations in prostate cancer. Front Biosci. 12:3287-301.

Saramäki OR, Harjula AE, Martikainen PM, Vessella RL, Tammela TL, Visakorpi T. (2008) TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. Clin Cancer Res. 14:3395-400.

Sato N, Sadar MD, Bruchovsky N, Saatcioglu F, Rennie PS, Sato S, Lange PH, Gleave ME. (1997) Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. J Biol Chem. 272(28):17485-94.

Savinainen KJ, Linja MJ, Saramäki OR, Tammela TL, Chang GT, Brinkmann AO, Visakorpi T. (2004) Expression and copy number analysis of TRPS1, EIF3S3 and MYC genes in breast and prostate cancer. Br J Cancer. 90(5):1041-6.

Savinainen KJ, Helenius MA, Lehtonen HJ, Visakorpi T. (2006) Overexpression of EIF3S3 promotes cancer cell growth. Prostate. 66(11):1144-50.

Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, Miner JN, Diamond MI. (2005): The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. Proc Natl Acad Sci USA 102: 9802-9807.

Scher HI, Buchanan G, Gerald W, Butler LM, Tilley WD. (2004) Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. Endocr Relat Cancer. 11(3):459-76.

Schröder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M, Denis LJ, Recker F, Berenguer A, Määttänen L, Bangma CH, Aus G, Villers A, Rebillard X, van der Kwast T, Blijenberg BG, Moss SM, de Koning HJ, Auvinen A; ERSPC Investigators. (2009) Screening and prostate-cancer mortality in a randomized European study. N Engl J Med. 360(13):1320-8.

Seppälä EH, Ikonen T, Autio V, Rökman A, Mononen N, Matikainen MP, Tammela TL, Schleutker J. (2003) Germ-line alterations in MSR1 gene and prostate cancer risk. Clin Cancer Res. 9(14):5252-6.

Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, Miller K, Lein M, Kristiansen G, Jung K. (2010) Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer. 126(5):1166-76.

Sharma S, Pirilä P, Kaija H, Porvari K, Vihko P, Juffer AH. (2005) Theoretical investigations of prostatic acid phosphatase. Proteins. 58(2):295-308.

Shatkina L, Mink S, Rogatsch H, Klocker H, Langer G, Nestl A, Cato AC. (2003) The cochaperone Bag-1L enhances androgen receptor action via interaction with the NH2-terminal region of the receptor. Mol Cell Biol. 23(20):7189-97.

Shi XB, Xue L, Yang J, Ma AH, Zhao J, Xu M, Tepper CG, Evans CP, Kung HJ, deVere White RW. (2007) An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. Proc Natl Acad Sci U S A. 104(50):19983-8.

Simental JA, Sar M, Lane MV, French FS, Wilson EM. (1991): Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 266: 510-518.

Simon JA, Lange CA. (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res. 647(1-2):21-9.

Sirotnak FM, She Y, Khokhar NZ, Hayes P, Gerald W, Scher HI. (2004) Microarray analysis of prostate cancer progression to reduced androgen dependence: studies in unique models contrasts early and late molecular events. Mol Carcinog. 41(3):150-63.

Song CS, Jung MH, Supakar PC, Chatterjee B, Roy AK. (1999) Negative regulation of the androgen receptor gene promoter by NFI and an adjacently located multiprotein-binding site. Mol Endocrinol. 13(9):1487-96.

Sotelo J, Esposito D, Duhagon MA, Banfield K, Mehalko J, Liao H, Stephens RM, Harris TJ, Munroe DJ, Wu X. (2010) Long-range enhancers on 8q24 regulate c-Myc. Proc Natl Acad Sci U S A. [Epub ahead of print]

Squire JA. (2009) TMPRSS2-ERG and PTEN loss in prostate cancer. Nat Genet. 41(5):509-10.

Spahn M, Kneitz S, Scholz CJ, Nico S, Rüdiger T, Ströbel P, Riedmiller H, Kneitz B. (2009) Expression of microRNA-221 is progressively reduced in aggressive prostate cancer and metastasis and predicts clinical recurrence. Int J Cancer. Jul 7. [Epub ahead of print]

Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. (1987) Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med. 317(15):909-16.

Steele VE, Arnold JT, Lei H, Izmirlian G, Blackman MR. (2006) Comparative effects of DHEA and DHT on gene expression in human LNCaP prostate cancer cells. Anticancer Res. 26(5A):3205-15.

Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J. (2002) Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer. 100(3):309-17.

Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J. (1993) Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol. 46(6):759-65.

Suzuki H, Akakura K, Komiya A, Aida S, Akimoto S, Shimazaki J. (1996) Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. Prostate. 29(3):153-8.

Swinnen JV, Heemers H, van de Sande T, de Schrijver E, Brusselmans K, Heyns W, Verhoeven G. (2004) Androgens, lipogenesis and prostate cancer. J Steroid Biochem Mol Biol. 92:273-9.

Szczyrba J, Löprich E, Wach S, Jung V, Unteregger G, Barth S, Grobholz R, Wieland W, Stöhr R, Hartmann A, Wullich B, Grässer F. (2010) The microRNA profile of prostate carcinoma obtained by deep sequencing. Mol Cancer Res. 8(4):529-38.

Takane KK, McPhaul MJ. (1996) Functional analysis of the human androgen receptor promoter. Mol Cell Endocrinol. 119:83-93.

Tammela T. (2004) Endocrine treatment of prostate cancer. J Steroid Biochem Mol Biol. 92(4):287-95.

Tamura K, Furihata M, Tsunoda T, Ashida S, Takata R, Obara W, Yoshioka H, Daigo Y, Nasu Y, Kumon H, Konaka H, Namiki M, Tozawa K, Kohri K, Tanji N, Yokoyama M, Shimazui T, Akaza H, Mizutani Y, Miki T, Fujioka T, Shuin T, Nakamura Y, Nakagawa H. (2007) Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. Cancer Res. 67(11):5117-25.

Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, French FS. (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol. 11(4):450-9

Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med. 332(21):1393-8.

Taplin ME, Ho SM. (2001) Clinical review 134: The endocrinology of prostate cancer. J Clin Endocrinol Metab. 86(8):3467-77.

Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ; Cancer and Leukemia Group B Study 9663. (2003) Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol. 21(14):2673-8.

Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Socci ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL, Gerald WL. (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell. 18(1):11-22.

Terada N, Shimizu Y, Yoshida T, Maeno A, Kamba T, Inoue T, Nakamura E, Kamoto T, Ogawa O. (2010) Antiandrogen withdrawal syndrome and alternative antiandrogen therapy associated with the W741C mutant androgen receptor in a novel prostate cancer xenograft. Prostate. 70(3):252-61.

Thomas M, Dadgar N, Aphale A, Harrell JM, Kunkel R, Pratt WB, Lieberman AP. (2004) Androgen receptor acetylation site mutations cause trafficking defects, misfolding, and aggregation similar to expanded glutamine tracts. J Biol Chem. 279(9):8389-95.

Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, Lieber MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman CA Jr. (2003) The influence of finasteride on the development of prostate cancer. N Engl J Med. 349(3):215-24.

Thompson J, Saatcioglu F, Jänne OA, Palvimo JJ. (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. Mol Endocrinol. 15(6):923-35.

Tilley WD, Buchanan G, Hickey TE, Bentel JM. (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res. 2(2):277-85.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 310(5748):644-8.

Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaiyan AM. (2006) TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res. 66(7):3396-400.

Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie JE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R, Chinnaiyan AM. (2007) Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature. 448(7153):595-9.

Tomlins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, Rubin MA, Schalken JA. (2009) ETS gene fusions in prostate cancer: from discovery to daily clinical practice. Eur Urol. 56(2):275-86.

Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL. (2009) Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science. 324(5928):787-90.

Trapman J, Klaassen P, Kuiper GG, van der Korput JA, Faber PW, van Rooij HC, Geurts van Kessel A, Voorhorst MM, Mulder E, Brinkmann AO. (1988): Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Commun 153: 241-248.

Ulrix W, Swinnen J, Heyns W and Verhoeven G. (1998) J. Biol. Chem. 273(8):4660-5

Vaarala MH, Porvari K, Kyllönen A, Lukkarinen O, Vihko P. (2001) The TMPRSS2 gene encoding transmembrane serine protease is overexpressed in a majority of prostate cancer patients: detection of mutated TMPRSS2 form in a case of aggressive disease. Int J Cancer. 94(5):705-10.

Wainstein MA, He F, Robinson D, Kung HJ, Schwartz S, Giaconia JM, Edgehouse NL, Pretlow TP, Bodner DR, Kursh ED (1994) CWR22: Androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res. 54:6049–6052

Wallén MJ, Linja M, Kaartinen K, Schleutker J, Visakorpi T. (1999) Androgen receptor gene mutations in hormone-refractory prostate cancer. J Pathol. 189(4):559-63.

Vander Griend DJ, D'Antonio J, Gurel B, Antony L, Demarzo AM, Isaacs JT.(2010) Cell-autonomous intracellular androgen receptor signaling drives the growth of human prostate cancer initiating cells. Prostate. 70(1):90-9.

van de Wijngaart DJ, Molier M, Lusher SJ, Hersmus R, Jenster G, Trapman J, Dubbink HJ. (2010) Systematic Structure-Function Analysis of Androgen Receptor Leu701 Mutants Explains the Properties of the Prostate Cancer Mutant L701H. J Biol Chem. 285(7):5097-105.

van Leenders GJ, Schalken JA. (2003) Epithelial cell differentiation in the human prostate epithelium: implications for the pathogenesis and therapy of prostate cancer. Crit Rev Oncol Hematol. 46 Suppl:S3-10.

Van Poppel H, Joniau S, Van Cleynenbreugel B, Mottaghy FM, Oyen R. (2009). Diagnostic evaluation of PSA recurrence and review of hormonal management after radical prostatectomy. Prostate Cancer Prostatic Dis. 12(2):116-23.

Wang LG, Ossowski L, Ferrari AC. (2004) Androgen receptor level controlled by a suppressor complex lost in an androgen-independent prostate cancer cell line. Oncogene. 23:5175-5184.

Wang Q, Carroll JS, Brown M. (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell. 19(5):631-42.

Wang Q, Li W, Liu XS, Carroll JS, Jänne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M. (2007a) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell. 27(3):380-92.

Wang XD, Wang BE, Soriano R, Zha J, Zhang Z, Modrusan Z, Cunha GR, Gao WQ. (2007b) Expression profiling of the mouse prostate after castration and hormone replacement: implication of H-cadherin in prostate tumorigenesis. Differentiation. 75(3):219-34.

Wang H, Mannava S, Grachtchouk V, Zhuang D, Soengas MS, Gudkov AV, Prochownik EV, Nikiforov MA. (2008) c-Myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle. Oncogene. 27(13):1905-15.

Wang X, Kruithof-de Julio M, Economides KD, Walker D, Yu H, Halili MV, Hu YP, Price SM, Abate-Shen C, Shen MM. (2009a) A luminal epithelial stem cell that is a cell of origin for prostate cancer. Nature. 461(7263):495-500.

Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, Chen Z, Beroukhim R, Wang H, Lupien M, Wu T, Regan MM, Meyer CA, Carroll JS, Manrai AK, Jänne OA, Balk SP, Mehra R, Han B, Chinnaiyan AM, Rubin MA, True L, Fiorentino M, Fiore C, Loda M, Kantoff PW, Liu XS, Brown M. (2009b) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell. 138(2):245-56.

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer.Nature. 419(6907):624-9.

Vasudevan S, Tong Y, Steitz JA. (2008) Cell-cycle control of microRNA-mediated translation regulation. Cell Cycle. 7(11):1545-9.

Watanabe M, Ushijima T, Shiraishi T, Yatani R, Shimazaki J, Kotake T, Sugimura T, Nagao M. (1997) Genetic alterations of androgen receptor gene in Japanese human prostate cancer. Jpn J Clin Oncol. 27(6):389-93.

Weckermann D, Harzmann R. Hormone therapy in prostate cancer: LHRH antagonists versus LHRH analogues. Eur Urol. 2004;46(3):279-83.

Veeramani S, Yuan TC, Chen SJ, Lin FF, Petersen JE, Shaheduzzaman S, Srivastava S, MacDonald RG, Lin MF. (2005) Cellular prostatic acid phosphatase: a protein tyrosine phosphatase involved in androgen-independent proliferation of prostate cancer. Endocr Relat Cancer. 12(4):805-22.

Velasco AM, Gillis KA, Li Y, Brown EL, Sadler TM, Achilleos M, Greenberger LM, Frost P, Bai W, Zhang Y. (2004) Identification and validation of novel androgen-regulated genes in prostate cancer. Endocrinology. 145(8):3913-24.

Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E. (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun. 173(2):534-40.

Vihko P. (1979) Human prostatic acid phosphatases: purification of a minor enzyme and comparisons of the enzymes. Invest Urol. 16(5):349-52.

Williams K, Fernandez S, Stien X, Ishii K, Love HD, Lau YF, Roberts RL, Hayward SW. (2005) Unopposed c-MYC expression in benign prostatic epithelium causes a cancer phenotype. Prostate. 63(4):369-84.

Visakorpi T, O.P Kallioniemi, A Heikkinen, T Koivula, J Isola. (1992) Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. J. Natl. Cancer Inst. 84: 883-7

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. (1995) In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet. 9(4), 401-6.

Vogelstein B, Kinzler KW. (1993) The multistep nature of cancer. Trends Genet. 9(4):138-41.

Wolf DA, Schulz P, Fittler F. (1992) Transcriptional regulation of prostate kallikrein-like genes by androgen. Mol Endocrinol. 6(5):753-62.

Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, Carter KC, Srivastava S. (2000) Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. J Urol. 163(3):972-9.

Xu J, Wu RC, O'Malley BW. (2009a) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer. 9(9):615-30.

Xu K, Shimelis H, Linn DE, Jiang R, Yang X, Sun F, Guo Z, Chen H, Li W, Chen H, Kong X, Melamed J, Fang S, Xiao Z, Veenstra TD, Qiu Y. (2009b) Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. Cancer Cell. 15(4):270-82.

Yeap BB, Voon DC, Vivian JP, McCulloch RK, Thomson AM, Giles KM, Czyzyk-Krzeska MF, Furneaux H, Wilce MC, Wilce JA, Leedman PJ. (2002) Novel binding of HuR and poly(C)-binding protein to a conserved UC-rich motif within the 3'-untranslated region of the androgen receptor messenger RNA. J Biol Chem. 277:27183-27192.

York TP, Plymate SR, Nelson PS, Eaves LJ, Webb HD, Ware JL.(2005) cDNA microarray analysis identifies genes induced in common by peptide growth factors and androgen in human prostate epithelial cells. Mol Carcinog. 44(4):242-51.

Yoshida T, Kinoshita H, Segawa T, Nakamura E, Inoue T, Shimizu Y, Kamoto T, Ogawa O. (2005) Antiandrogen bicalutamide promotes tumor growth in a novel androgen-dependent prostate cancer xenograft model derived from a bicalutamide-treated patient. Cancer Res. 65(21):9611-6.

Yegnasubramanian S, Kowalski J, Gonzalgo ML, Zahurak M, Piantadosi S, Walsh PC, Bova GS, De Marzo AM, Isaacs WB, Nelson WG. (2004) Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res. 64(6):1975-86.

Yoon HG, Wong J. (2006) The corepressors silencing mediator of retinoid and thyroid hormone receptor and nuclear receptor corepressor are involved in agonist- and

antagonist-regulated transcription by androgen receptor. Mol Endocrinol. 20(5):1048-60.

Young CY, Andrews PE, Montgomery BT, Tindall DJ. (1992) Tissue-specific andhormonal regulation of human prostate-specific glandular kallikrein. Biochemistry. 31(3), 818-24.

Yu X, Gupta A, Wang Y, Suzuki K, Mirosevich J, Orgebin-Crist MC, Matusik RJ. (2005) Foxa1 and Foxa2 interact with the androgen receptor to regulate prostate and epididymal genes differentially. Ann N Y Acad Sci. 1061:77-93.

Zhang X, Lee C, Ng PY, Rubin M, Shabsigh A, Buttyan R. (2000) Prostatic neoplasia in transgenic mice with prostate-directed overexpression of the c-myc oncoprotein. Prostate 43(4): 278–285.

Zhang L, Deng T, Li X, Liu H, Zhou H, Ma J, Wu M, Zhou M, Shen S, Li X, Niu Z, Zhang W, Shi L, Xiang B, Lu J, Wang L, Li D, Tang H, Li G. (2010) microRNA-141 is involved in a nasopharyngeal carcinoma-related genes network. Carcinogenesis. 31(4):559-66.

Zheng Z, Cai C, Omwancha J, Chen SY, Baslan T, Shemshedini L. (2006) SUMO-3 enhances androgen receptor transcriptional activity through a sumoylation-independent mechanism in prostate cancer cells. J Biol Chem. 281(7):4002-12.

Zhao XY, Boyle B, Krishnan AV, Navone NM, Peehl DM, Feldman D. (1999) Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. J Urol. 162(6):2192-9.

Zhou ZX, Kemppainen JA, Wilson EM. (1995) Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol Endocrinol. 9(5), 605-15.

Zhu Y, Stevens RG, Hoffman AE, Fitzgerald LM, Kwon EM, Ostrander EA, Davis S, Zheng T, Stanford JL.(2009) Testing the circadian gene hypothesis in prostate cancer: a population-based case-control study. Cancer Res. 69(24):9315-22.

Zimmerman RA, Dozmorov I, Nunlist EH, Tang Y, Li X, Cowan R, Centola M, Frank MB, Culkin DJ, Lin HK. (2004) 5alpha-Androstane-3alpha,17beta-diol activates pathway that resembles the epidermal growth factor responsive pathways in stimulating human prostate cancer LNCaP cell proliferation. Prostate Cancer Prostatic Dis. 7(4):364-74.

Zou JX, Zhong Z, Shi XB, Tepper CG, deVere White RW, Kung HJ, Chen H. (2006) ACTR/AIB1/SRC-3 and androgen receptor control prostate cancer cell proliferation and tumor growth through direct control of cell cycle genes. Prostate. 66(14):1474-86.

Zou JX, Guo L, Revenko AS, Tepper CG, Gemo AT, Kung HJ, Chen HW. (2009) Androgen-induced coactivator ANCCA mediates specific androgen receptor signaling in prostate cancer. Cancer Res. 69(8):3339-46.

ORIGINAL COMMUNICATIONS I-III

The Prostate 66:1585 – 1591 (2006)

Mutation Screening of the Androgen Receptor Promoter and Untranslated Regions in Prostate Cancer

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BACKGROUND. Mechanisms, other than gene amplification, leading to overexpression of *AR* in androgen ablation-resistant prostate cancer remain unknown and could include genetic alterations in the promoter or untranslated regions (UTR) of the *AR* gene.
MATERIALS AND METHODS. DNAs from five prostate cancer cell lines, 19 LuCaP xenografts, 44 clinical tumors, and 36 non-malignant controls were used for screening mutations in the upstream regulatory region, promoter and the 5′- and 3′-UTRs of the *AR* gene with denaturating high performance liquid chromatography (DHPLC) and sequencing.
RESULTS. Ten different sequence variations were found in prostate cancer cell lines and xenografts. However, none of them were recurrent or were found in clinical prostate cancer specimens or in normal controls.
CONCLUSIONS. Recurrent mutations in the promoter or UTRs of *AR* seem to be rare, and thus not likely mechanisms for the increased expression of the gene in the androgen ablation-resistant prostate cancer. *Prostate 66: 1585–1591, 2006*.

KEY WORDS: **genetic alterations; neoplasia; prostatic; carcinoma**

INTRODUCTION

The growth of prostate cancer is dependent on androgens. Therefore, endocrine therapy has been a standard treatment in advanced prostate cancer for more than a half century [1]. During the hormonal therapy, an ablation-resistant or androgen-independent tumor clone eventually emerges leading to the clinical progression of the disease.

Recent findings suggest that *AR* plays a major role in the emergence of the hormone-refractory prostate carcinoma. We have shown that about 30% of ablation-resistant prostate cancers contain *AR* gene amplification leading to overexpression of the gene [2,3]. Mutations in the coding region of the *AR* have been found in 10–30% of the ablation-resistant prostate cancer treated with antiandrogens, such as flutamide or bicalutamide [4–6]. In addition, Chen and co-workers have shown that overexpression of *AR* is required and sufficient to transform androgen-dependent prostate cancer xenografts to ablation-resistant ones [7]. The study indicated also that the only common nominator in the expression profiles of androgen-dependent and-independent xenografts was the increased expression of *AR* in the independent counter parts. The finding is

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in a good agreement with studies showing an overall increased expression of *AR* in a majority of ablation-resistant clinical prostate cancer specimens [2,3,8–11]. Although, when a large series of bone metastases from patients who had expired from advanced androgen-independent disease were examined by immunohistochemistry there was extreme variability in expression levels of the androgen receptor among the lesions in individual patients [12].

The regulatory regions of the *AR* gene are incompletely known (Fig. 1). The core promoter (−74 to +87) of *AR* has no TATA or CAAT box, but does have an SP1-binding site (−52–57) and a palindromic homopurine (−129–70) repeat. Two *AR* transcription initiation sites, ARTIS I (−12/−11/−10) and ARTIS II (−1/+1), have been demonstrated to function as independent overlapping pathways, where SP1-binding is inducing the transcription through ARTIS II, but has no influences on ARTIS I [13,14]. Regulation and the role of these two overlapping pathways are still unclear. Upstream several putative positively regulating *cis*-acting elements can be found [15,16]. Functional studies of the promoter have shown that the palidromic homopurine repeat is important for *AR* transcription and may facilitate transcription initiation from the GC-box [15,17]. Mizokami et al. [15] identified a cAMP responsive element −518 bp upstream of the core promoter. They also found a putative suppression region from −540 to −150 bp from core promoter and another *cis*-acting region(s) in −1,390 to −940 bp. Other functional regulatory elements, which may alter the *AR* transcription, have been found for HL (helix-loop-helix-like) motifs 1 and 2, −179 and −37 bp upstream from the core promoter [16], and for NF-κB and TNFα in distal part of rat *AR* promoter [18].

AR is known to have a long (1.1 kb) 5′-UTR and a very long (∼7 kb) 3′-UTR [19–21]. The 5′-UTR contains a stem-loop structure and is essential for *AR* translation [21]. Recently the androgen-independent LNCaP-AI cell line, which overexpresses *AR*, was found to have a loss of an unidentified suppressor complex that binds to a suppressor element 313 bp downstream from ARTIS II [22]. 3′-UTR contains highly conserved UC-rich motifs and 3′-CCCUCCC poly(C)-binding protein (CP) motifs 4,036 and 4,071 bp downstream of the ARTIS. The UC-rich region is a target for Elav/Hu family of RNA binding proteins, such as HuR, that are involved in stabilation of several AU-rich elements containing mRNAs. UC-rich region binds also simultaneously CP1 and 2, which have a role in control of mRNA turnover and translation rate, and thus these proteins are suggested to have a co-operative role in controlling *AR* expression in prostate cancer [23].

Two single cases of germ-line alterations have been identified in the 5′-UTR and one in the promoter region, two of these in prostate cancer patients [24], and one in a healthy male blood donor [25]. The alterations that were found in one prostate cancer patient were G-10T within the ARTIS I and C203A within a GC rich region of the 5′-UTR [24]. The third alteration ,which was found in the one healthy man out of 100 blood donors, was 25delT in a conserved region of 5′-UTR. In the same study no germ-line alterations were found among 92 prostate cancer patients [25]. In addition, one somatic change in 3′-UTR of a prostate cancer specimen has been reported [26].

Since the amplification of *AR* gene can explain only partly the overexpression of the gene in the ablation-resistant prostate cancer, we decided to screen mutations in prostate cancer cell lines, xenografts, and clinical samples, the promoter and the 5′-UTR regions, which could alter the transcriptional rate of *AR* gene, and the 3′-UTR, which could affect the stability of the androgen receptor mRNA.

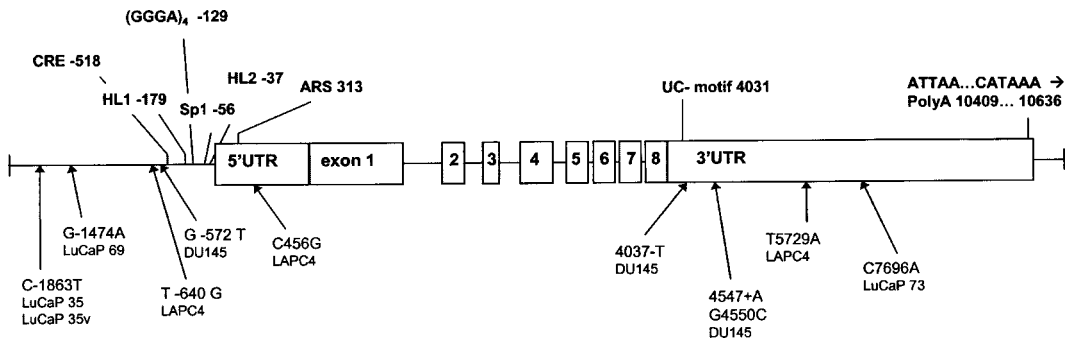

Fig. 1. Schematic structure of the *AR* promoter (2.3 kb), 5′ - and 3′ -UTR regions (1.1 and 6.8 kb, respectively) and coding regions (exons 1–8, 2.7 kb). The core promoter region (−74 to +87) that includes Sp1 and HL2 binding sites, and other functionally known motifs are marked above, and the alterations found in the present study are marked with arrows below the schematic structure of the gene. The coordinates are calculated from transcription initiation site (ARTIS II) using GenBank sequences (accession numbers NM.000044 and AL049564 for upstream regulatory region and AL356358 for the 3′UTR).

MATERIALS AND METHODS

Materials

DU145, LNCaP, PC-3, and 22Rv1 prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and LAPC4 was kindly provided by Dr. Charles Sawyers (UCLA, Los Angeles, CA) and were cultured under the recommended conditions. Freshly frozen samples of 19 xenografts (LuCaP 23.1, 23.8, 23.12, 35, 35 V, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 105, 115) were made available for the analyses by one of the co-authors (R.L.V.). Freshly frozen clinical samples (6 benign prostate hyperplasia, 30 untreated and 14 ablation-resistant prostate cancers) were obtained form the Tampere University Hospital (Tampere, Finland). The specimens were histologically examined for the presence of tumor tissue (>50% of cells) using hematoxylin and eosin-stained slides. Among the untreated carcinomas, the distribution of WHO-grade among was: 9 grade I, 14 grade II, and 7 grade III. The TNM-stage distribution was: 14 T2N0M0, 1 T2N1M0, 10 T3N0M0,

TABLE I. Primers for DHPLC and Sequencing

Primers[a]	Binding region from ARTIS II[b]	Fragment size (bp)	Annealing temperature (°C)	Sequence in 5′ → 3′ orientation
PRO6for	−2,264–2,284	400	58/55	CTGCAAAGAACAGGAGGAGAA
PRO6rev	−1,885–1,904		58/55	ATGTTGCTTCCACATCACCA
PRO5for	−1,923–1,943	436	58/55	GGCAACAGTTTTCAGATGTGG
PRO5rev	−1,508–1,572		58/55	ATGGCTACAGCCTTCCTTGA
PRO4for	−1,589–1,609	531	58/55	TGTGGTGGGATTAAATGTTGC
PRO4rev	−1,079–1,098		58/55	ATTCTGGGGAGGCTCTCTGT
PRO3for	−1,119–1,138	572	58/55	CAAAAGAGACCCAGGCAAAA
PRO3rev	−567–586		58/55	CTCAGCCCCAAGAATCAGAG
PRO2for	−659–678	525	58/55	GGGTGATTTTGCCTTTGAGA
PRO2rev	−154–173		58/55	GGCTTTGGAGAAACAAGTGC
PRO1for	−228–247	527	60/58	GCAGGAGCTATTCAGGAAGC
PRO1rev	+261–280		60/58	CTACCAGGCACTTTCCTTGC
5′UTR-1for	+240–259	400	60/58	GCTGCTAAAGACTCGGAGGA
5′UTR-1rev	+620–639		60/58	CAGGAGGAGGTGGAGAGAGA
5′UTR2 for	+507–526	192	60/58	CACATTGCAAAGAAGGCTCT
5′UTR2 rev	+678–698		60/58	GAAGACCTGACTGCCTTTTCA
5′UTR-3 for	+658–677	272	58/55	CGGAGCCAGAGATCAAAAGA
5′UTR-3 rev	+910–929		58/55	CGTGCAGAAGAAGACACACG
5′UTR-4 for	+886–905	388	58/55	CCTAGCAGGGCAGATCTTGT
5′UTR-4 rev	+1,170–1,189		58/55	TTCTGGAAAGCTCCTCGGTA
3′UTR-1for	3,822–3,841	828	58	CAAGTGCCCAAGATCCTTTC
3′UTR-1rev	4,631–4,650		58	GGCAGGTACTGATGCTCCAT
3′UTR-2afor	4,445–4,464	623	58	CCCAAAGAGGCCAATAGTGA
3′UTR-2arev	5,049–5,068		58	AGGTGGGGAAAAGGTAGTGG
3′UTR-2bfor	4,971–4,990	691	58	TGGAGCCAGAGGAGAAGAAA
3′UTR-2brev	5,643–5,662		58	CATCTGGCTTTAGGCTTTGC
3′UTR-3for	5,455–5,474	750	58	AGCTAAAGGGGCTACCCAGA
3′UTR-3rev	6,186–6,205		58	TAGGTTCCCCTCTCCCTTGT
3′UTR-4for	6,112–6,131	875	58	ATCCACAAGGGTTTCCTTCC
3′UTR-4rev	6,968–6,987		58	TGCCAACTTGTTTGGAGATG
3′UTR-5for	6,901–6,920	758	58	GCCACTCAGACCCACTTAGC
3′UTR-5rev	7,640–7,659		58	CCTTTATGCCCTGCCAGATA
3′UTR-6afor	7,534–7,553	592	58	TCCACATGATGCACAAATGA
3′UTR-6arev	8,107–8,126		58	CCCCTGCCCTTATGAATTTT
3′UTR-6bfor	7,990–8,009	539	58	AGGCAGATCTGTTCTCACCA
3′UTR-6brev	8,510–8,529		58	CATCCAAAGTGGGCAGAAAT

[a]Primers PRO1 to PRO6 and 5′UTR1 to 5′UTR4 were used for DHPLC analysis of promoter and 5′-UTR regions, respectively. Primers 3′UTR1 to 3′UTR6b were used to sequencing analysis of 3′UTR sequence.
[b]According to NM_000044 and deduced from AL049564 for promoter and AL356358 for 3′-UTR.

TABLE II. DHPLC Running Conditions

Fragment	Size (bp)	Temperatures (°C)	Gradient (% of Buffer B[a] in 8 min)
PRO6	400	53, 55, 57	55–67
PRO5	436	56, 58, 59	59–71
PRO4	531	56, 58, 59	56–68
PRO3	572	57, 59, 61	57–67
PRO2	525	59, 61, 63	55–67
PRO1	527	64, 65, 66	55–67
UTR1	400	61, 64, 65	58–70
UTR2	192	61, 63, 65	49–61
UTR3	272	57, 59, 60	55–67
UTR4	388	62, 63, 65	57–69

[a]Helix^{TM} buffer B, 25% acetonitrile, 1% triethylamine, 0.6% acetic acid, 0.01% ethylenediaminetetraacetic acid disodium salt (Varian, Inc., Palo Alto, CA).

1 T3NXM0, 1 T3NXM1, 1 T4NXM0, 1 T4NXM1, and 1 TXN0M0. The ablation-resistant samples were from patients who had experienced a local progression of the disease during hormonal therapy. The therapy modalities were: four orchiectomy, three LHRH (luteinizing hormone-releasing hormone) analog, two estrogen, two orchiectomy and estrogen, one orchiectomy and bicalutamide, one LHRH analog and bicalutamide, and one unknown. The median time from the onset of the androgen ablation to the progression was 38 months (range: 15–68).

Thirty normal samples were obtained from healthy Finnish female blood donors. DNA samples, extracted with routine techniques, were first amplified with GenomiPhi^{TM} - DNA amplification kit (Amersham, GE Healthcare, UK) according to the manufacturer's instructions and diluted 1:5 for the subsequent PCR reactions.

PCR

The primers and annealing temperatures used are listed in Table I. All primers were designed with Primer3-program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ www.cgi). Amplification of the promoter and the 5′-UTR fragments was done with Accutype polymerase (Stratagene, La Jolla, CA) in detergent-free buffer according to the manufacturer's instructions. After 5 min denaturation at 98°C, the 10 cycles consisted of denaturation at 98°C for 50 sec, annealing at 60°C or 58°C for 40 sec, and elongation at 72°C for 2 min, followed by additional 25 cycles consisting of denaturation at 98°C for 50 sec, annealing at 57 or 55°C for 40 sec, and elongation at 72°C initially for 2 min, and subsequently additional 10 sec in each cycle followed by final elongation at 72°C for 10 min. Amplification of the 3′-UTR fragments was done with Platinum Taq (Invitrogen^{TM}, Carlsbad, CA) added with 1:4 ratio of Pfu polymerases (Fermentas, Inc. Hanover, MD) in 1.0 or 1.5 mM $MgCl_2$ supplemented Platinum-buffer according to the manufacturer's instructions. After 3 min denaturation at 96°C, the 35 cycles consisted of denaturation at 96°C for 40 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 1 min followed by final elongation at 72°C for 2 min.

Denaturating High Performance Liquid Chromatograph (DHPLC)

For heteroduplex analysis, each fragment was mixed in a 1:1 ratio with a normal correspondingly amplified

TABLE III. Sequence Alterations in *AR* Promoter and UTR-regions

Sample	Region	Base pairs from ARTIS II[a]	Change	Sequence variations (changed base bolded)
DU145	Promoter	−572	G → T	GATTCTTGGG**T**CTGAGGGTT
DU145	3′UTR	4,037	Del T	CTATTTGCTGGGC-TTTTTTTTCTCT
DU145	3′UTR	4,547	Ins A	TTCTGCCAAA**A**TGCCTATTGC
DU145	3′UTR	4,550	G → C	TTCTGCCAAATG**C**CTATTGC
LAPC4	Promoter	−640	T → G	GAGAAATGCA**G**GGTTAAAGG
LAPC4	5′UTR	456	C → G	GCTGCCAGCC**G**GAGTTTGCA
LAPC4	3′UTR	5,729	T → A	ACATTGCCCA**A**ACTCACTCA
LuCaP 35	Promoter	−1,863	C → T	TCTTTCAGAC**T**CAGGTTTGA
LuCaP 35V	Promoter	−1,863	C → T	TCTTTCAGAC**T**CAGGTTTGA
LuCaP 69	Promoter	−1,474	G → A	CACCTCCTCA**A**GTGAAAGGG
LuCaP 73	3′UTR	7,696	C → A	CAGCCCTGCA**A**CAAAGCTGC

[a]*AR* transcription initiation site. Base pair position according to according to NM_000044 and deduced from AL049564 for promoter and upstream regulator regions and for AL356358 for 3′-UTR.

DNA fragment and denatured at 95°C for 5 min and then reannealed over 30 min using a temperature gradient from 95 to 65°C. DHPLC analysis was performed using Agilent 1100 LC HPLC instrumentation (Agilent Technologies, Palo Alto, CA) equipped with Varian CP28353 Helix DNA Column (50 mm × 3.0 mm) (Varian, Inc., Palo Alto, CA) in a 12% gradient at suitable temperatures for 8 min. The optimal melting temperatures and gradients (Table II) for each PCR amplicon were obtained by analysis of the wild type sequence, using the DHPLC Melt Program at the Stanford Genome Technology Center web site (http://insertion.stanford.edu/), and by empirical testing.

Sequencing

For sequencing analysis, the PCR reactions were purified using either QIAquick PCR purification columns (Qiagen, Inc. Valencia, CA) or MultiScreen™ Filtration System (Millipore, Billerica, MA). Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI PRISM® 3100 sequencer (Applied Biosystems) according to the manufacturer's instructions. All found alterations were confirmed by an independent amplification of the PCR fragment and subsequent re-sequencing from the original non-whole genome amplified samples.

Controls

For DHPLC analysis, commercially available DYS271-control (Varian, Inc.) and one base substitution primers for each fragment were used to test the suitability of the column and assay conditions.

RESULTS AND DISCUSSION

Although mutations in the coding region of *AR* have thoroughly been studied in prostate cancer [4–6,27,28], alterations in the regulator regions of the gene have not been systematically analyzed. However, the common overexpression of *AR* in ablation-resistant prostate cancer suggests that such alterations could be common. In order to screen for mutations in the regulatory regions of *AR*, we first utilized prostate cancer cell lines and xenografts (n = 24), since almost all of them derive from ablation-resistant prostate cancers. Also, these models contain high frequency of genetic alterations suggesting that common mutations, if present in ablation-resistant prostate cancer, should be found in the cell lines and xenografts [29,30].

In the promoter and upstream regulatory region, four single base changes were found in the cell lines

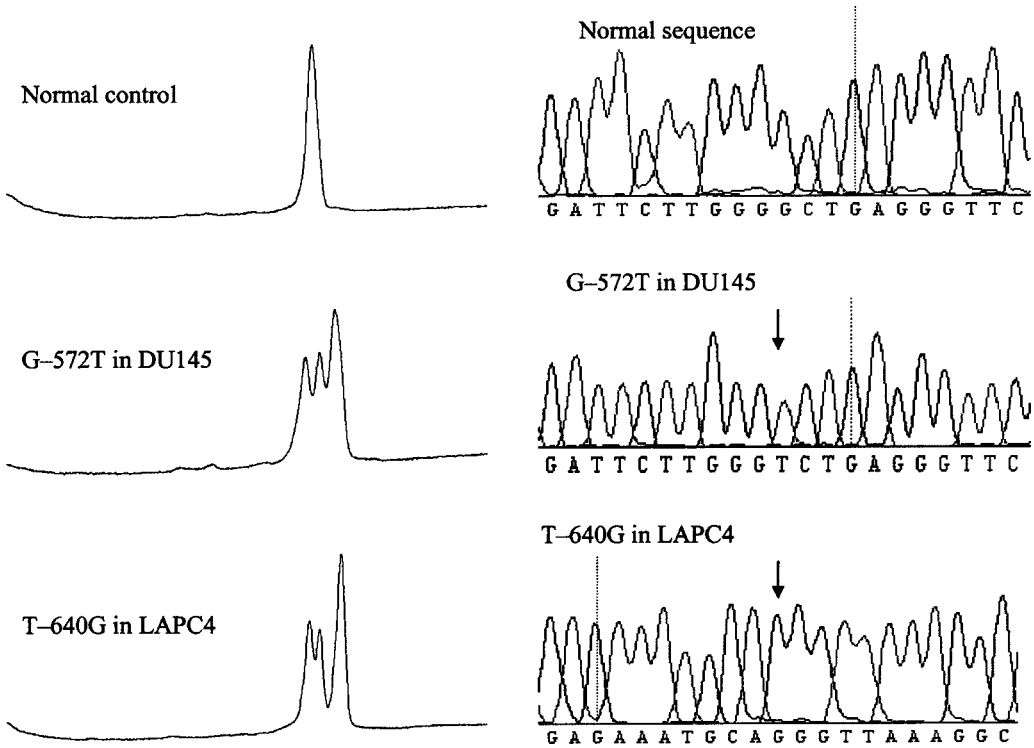

Fig. 2. Analyses of heteroduplexes with DHPLC. Above is shown a normal homoduplex sample (one peak curve) and below two mutation samples (three peak curves). Both changes are single base substitutions. On the right side are shown the exact sequences of each fragment.

and xenografts (Table III). One (G-572T) was found in *AR* negative cell line DU145 and another (T-640G) in *AR* positive LAPC4 cell line. Two other alterations, C-1863T and G-1474A, were found in xenografts LuCaP 35 and 69, respectively, which both express high-levels of *AR*, and also contain amplification of the *AR* gene [3]. The alteration found in the LuCaP 35 was also present in the subline of the xenograft (LuCaP 35 V). In the 5′-UTR, only one change, C456G, was found in LAPC4 cell line. None of the alterations are located in the known functional regions of the promoter or the 5′-UTR.

AR contains a very long but poorly characterized 3′-UTR. The databases, such as GenBank, recognize only a 4313 bp long mRNA, whereas according to Northern analysis, the size of the major form of the mRNA is about 11 kb, indicating about 6.8 kb long 3′-UTR [18]. However, only a small part of the 3′-UTR has been sequenced from cDNA, and thus the sequence has to be inferred from the genomic sequence. Here, we analyzed 4.6 kb of the putative 3′-UTR. Three substitutions, G4550C in DU145, T5729A in LAPC4, and C7696A in LuCaP 73 were found. In addition, there was one single base deletion (4037delT) and single base insertion (4547insA) in DU145. Except the 4037delT, the mutations are not located in the known functionally active elements or motifs [14–17,21–23] (Fig. 1). The functional analyses by Yeap et al. [23] showed that one base substitution in the $C(U)_9C$–motif, the region where delT was found in DU145, has no effect on the stability of the mRNA-protein interaction. Thus, the functional significance of these rare alterations, if any, remains unpredictable.

To find out the frequency of each alteration in clinical prostate cancer as well as to deduce whether they are polymorphisms, 44 prostate cancer specimens, six BPH specimens, and 30 (60 chromosomes) normal female germ-line DNAs were analyzed for these variants. None of these alterations were in these samples indicating that the variants are rare mutations. Of the 10 mutations, 4 were found in cell line DU145 and 3 in LAPC4 suggesting that these cell lines are possible genetically instable, and thus, contain high frequency of sequence alterations. Indeed, DU145 has been shown to lack functional MLH1 leading to loss of mismatch repair [31].

To screen mutations in the promoter and 5′-UTR of the *AR*, we utilized DHPLC. It is a very specific and sensitive HPLC-based heteroduplex-analysis at partly denaturing temperatures in a gradient of organic diluent [32]. The sensitivity and specificity of DHPLC have been reported to be from 96% up to as high as 100% in blind analysis [32]. We analyzed all samples in a gradient of 1.5% per minute of Helix^{TM} Buffer B for 8 min in three different temperatures suggested by the DHPLC-melt program and empirical testing (Table II). All samples showing additional peaks in DHPLC (Fig. 2) were sequenced from non-GenomiPhi^{TM} amplified template. Also 80 other PCR fragments were screened by both DHPLC and sequencing with fully concordant results. In addition, synthetic mutation controls were constructed for all fragments and they were all detected by DHPLC. Due to the heterogenic distribution of AT-repetitive sequences in 3′-UTR, which would have make the DHPLC analysis practically impossible, we used direct sequencing for the 3′-UTR mutation screening. Altogether, we believe our analyses should have been sensitive enough to detect most, if not all, sequence variations present in the samples. However, it is naturally possible that mutations, affecting the expression of *AR*, exist outside the regions that were analyzed here.

CONCLUSIONS

Altogether, only five cancer samples showed non-recurrent sequence alterations in the promoter as well as 5′- and 3′-UTR of *AR*. The data indicate that recurrent mutations in these regions are rare in prostate cancer and thus not likely to contribute to the common over-expression of *AR* in ablation-resistant prostate cancers.

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REFERENCES

1. Huggins C, Hodge CV. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1941;1:293–297.
2. Visakorpi T, Hyytiäinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9:401–406.
3. Linja M, Savinainen K, Saramäki O, Tammela T, Vessella R, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61:3550–3555.
4. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ. Cancer and Leukemia Group B Study 9663. Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 2003;21:2673–2678.
5. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ. Androgen receptor mutations in prostate cancer. Cancer Res 2000;60:944–949.
6. Haapala K, Hyytinen ER, Roiha M, Laurila M, Rantala I, Helin HJ, Koivisto PA. Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. Lab Invest 2001;81:1647–1651.

7. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10(1):33–39.
8. Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, Delprado W, Stricker PD, Grygiel JJ, Sutherland RL. Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer Res 2001;61:423–427.
9. Edwards J, Krishna NS, Grigor KM, Bartlett JM. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 2003;89:552–556.
10. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP. Androgen receptor gene amplification: A possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57:314–319.
11. Latil A, Bieche I, Vidaud D, Lidereau R, Berthon P, Cussenot O, Vidaud M. Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res 2001;61:1919–1926.
12. Roudier MP, True LD, Higano CS, Vesselle H, Ellis W, Lange P, Vessella RL. Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. Hum Pathol. 2003;34:646–653.
13. Faber PW, van Rooij HC, van der Korput HA, Baarends WM, Brinkmann AO, Grootegoed JA, Trapman J. Characterization of the human androgen receptor transcription unit. J Biol Chem 1991;266:10743–10749.
14. Faber PW, van Rooij HC, Shipper HJ, Brinkmann AO, Grootegoed JA, Trapman J. Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen promoter. J Biol Chem. 1993;268:9296–9301.
15. Mizokami A, Yeh SY, Chang C. Identification of 3′,5′-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. Mol Endocrinol 1994;8:77–88.
16. Takane KK, McPhaul MJ. Functional analysis of the human androgen receptor promoter. Mol Cell Endocrinol 1996;119:83–93.
17. Chen S, Supakar PC, Vellanoweth RL, Song CS, Chatterjee B, Roy AK. Functional role of a conformationally flexible homopurine/homopyrimidine domain of the androgen receptor gene promoter interacting with SP1 and a pyrimidine single strand DNA-binding protein. Mol Endocrinol 1997;11:3–15.
18. Delfino FJ, Boustead JN, Fix C, Walker WH. NF-kappaB and TNF-alpha stimulate androgen receptor expression in Sertoli cells. Mol Cell Endocrinol 2003;201(1-2):1–12.
19. Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 1988;240:327–330.
20. Trapman J, Klaassen P, Kuiper GG, van der Korput JA, Faber PW, van Rooij HC, Geurts van Kessel A, Voorhorst MM, Mulder E, Brinkmann AO. Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Commun 1988;153:241–248.
21. Mizokami A, Chang C. Induction of Translation by the 5′untranslated region of human androgen receptor mRNA. J Biol Chem 1994;269:25655–25659.
22. Wang LG, Ossowski L, Ferrari AC. Androgen receptor level controlled by a suppressor complex lost in an androgen-independent prostate cancer cell line. Oncogene 2004;23:5175–5184.
23. Yeap BB, Voon DC, Vivian JP, McCulloch RK, Thomson AM, Giles KM, Czyzyk-Krzeska MF, Furneaux H, Wilce MC, Wilce JA, Leedman PJ. Novel binding of HuR and poly(C)-binding protein to a conserved UC-rich motif within the 3′-untranslated region of the androgen receptor messenger RNA. J Biol Chem 2002;277:27183–27192.
24. Crocitto LE, Henderson BE, Coetzee GA. Identification of two germline point mutations in the 5′UTR of the androgen receptor gene in men with prostate cancer. J Urol 1997;158:1599–1601.
25. Cabral DF, Santos A, Ribeiro ML, Mesquita JC, Carvalho-Salles AB, Hackel C. Rarity of DNA sequence alterations in the promoter region of the human androgen receptor gene. Braz J Med Biol Res 2004;37:1789–1794.
26. Paz A, Lindner A, Zisman A, Siegel Y. A genetic sequence change in 3′-noncoding region of the androgen receptor gene in prostate carcinoma. Eur Urol 1997;31:209–215.
27. Wallén MJ, Linja M, Kaartinen K, Schleutker J, Visakorpi T. Androgen receptor gene mutations in hormone-refractory prostate cancer. J Pathol 1999;189:559–563.
28. Gottlieb B, Beitel LK, Wu JH, Trifiro M. The androgen receptor gene mutations database (ARDB): 2004 update. Hum Mutat 2004;23:527–533.
29. Laitinen S, Karhu R, Sawyers CL, Vessella RL, Visakorpi T. Chromosomal aberrations in prostate cancer xenografts detected by comparative genomic hybridization. Genes Chromosome Cancer 2002;35:66–73.
30. Nupponen NN, Hyytinen ER, Kallioniemi AH, Visakorpi T. Genetic alterations in prostate cancer cell lines detected by comparative genomic hybridization. Cancer Genet Cytogenet 1998;101:53–57.
31. Chen Y, Wang J, Fraig MM, Metcalf J, Turner WR, Bissada NK, Watson DK, Schweinfest CW. Defects of DNA mismatch repair in human prostate cancer. Cancer Res 2001;61:4112–4121.
32. Xiao W, Oefner PJ. Denaturating high-performance liquid chromagraphy: a review. Hum Mutat 2001;17:439–474.

Increased Expression of Androgen Receptor Sensitizes Prostate Cancer Cells to Low Levels of Androgens

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Abstract

Androgen receptor (AR) is known to be overexpressed in castration-resistant prostate cancer. To interrogate the functional significance of the AR level, we established two LNCaP cell sublines expressing in a stable fashion two to four times (LNCaP-ARmo) and four to six times (LNCaP-ARhi) higher level of AR than the parental cell line expressing the empty vector (LNCaP-pcDNA3.1). LNCaP-ARhi cell line grew faster than the control line in low concentrations, especially in 1 nmol/L 5α-dihydrotestosterone (DHT). Microarray-based transcript profiling and subsequent unsupervised hierarchical clustering showed that LNCaP-ARhi cells clustered together with VCaP cells, containing endogenous *AR* gene amplification and overexpression, indicating the central role of AR in the overall regulation of gene expression in prostate cancer cells. Two hundred forty genes showed >2-fold changes on DHT treatment in LNCaP-ARhi at 4 h time point, whereas only 164 and 52 showed changes in LNCaP-ARmo and LNCaP-pcDNA3.1, respectively. Many androgen-regulated genes were upregulated in LNCaP-ARhi at 10-fold lower concentration of DHT than in control cells. DHT (1 nmol/L) increased expression of several cell cycle–associated genes in LNCaP-ARhi cells. ChIP-on-chip assay revealed the presence of chromatin binding sites for AR within ±200 kb of most of these genes. The growth of LNCaP-ARhi cells was also highly sensitive to cyclin-dependent kinase inhibitor, roscovitine, at 1 nmol/L DHT. In conclusion, our results show that overexpression of AR sensitizes castration-resistant prostate cancer cells to the low levels of androgens. The activity of AR signaling pathway is regulated by the levels of both ligand and the receptor. [Cancer Res 2009;69(20):8141–9]

Introduction

Prostate cancer is the most common male malignancy in many western countries (1, 2). The growth and the differentiation of normal prostate epithelial cells as well as development of prostate cancer are dependent on androgens (3). Androgen ablation, the gold standard treatment for advanced prostate cancer, initially inhibits tumor growth but ultimately fails and leads to emergence of castration-resistant prostate cancer (CRPC), which has also been called as androgen-independent prostate cancer. However, a recent clinical trial on abiraterone indicated directly that CRPC is still androgen-dependent (4). It has been shown that CRPC cells upregulate the expression of many of enzymes involved in steroidogenesis, suggesting that cancer cells themselves produce androgens during androgen withdrawal (5–7). In addition, the experimental models of CRPC have shown that many of the androgen-regulated genes are upregulated in CRPC (8–11).

Androgen action is mediated by the androgen receptor (AR; ref. 12). It has been shown that AR is overexpressed in vast majority of CRPC (13, 14). In addition, ~30% of CRPC carry *AR* gene amplification (15). Somatic mutations of *AR* in prostate cancer have also been extensively studied. The mutations seem to be rare in untreated tumors but are found in 10% to 30% of tumors treated with antiandrogens, such as flutamide and bicalutamide (16, 17). Receptor mutations may broaden the ligand specificity converting even the antagonist effect of antiandrogens to agonist one (10, 18). It has also been suggested that crosstalk between AR signaling and other pathways, such as mitogen-activated protein kinase, epidermal growth factor receptor, and Akt pathways, takes place, especially in androgen-depleted environment (12). In addition, alterations in the expression of AR coregulators have been suggested, but not proven, to be involved in the progression of prostate cancer (19). Functional evidence that AR is involved in the emergence of CRPC was presented by Chen and colleagues (20) who showed that ectopic expression of a high AR content was sufficient to transform androgen-dependent prostate cancer cells to androgen-independent ones. Also, Kokontis and colleagues (21) have shown previously that adaptation of LNCaP cell line to low levels of androgens is associated with increased expression of endogenous mutant AR. Together with the findings that AR overexpression is common in CRPC, the experimental data suggest that the overexpression of this receptor is a key mechanism for the progression of prostate cancer.

To mimic the conditions of high AR expression in CRPC, we have established a cell line model by a stable transfection of *AR* into an androgen-responsive prostate cancer cell line, LNCaP. Two sublines with moderate (LNCaP-ARmo) and high (LNCaP-ARhi) levels of AR overexpression were produced. The model cell lines were subsequently used to examine the influence of AR levels on cell growth and expression of downstream genes of the AR signaling pathway. The aim was to investigate whether AR overexpression hypersensitizes cells to the low levels of androgens, as we have suggested previously (15), as well as to identify the candidate downstream genes that are involved in the emergence of CRPC.

Materials and Methods

Cell culture protocols and transfections. LNCaP cells (American Type Culture Collection) were cultured under the recommended conditions.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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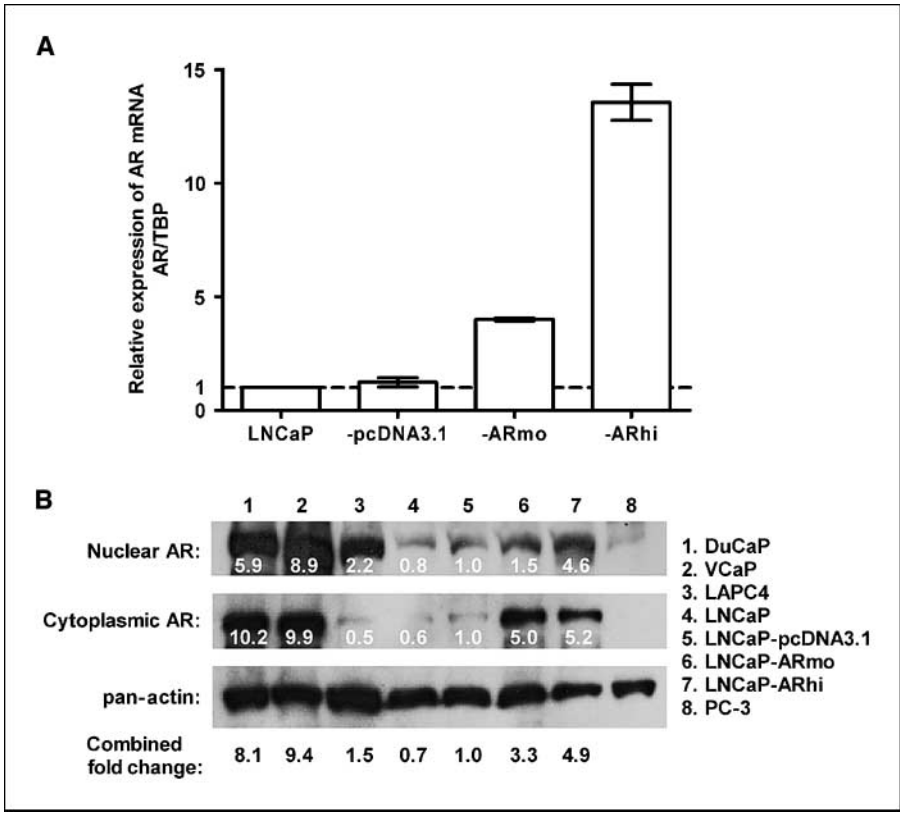

Figure 1. *A,* relative expression levels of *AR* mRNA (*AR/TBP*) as measured by Q-RT-PCR. The *AR* mRNA level is ~13 times higher in LNCaP-ARhi and 4 times higher in LNCaP-ARmo cells than in controls (parental LNCaP and LNCaP-pcDNA3.1). Mean ± SE of three replicates. *B,* Western analysis of the AR protein levels. LNCaP-ARhi cells had 4- to 6-fold and LNCaP-ARmo cells had 2- to 4-fold higher AR protein levels than control cells (LNCaP and LNCaP-pcDNA3.1). Nuclear and cytoplasmic protein levels were quantified by ImageJ software program and normalized against pan-actin (loading control). The combined fold change is also shown. PC-3 cells were used as a negative control.

Either pcDNA3.1(+) empty expression vector (Invitrogen) or pcDNA3.1(+) inserted with the *AR* coding region [accession #_M23263; digested with *Sal*I and *Nhe*I from pSG5 expression vector and subcloned pTarget vector (Invitrogen) and finally into *Not*I/*Bam*HI site in pcDNA3.1] were stable transfected into LNCaP with Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's instructions. Transfected clones were selected with 400 μg/mL geneticin (G418; Invitrogen), and several clones were expanded. *AR* mRNA level was determined by using Northern blot analysis and quantitative real-time reverse transcription-PCR (Q-RT-PCR). Subsequently, dozen clones, showing the highest overexpression of AR mRNA, were analyzed further for their AR protein levels using Western blotting. Finally, two clones, expressing moderately (LNCaP-ARmo) and highly (LNCaP-ARhi) increased levels of AR protein, were selected for further analyses. Cells transfected with an empty vector (LNCaP-pcDNA3.1) were used as a control. The transfected cells were cultured in medium containing geneticin (200 μg/mL). DuCaP and VCaP cells were kindly provided by Dr. Jack Schalken (Radboud University Nijmegen Medical Center) and grown under the recommended conditions. LAPC4 cell line was kindly provided by Dr. Charles Sawyers (Sloan-Kettering Institute) and cultured under the recommended conditions.

Before each experiment with hormone exposure, cells were grown in charcoal-stripped serum (CSS; Hyclone) in medium without phenol red for 4 days. Subsequently, the medium was replaced for the experiment with that containing various concentrations of 5α-dihydrotestosterone (DHT; Steraloids) or roscovitine (Calbiochem, EMD Chemicals).

Cell proliferation assays. After 3 days of incubation in charcoal-stripped serum medium, the cells were trypsinized, counted, and placed in 12-well dishes in charcoal-stripped serum medium with desired concentration of DHT. The amount of cells at each time point was analyzed using Alamar Blue reagent (AbD Serotec) and luminometric detection using a fluorometer (Wallac 1420 Victor; Perkin-Elmer). Alternatively, the cells were trypsinized and the number of cells was calculated with Beckman Coulter Z2-series particle counter according to the manufacturer's instructions. Each experiment was done in quadruplicate for DHT-induced growth analysis and in triplicate for roscovitine exposures. For the relative growth curves, the luminometric values or the number of the cells in each well in each follow-up day were divided by the mean values or number at day 1.

Q-RT-PCR. Subconfluent cells were collected from dishes and their total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out from total RNA using AMV reverse transcriptase (Finnzymes) according to the manufacturer's instructions. The primers for Q-RT-PCR were designed with Primer3 program.[3] Primer sequences are listed in Supplementary Table S1. SYBR Green II-Fast Start kit (Roche Diagnostics) and Light Cycler apparatus (Roche Diagnostics) were used for Q-RT-PCR essentially as described previously (13). *TBP* (TATA box binding protein) mRNA was used as a reference. The specificity of the reactions was confirmed, in addition to the melting curve analysis, with 1.5% agarose gel electrophoresis.

Western blot. The soluble cytoplasmic and nuclear proteins were extracted from subconfluent cells using the modified method of Dignam and colleagues (22). Both cytoplasmic and nuclear proteins (12.5 μg each) were separated in 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane (Immobilon-P; Millipore). After blocking, membranes were incubated with the primary antibody (mouse anti-AR 441 and mouse anti-pan-actin, clone ACTN05; NeoMarkers), washed, and incubated with the secondary antibody (anti-mouse IgG-horseradish peroxidase conjugate; DAKO). After washing, the protein bands were visualized on autoradiography film (Kodak) using chemiluminescence detection (Western Blotting Luminol reagent; Santa Cruz Biotechnology). Intensity differences were quantified by ImageJ image analysis software program.[4] Equal loading was confirmed by staining with antibody against pan-actin.

Microarray analysis. Microarray hybridizations were done in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. First,

[3] http://frodo.wi.mit.edu/primer3/input.htm
[4] http://rsb.info.nih.gov/ij/index.html

300 ng total RNA of each sample was transcribed *in vitro*, biotinylated and amplified with Illumina RNA TotalPrep Amplification kit (Ambion), and hybridized to Illumina Sentrix HumanRef-8_V2 Expression BeadChip according to the manufacturer's instructions. The probes of Illumina HumanRef-8 v2 chip are based on the content from the National Center for Biotechnology Information RefSeq database 1, release 17 containing >22,000 well-annotated transcripts. The data were analyzed with GeneSpring Analysis Platform version GX 7.3.1 (Agilent Technologies). First, lowest signal value was set to be 3-fold higher compared with negative control (water) signals. All individual values below that were set to this lowest signal value.

For unsupervised hierarchical clustering, samples were normalized per chip by the 50th percentile and per gene by the median. Average linkage method was used for clustering, and the similarities were estimated with Pearson's correlation. For the analyses of DHT dose responses, data were normalized with repeated median polishing per chip and gene. Subsequently, signal values in each treatment were divided by the signal value of the 0 nmol/L DHT treatment of the same cell line at the same time point. To identify differently expressed genes in the faster-growing LNCaP-ARhi cells at 1 nmol/L DHT, the data were normalized with intensity dependent Lowess normalization. Twenty percent of the data were used to calculate the Lowess fit at each point. This curve was used to adjust the control value (LNCaP-pcDNA3.1 or LNCaP-ARmo were used as control samples for LNCaP-ARhi) for each measurement. For ontology classifications, all gene ontology lists containing at least 10 genes with $P < 0.001$ (hypergeometric P value without multiple testing correction) in either LNCaP-pcDNA3.1, or LNCaP-ARmo, or LNCaP-ARhi were filtered and organized with GeneSpring Ontology browser.

The array data were submitted using MIAMExpress to the ArrayExpress database (accession number E-MEXP-2286).

ChIP-on-chip assays. ChIP-on-chip assays were done with anti-AR antibody (BJ14-AR3; ref. 23) in nontransfected LNCaP-1F5 cells that were cultured in the absence of hormone for 4 days and then exposed to 100 nmol/L DHT or vehicle for 2 h. The immunoprecipitation enriched and input chromatin samples were amplified by ligation-mediated PCR followed by fragmentation and labeling of DNA. Three micrograms of samples from input and immunoprecipitated samples were hybridized to Affymetrix whole-genome tiling arrays (GeneChip Human Tiling 2.OR Array Set; Affymetrix). The regions enriched for AR-binding sites were identified by MAT algorithm (24) and mapped to the most recent human genome sequence (Hg18), and sites that were enriched above input were scored at a P value of 10^{-4}. These AR-binding sites were then subsequently mapped to the differentially expressed genes in the AR overexpression model.

Results

Establishment of AR-overexpressing LNCaP. Two AR-overexpressing clones were selected for experiments. These were LNCaP-ARmo, with ~4-fold higher AR mRNA and ~2- to 4-fold higher AR protein level, and LNCaP-ARhi, with 13-fold higher AR

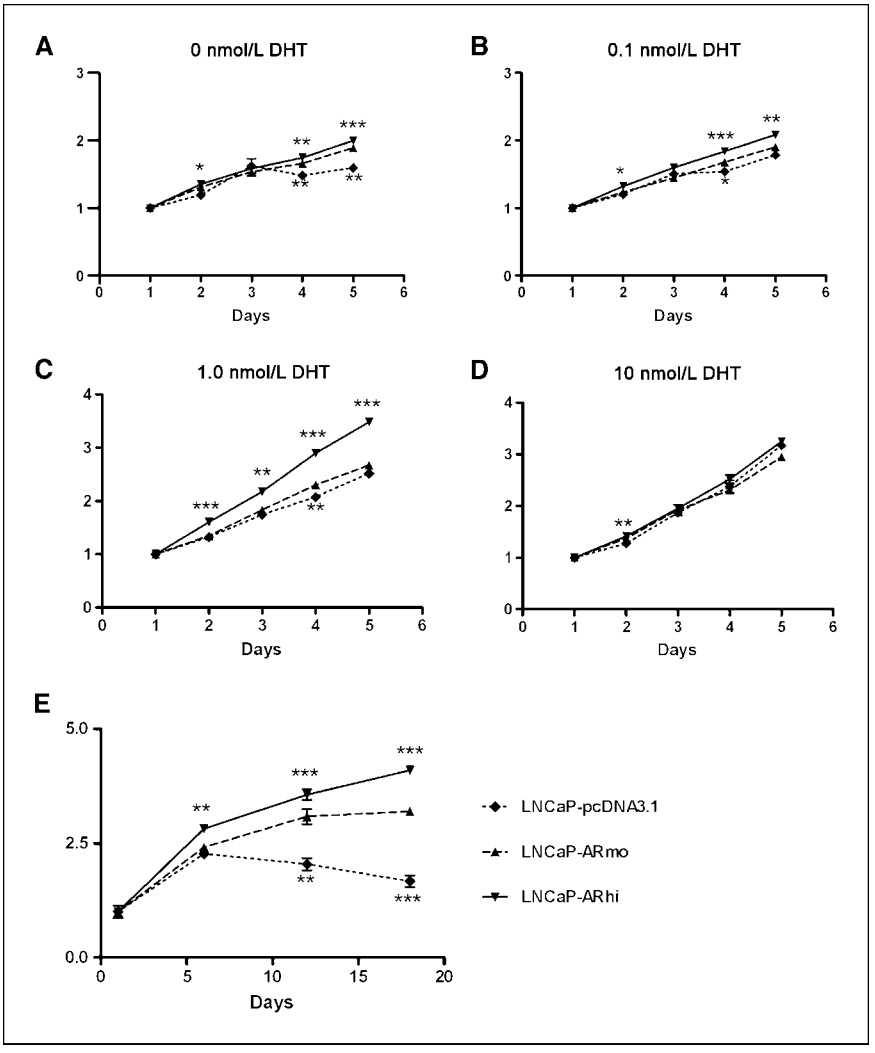

Figure 2. Relative growth of the LNCaP-AR model cells in the presence of (*A*) 0 nmol/L DHT, (*B*) 0.1 nmol/L DHT, (*C*) 1 nmol/L DHT, and (*D*) 10 nmol/L DHT. The greatest advantage in proliferation rate for AR-overexpressing cells was seen in 1.0 nmol/L DHT. *E,* growth of cells in charcoal-stripped serum medium without androgens during 3 wk. The highest growth rate was seen in LNCaP-ARhi cells. The growth of LNCaP-ARmo was between LNCaP-ARhi and control cells. Mean ± SE of four replicates. *Y axis,* relative growth against day 1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, below the curves for LNCaP-ARmo versus LNCaP-pcDNA3.1 and above the curves for LNCaP-ARhi versus LNCaP-pcDNA3.1.

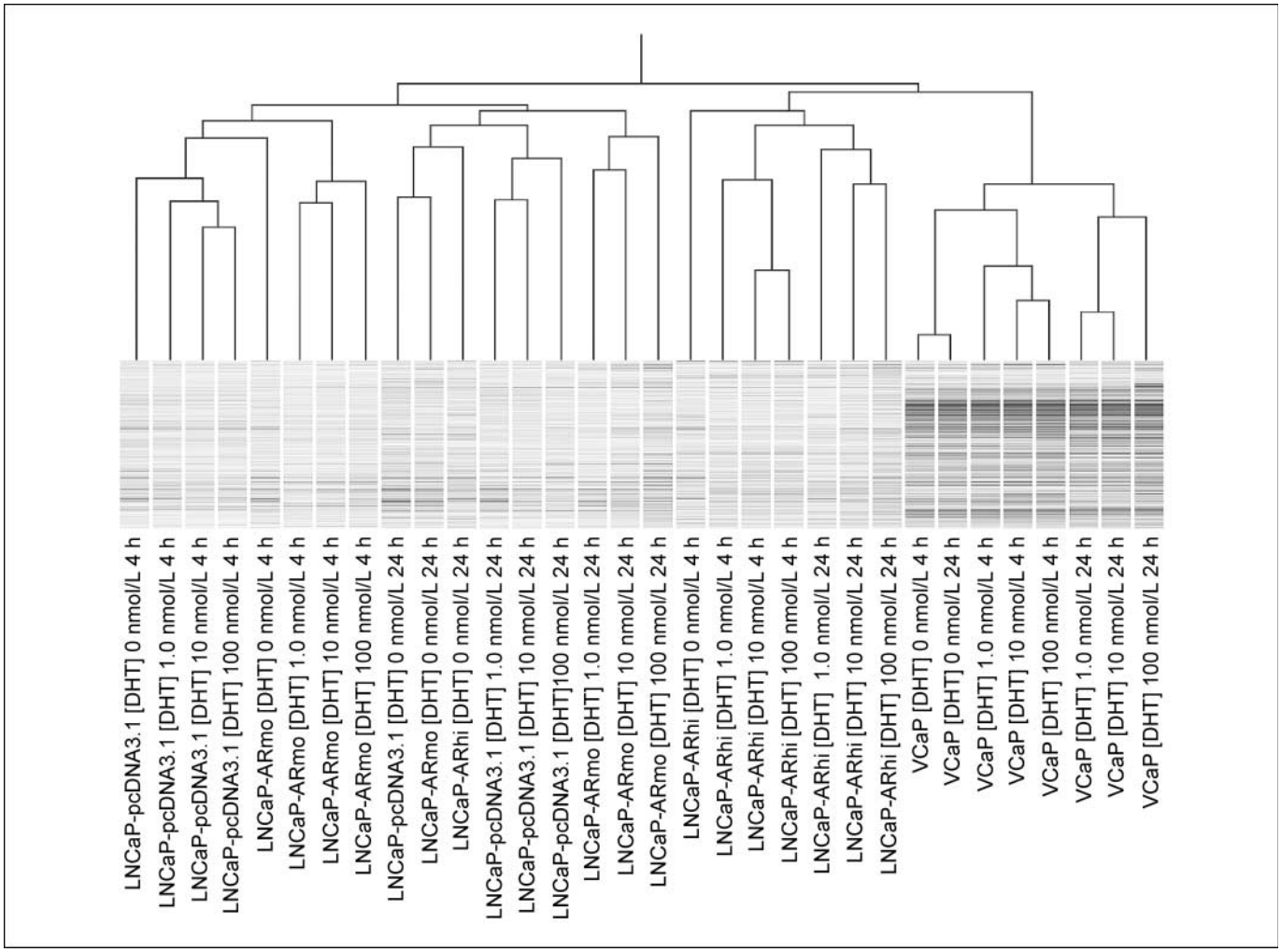

Figure 3. Unsupervised hierarchical clustering of mRNA expression in DHT-treated LNCaP-AR and VCaP cells. LNCaP-ARhi cells cluster together with VCaP cells (except 0 mol/L DHT treated), and LNCaP-pcDNA3.1 cells together with LNCaP-ARmo cells.

mRNA and 4- to 6-fold higher AR protein level, than those in the control LNCaP-pcDNA3.1 cells (Fig. 1). Immunofluorescence staining showed that the AR protein is located in nucleus when cells are grown in the presence of androgens (Supplementary Fig. S1). In addition, overexpression of AR seemed to enhance the nuclear transport after DHT exposure. The most intense nuclear staining was seen in the LNCaP-ARhi cells after 1 h exposure to 10 nmol/L DHT.

Growth curve analyses. The proliferation rates of these cells were analyzed in the presence of various DHT concentrations. The growth of LNCaP-ARhi cells was stimulated with lower concentrations of DHT than control LNCaP-pcDNA3.1 or LNCaP-ARmo cells (Fig. 2). Especially, in the presence of 1 nmol/L DHT, LNCaP-ARhi cells grew clearly faster than LNCaP-ARmo or LNCaP-pcDNA3.1 cells ($P < 0.01$). The cells overexpressing AR were also capable of growing better in charcoal-stripped serum medium without DHT (Fig. 2*E*). However, the growth finally plateaued in all cell lines.

Expression profiling. For genome-wide expression profiling, LNCaP-ARhi, LNCaP-ARmo, and LNCaP-pcDNA3.1 cells were grown in the presence of various DHT concentrations (0, 1, 10, and 100 nmol/L), and total RNA was extracted at 4 and 24 h time points. In addition, VCaP cells that contain endogenous *AR* gene amplification and strong overexpression of AR (ref. 24; Fig. 1) were treated and analyzed in a similar fashion. Unsupervised hierarchical clustering of androgen-regulated transcripts revealed that VCaP and LNCaP-ARhi cells clustered together, whereas androgen-dependent transcripts of LNCaP-ARmo cells clustered together with those of LNCaP-pcDNA3.1 cells (Fig. 3).

More genes were upregulated than downregulated in the LNCaP-AR cells by DHT. Venn diagrams for upregulated and downregulated genes are shown in Fig. 4. In LNCaP-pcDNA3.1 cells, expression of 52 and 118 genes was changed >2-fold at any DHT concentration compared with vehicle at 4 and 24 h, respectively. In LNCaP-ARmo and LNCaP-ARhi, the number of genes with altered expression was higher; in ARmo 164 and 379 at 4 and 24 h time points, respectively, and in ARhi 240 and 475 at 4 and 24 h time points, respectively ($P < 0.0001$, χ^2 test). In VCaP cells, expression of 430 genes was changed >2-fold at the 4 h time point and 428 genes at the 24 h time point. The upregulated and downregulated genes by DHT exposure in LNCaP-pcDNA3.1, LNCaP-ARmo, LNCaP-ARhi, and VCaP are listed in Supplementary Tables S2 to S5.

Next, we analyzed gene ontology categories for all genes upregulated and downregulated at 24 h of DHT treatments. In

LNCaP-pcDNA3.1 cells, DHT upregulated transcripts belonged to the following five gene ontology categories (at least 10 upregulated genes with $P < 0.001$): intracellular signaling cascade, cell cycle, cell division, protein metabolism, and DNA metabolism (Supplementary Table S6). In LNCaP-ARhi and LNCaP-ARmo cells, the same five gene ontology categories were also upregulated as those in LNCaP-pcDNA3.1 cells, but the number of upregulated genes was significantly higher in each category. In addition, six other main ontologies were enriched in LNCaP-ARmo and/or LNCaP-ARhi cells. These categories were lipid metabolism, secretory pathway, cell organization and biogenesis, chromosome segregation, response to endogenous stimulus, and cell proliferation (Supplementary Table S6). Ontologies that showed highly significantly ($P < 0.0001$, χ^2 test) more upregulated genes in LNCaP-ARhi cells compared with LNCaP-pcDNA3.1 or LNCaP-ARmo cells were genes associated with mitotic cell cycle, regulation of progression through cell cycle, organelle organization and biogenesis, cellular protein metabolism, and DNA metabolism (Supplementary Table S6). Of upregulated ontologies, intracellular signaling cascade, cell cycle, and lipid metabolism were also upregulated significantly ($P < 0.01$) already at 4 h time point. Only one ontology category, apoptosis, was significantly upregulated at 4 h time point but not at 24 h time point. The downregulated genes were not enriched in any of the categories with more than two genes in any of the cell lines.

When upregulated ontologies were examined separately at various DHT concentrations (Fig. 5), particularly DNA metabolism, cell cycle, cell organization and biogenesis, cell division, and intracellular signaling cascade were found to be highly significantly upregulated in LNCaP-ARhi cells already in 1 nmol/L DHT concentration (Fig. 5*A*). At 10 and 100 nmol/L DHT concentrations, the same ontologies were upregulated but with higher number of genes. At 10 and 100 nmol/L DHT concentrations, cell cycle, DNA metabolism, lipid metabolism, cell organization and biogenesis, cell division, and chromosome segregation were highly significantly upregulated also in LNCaP-pcDNA3.1 or LNCaP-ARmo cells. The number of upregulated genes in these categories were, however, always highest in LNCaP-ARhi cells. The most significantly upregulated ontologies in LNCaP-ARmo cells were lipid metabolism and secretory pathway.

Expression of known AR target genes. Expression of well-known AR target genes, such as *PSA, TMPRSS2, NKX3-1*, and *TMEPAI*, was found to be increased by 4- to 10-fold in LNCaP-ARhi and LNCaP-ARmo compared with LNCaP-pcDNA3.1 at 4 and 24 h time points (Fig. 6). Likewise, expression of well-known downregulated AR target genes, such as *PAP* and *PSMA*, was attenuated more in LNCaP-ARhi and LNCaP-ARmo cells than in control cells. On average, for an equal level of upregulation of genes, a 10-fold higher DHT concentration was required for LNCaP-pcDNA3.1 cells than for LNCaP-ARhi or LNCaP-ARmo cells. Q-RT-PCR of *PSA* and six selected genes, unknown previously to be regulated by androgens, confirmed the microarray data (Supplementary Fig. S2).

LNCaP and VCaP cells contain genetic rearrangements affecting Ets transcription factors, *ETV1* and *ERG*, respectively (25, 26). In VCaP cells, *ERG* expression was upregulated by androgens

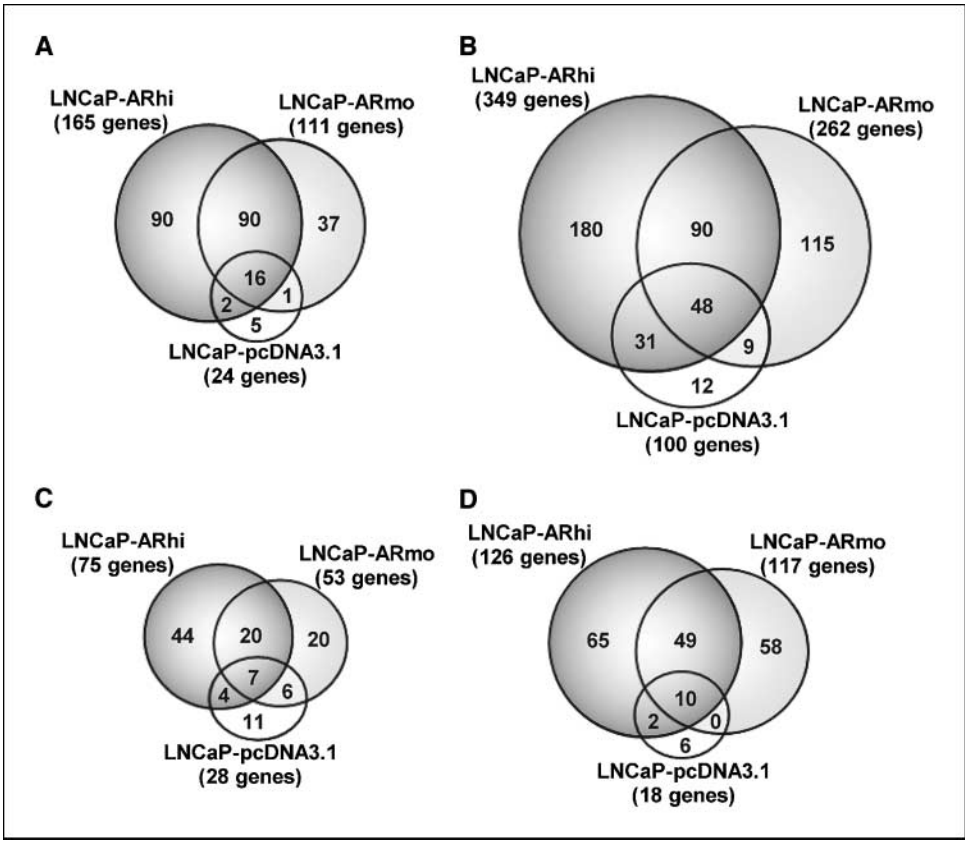

Figure 4. Venn diagrams of DHT responding genes in LNCaP-AR cell panel for upregulated genes at (*A*) 4 h and (*B*) 2 h. Ninety and 180 genes were found to be upregulated (>2-fold) only in LNCaP-ARhi at 4 and 24 h time points, respectively. Venn diagrams of genes downregulated by DHT in LNCaP-AR panel at (*C*) 4 h and (*D*) 24 h. Forty-four and 65 genes were found to be downregulated (>2-fold) only in LNCaP-ARhi at 4 and 24 h time points, respectively.

(Supplementary Fig. S3). Unfortunately, Illumina RefSeq8-v2 probes for *ETV1* failed to detect any expression. However, Q-RT-PCR analysis showed >2-fold increase of ETV1 mRNA (Supplementary Fig. S3). Of Ets family members, only *ELK4* mRNA level was increased with androgens in LNCaP-ARhi cells at 24 h time point (Supplementary Fig. S3). The *ELK4* gene is known to be an AR target in human prostate cancer cells with association to cell growth *in vitro* (27).

Kokontis and colleagues (21) have shown previously that LNCaP cells overexpressing endogenous AR show higher expression of *MYC* in low levels of androgens, and the expression is decreased in lower concentrations of the androgens than in the parental cell line. In similar fashion, here the highest *MYC* expression was found in VCaP and LNCaP-ARhi cells in the low DHT levels, and the expression was decreased in higher concentrations of DHT (Supplementary Fig. S4).

Identification of candidate AR downstream genes. Because the LNCaP-ARhi cells gained a growth benefit *in vitro* with 1 nmol/L DHT concentration compared with LNCaP-ARmo cells or empty vector–transfected LNCaP-pcDNA3.1 cells, we were particularly interested in the genes that are upregulated or downregulated in LNCaP-ARhi cells at 1 nmol/L DHT. Expression of 173 genes were found to be altered >2-fold (127 upregulated and 46 downregulated) in LNCaP-ARhi cells and compared with LNCaP-pcDNA3.1 or LNCaP-ARmo cell at 24 h after DHT exposure (Supplementary Table S7). Of these genes, we examined whether any of the upregulated genes were expressed in VCaP cells to the same or higher level than in LNCaP-ARhi cells. Ninety-nine such genes

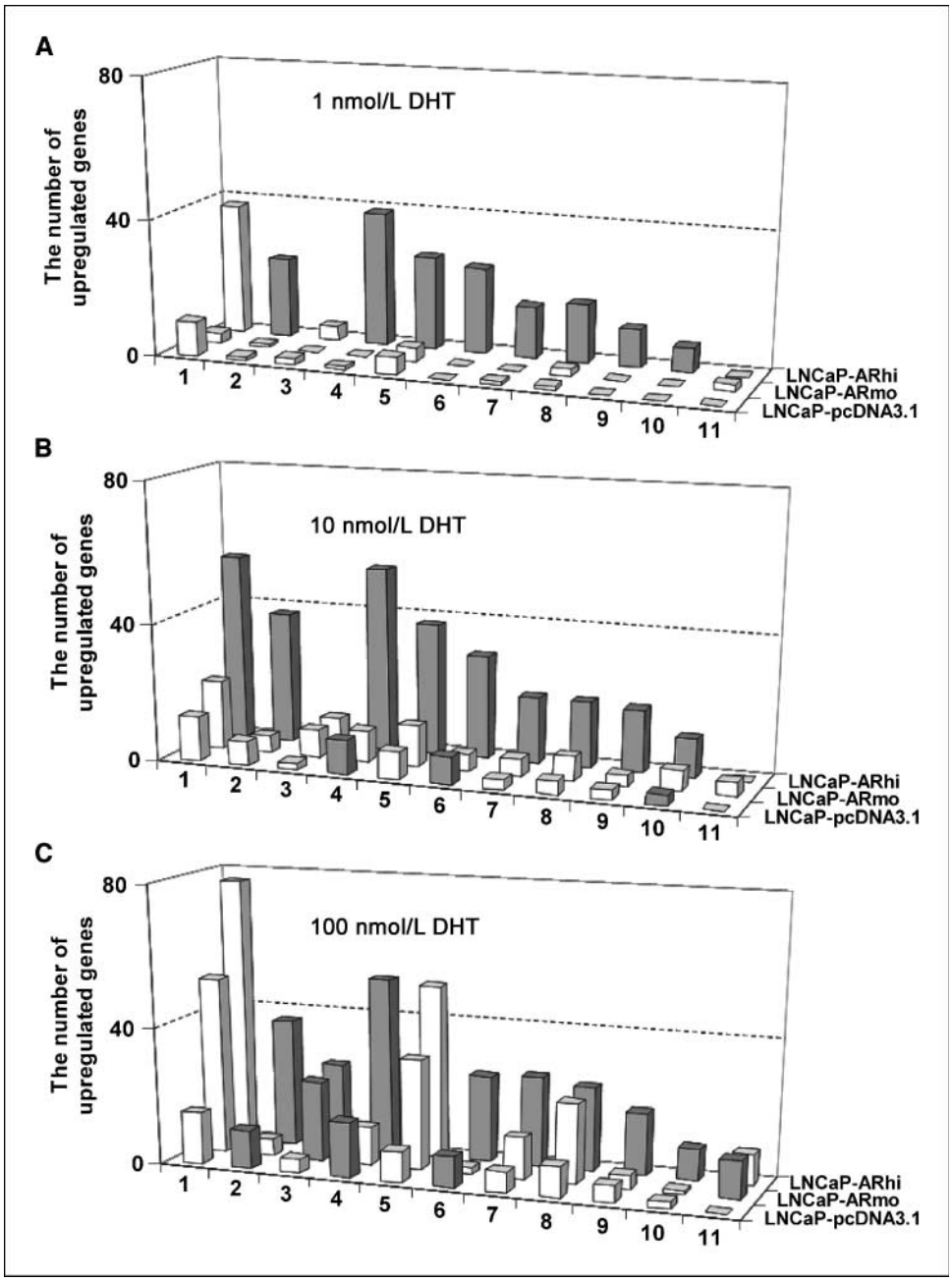

Figure 5. Androgen upregulated (>2-fold) gene ontology classes at different DHT concentrations. The histograms show the number of upregulated genes in LNCaP-pcDNA3.1, LNCaP-ARmo, and LNCaP-ARhi at 1 nmol/L (*A*), 10 nmol/L (*B*), and 100 nmol/L (*C*) DHT concentrations at the 24 h time point. *Gray columns,* highly significantly ($P < 0.001$, a hypergeometric *P* value without multiple testing correction) upregulated ontologies. Different ontology classes are numbered as follows: *1,* GO:19538 protein metabolism; *2,* GO:6259 DNA metabolism; *3,* GO:6629 lipid metabolism; *4,* GO:7049 cell cycle; *5,* GO:16043 cell organization and biogenesis; *6,* GO:51301 cell division; *7,* GO:8283 cell proliferation; *8,* GO:7242 intracellular signaling cascade; *9,* GO:9719 response to endogenous stimulus; *10,* GO:7059 chromosome segregation; *11,* GO:45045 secretory pathway.

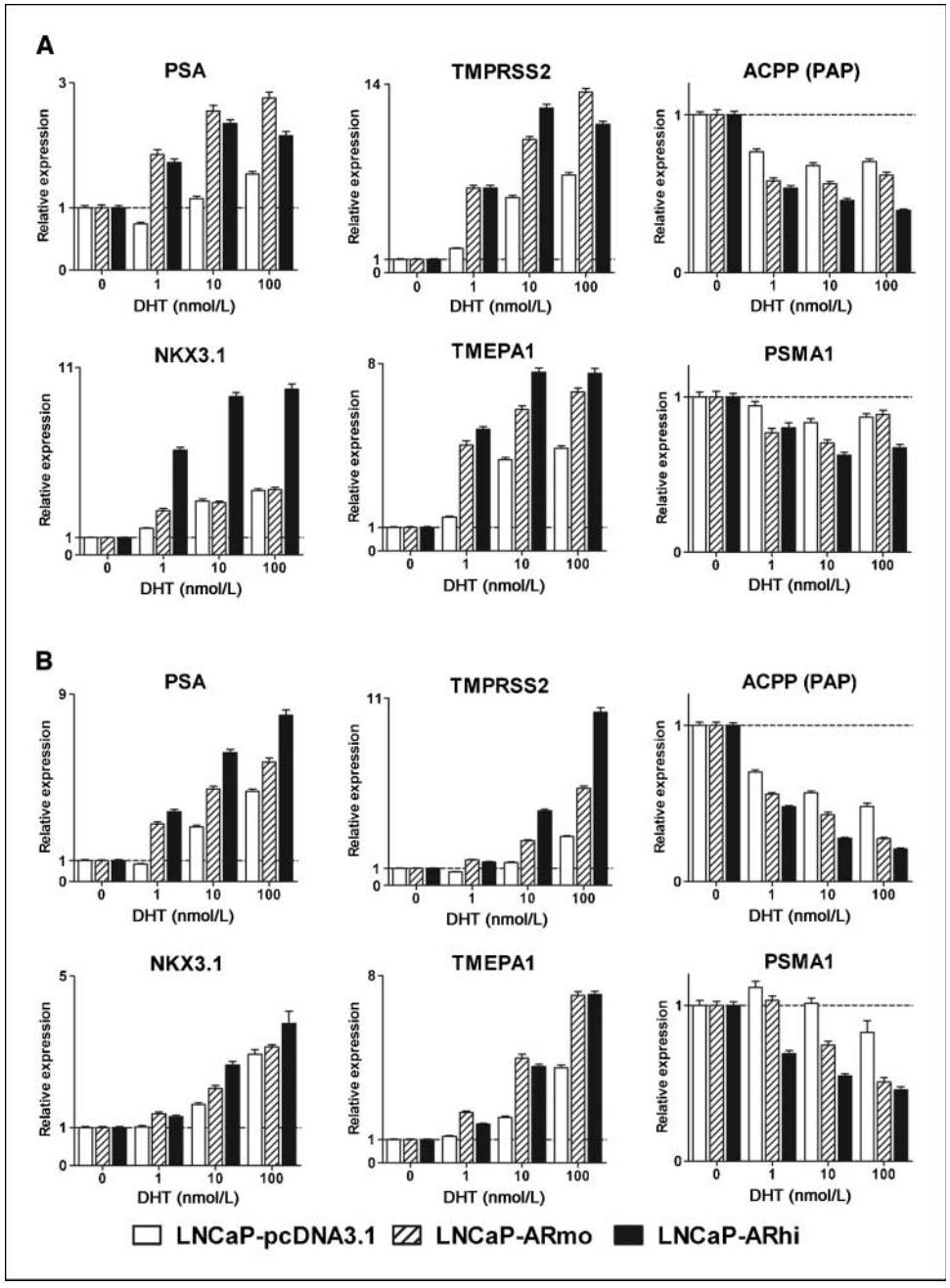

Figure 6. Expression of well-known androgen-regulated target genes (*PSA, TMPRSS2, TMEPA1, NKX3-1, ACPP*, and *PSMA*) in LNCaP-AR cell panel in different concentrations of DHT at (*A*) 4 h and (*B*) 24 h according to microarray analysis. Mean ± SE.

were found (Supplementary Table S8), and 66 of those were also >2-fold upregulated with 1.0 nmol/L DHT compared with vehicle-treated LNCaP-ARhi cells. The expression of 56 of these 66 genes were also >1.5-fold upregulated by DHT already at 4 h time point in LNCaP-ARhi. These genes were determined using Oncomine Research Edition.[5] Almost all of them (51 of 56, 91%) showed significantly upregulated expressions in prostate cancer, at least, in one of the data sets, and 49 of 56 (88%) genes were upregulated, at least, in two independent sets in metastasized prostate cancer compared with primary prostate cancers (Supplementary Table S9). ChIP-on-chip analysis of genome-wide AR binding in LNCaP-1F5 cells after a 2 h DHT exposure indicated that a majority of these 56 genes (34 of 56, 61%) possessed AR-binding site within a 200-kb window from transcription start sites of the genes (Supplementary Table S9). Two of 34 had an AR-binding site in the proximal promoter, 15 of 34 had an AR-binding site within -100 kb upstream of transcription start site, and 16 of 34 had AR-binding sites downstream of transcription start site. Eight of 34 had AR-binding site both upstream and downstream of transcription start site. The androgen response elements within the AR-binding sites were analyzed using MotifMatch (28). Using a high cutoff score of 9 and with a strict 3-bp spacing for typical class I canonical androgen response element (AGAACAnnnTGTTCT), we could find a canonical androgen response element in following genes: *NDC80, NCAPG, FANCI, C12orf48, PRIM1, IQGAP3, KIF20A, CDC2, HMGB2, SPAG5, TK1, PRC1, MCM4*, and *PCNA*.

[5] http://www.oncomine.org

Effect of roscovitine on growth of LNCaP-AR cells. Because the expression of both *CDC2* (alias CDK1) and CDK2 were increased in 1 nmol/L DHT, especially in LNCaP-ARhi, and the genes have AR-binding site according to ChIP-on-chip data, the effect of CDK1/2 inhibition was tested. The growth of LNCaP sublines was assayed in different concentrations of CDK1/2 inhibitor roscovitine and DHT. The growth of LNCaP-pcDNA3.1, LNCaP-ARmo, and LNCaP-ARhi cells was significantly ($P < 0.001$, unpaired two-tailed t test) inhibited with 15 μmol/L roscovitine in 10 nmol/L DHT (Supplementary Fig. S5). The LNCaP-ARhi cells showed the growth inhibition also by 7.5 μmol/L roscovitine ($P = 0.0002$). In addition, the growth of LNCaP-ARhi ($P = 0.0253$), but not LNCaP-pcDNA3.1 or LNCaP-ARmo, was suppressed by roscovitine in 1 nmol/L DHT.

Discussion

AR is a key protein in both development and progression of prostate cancer. It is expressed in almost all prostate carcinomas from the beginning of the disease to the castration-resistant stage (8, 13, 14). The standard treatment of advanced prostate cancer is hormone ablation. Although androgen withdrawal attenuates AR-mediated signaling and initially prevents tumor growth, several changes occur in AR action during the treatment that lead to reactivation of AR signaling and eventually to emergence of the lethal form of the disease, the CRPC (12). One of the key mechanisms in the emergence of CRPC is amplification of the *AR* gene leading to overexpression of AR protein (13, 15). This suggests that CRPC cells are not androgen-independent but may be hypersensitive to low androgen level. To study the functional consequences of AR overexpression and the role of the AR level in more detail, we generated two LNCaP cell lines that overexpressed AR to different levels.

AR overexpression seemed to increase the ability of prostate cancer cells to grow and proliferate in the absence of or at a low concentration of DHT. The level of AR affected the growth in different androgen concentrations. The LNCaP-ARhi cells with the highest level of AR expression had the fastest growth rate, whereas the growth of LNCaP-ARmo cells was between that of the LNCaP-ARhi and the control cells (LNCaP-pcDNA3.1). Both LNCaP-ARhi and LNCaP-ARmo were also able to grow longer in the medium without androgens. Instead, control cell proliferation ceased after the first week. The data indicate that increased expression of AR sensitized the growth of the cells to low hormone concentrations. Even a modest increase in AR expression level can help tumor cells to proliferate at a low androgen concentration as has also been suggested previously by Chen and colleagues (20). Kokontis and colleagues (21) have shown previously that androgens have biphasic effect on the growth of LNCaP cells. Androgens stimulate the growth, but in higher concentrations the induction of proliferation is diminished. In addition, they showed that, in the LNCaP cells overexpressing endogenous AR on adaptation to growth in low levels of androgens, the repression of proliferation takes place in lower androgen levels than in parental cell lines. Here, we found similar AR level-dependent biphasic effect of androgens (Supplementary Fig. S6). Thus, the optimal level of growth induction is dependent on the level of both ligand and the receptor.

Several microarray studies have addressed androgen regulation of gene expression in LNCaP cells (20, 29, 30). However, the data in these studies are only partially concordant. Reasons for the discrepant findings could be different time points used, different ligands, different ligand concentrations, and heterogeneity of the LNCaP cells themselves in different laboratories. For example, in our data, several genes, such as *TNFRSF10B, APRIN, TNFAIP3*, and *SGK*, which were upregulated strongly at the 4 h time point, showed very little, if any, upregulation anymore at 24 h. An important aspect is also that AR in the parental LNCaP cells is mutated allowing other steroids and even antiandrogens, such as flutamide, function as agonist (18). To alleviate these problems, we transfected wild-type AR cDNA into LNCaP cells and used the natural ligand at two different time points with four different concentrations. In our experimental model, we cannot fully separate the effect of wild-type and mutated AR. However, at least in LNCaP-ARmo and LNCaP-ARhi cells, the majority of AR is wild-type. Thus, the differences between these two sublines can be assumed to be due to the different levels of the expression of wild-type AR.

In our models, the number of androgen-responsive genes was clearly associated with AR expression level. LNCaP-ARhi cells possessed more androgen-responsive genes than LNCaP-ARmo cells that, in turn, had more those genes than LNCaP-pcDNA3.1 cells. We analyzed also VCaP cells, which contain high-level amplification of the *AR* gene leading to strong (up to 12-fold) overexpression of AR protein (ref. 24; Fig. 1). Its growth is androgen-sensitive (31). Interestingly, the number of the androgen-responsive genes in VCaP cells was even higher than in LNCaP-ARhi. In unsupervised hierarchical clustering, VCaP and LNCaP-ARhi cells clustered together despite that they are different cell lines with different genetic backgrounds. This indicates a very strong influence of AR to the genome-wide expression of prostate cancer cells. Of the well-known androgen-regulated genes, such as *PSA* and *TMPRSS2*, induction of gene expression took place at a 10-fold lower concentrations of DHT in LNCaP-ARhi and LNCaP-ARmo cells than in LNCaP-pcDNA3.1 cells. Thus, it seems that the level of AR sensitizes the cells to androgens not only in terms of growth but also by increasing number of genes responding to DHT.

Because the growth advantage in LNCaP-ARhi cells was especially prominent at 1 nmol/L DHT, we were interested in genes whose expression was altered at that concentration. Because VCaP cells are also androgen-sensitive and contain *AR* gene amplification, we postulated that androgen target genes that are important for progression of CRPC should be detected in both LNCaP-ARhi and VCaP cells. All in all, 56 genes were induced in 1 nmol/L DHT in LNCaP-ARhi (but not in LNCaP-ARmo or LNCaP-pcDNA3.1) already at 4 h and were also highly expressed in VCaP. According to Oncomine data resource, 51 of 56 (91%) of these genes have been shown to exhibit significant upregulation in primary prostate cancer and/or metastatic prostate cancer samples (Supplementary Table S9). Unfortunately, the Oncomine data from studies comparing directly hormone-naive and castration-resistant cancers were not available for all of those genes. The list of genes consists of cell cycle genes, for example, *CDK1, CDK2, cyclin B, cyclin E*, and *aurora kinase A* and *B*. All of them are known to have an effect on cell proliferation or chromosome condensation and be upregulated in prostate cancer. ChIP-on-chip assay revealed that the majority (61%) of these genes have AR-binding sites within a 200-kb window from transcription start sites. Our experiments with roscovitine, a CDK1/2 inhibitor, showed that LNCaP-ARhi cells were more sensitive to the inhibition than the LNCaP-pcDNA3.1 and LNCaP-ARmo, indicating the importance of these androgen-regulated genes for the growth benefit of the AR-overexpressing cells. Because it has been shown previously that CDK1 phosphorylates and stabilizes AR (32), there may be a positive feedback mechanism between expression of AR and CDK1.

Previous studies have shown that increased AR expression is associated with the growth of castration-resistant cancers and with

transformation of androgen-dependent prostate cancer cells to androgen-independent ones (10, 20, 21). In addition, association of AR expression level with cell invasion has also been suggested (11). These studies have been performed with different cell lines or xenograft models. In our LNCaP-AR model cells, high AR level was significantly associated with increased proliferation at a low androgen concentration and with an increased number of genes being associated to cell cycle and DNA replication. The finding that only LNCaP-ARhi cells showed the growth advance, and high number of cell proliferation associated genes that were androgen-responsive, is in good agreement with observations that clinical CRPC often have >10-fold overexpression of *AR* (13, 14).

The results also indicated that AR can have an effect on different cellular processes depending on the receptor level. In LNCaP-ARmo cells, the number of responding genes associated with cell cycle was not increased; instead, genes in other ontology categories, such as lipid, sterol, and cholesterol biosynthesis, were highly significantly enriched. For example, the androgen-regulated lipid metabolism genes reviewed by Chen and colleagues (33) were all androgen-regulated, at least, in one of the cell line used in our data. Of those lipid metabolism genes, especially *DHCR24, FASN, HMGCS1, LDLR, PPAP2A*, and *SCAP* were strongly androgen-upregulated in LNCaP-ARmo. Also, the ontology category of secretory pathway, including endoplasmic reticulum to Golgi transport genes, showed higher number of responsive genes in LNCaP-ARmo compared with LNCaP-ARhi. These observations suggest that the amount of AR may also have other effects rather than simple sensitization of cells to lower levels of androgens.

In conclusion, increased expression of AR seems to sensitize prostate cancer cells in multiple ways and give them several biological benefits during hormone ablation. High AR protein level helped the cells to sustain and increase their proliferation in environment with no androgen or a low androgen concentration. Microarray analyses of AR-regulated genes gave further evidence for the biological benefits of AR overexpression. They showed enhanced expression of several cell cycle–associated genes at 1 nmol/L DHT, especially in LNCaP-ARhi cells. In addition, expression of genes associated with biosynthesis of lipids and other cellular structures was elevated in LNCaP-ARhi cells. AR expression level seemed also to predict the activity of AR; the more AR expression, the more androgen responsive genes. Further studies are warranted to investigate whether the genes upregulated at low androgen concentrations could functions as drug targets for CRPC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Curado MP, Edwards B, Shin HR, et al. Cancer incidence in five continents, vol. IX. IARC Scientific Publications No. 160. Lyon: IARC; 2007.

2. Coleman MP, Quaresma M, Berrino F, et al.; CONCORD Working Group. Cancer survival in five continents: a worldwide population-based study (CONCORD). Lancet Oncol 2008;9:730–56.

3. Isaacs JT, Isaacs WB. Androgen receptor outwits prostate cancer drugs. Nat Med 2004;10:26–7.

4. Attard G, Reid AH, Yap TA, et al. Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J Clin Oncol 2008;26:4563–71.

5. Holzbeierlein J, Lal P, LaTulippe E, et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 2004; 164:217–27.

6. Montgomery RB, Mostaghel EA, Vessella R, et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 2008;68:4447–54.

7. Locke JA, Guns ES, Lubik AA, et al. Androgen levels increase by intratumoral *de novo* steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68:6407–15.

8. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995; 55:3068–72.

9. Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. Cancer Res 1998;58:5718–24.

10. Hara T, Nakamura K, Araki H, Kusaka M, Yamaoka M. Enhanced androgen receptor signaling correlates with the androgen-refractory growth in a newly established MDA PCa 2b-hr human prostate cancer cell subline. Cancer Res 2003;63:5622–8.

11. Hara T, Miyazaki H, Lee A, Tran CP, Reiter RE. Androgen receptor and invasion in prostate cancer. Cancer Res 2008;68:1128–35.

12. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25:276–308.

13. Linja MJ, Savinainen KJ, Saramäki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61:3550–5.

14. Latil A, Bièche I, Vidaud D, et al. Evaluation of androgen, estrogen (ERα and ERβ), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res 2001;61:1919–26.

15. Visakorpi T, Hyytinen E, Koivisto P, et al. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9:401–6.

16. Marcelli M, Ittmann M, Mariani S, et al. Androgen receptor mutations in prostate cancer. Cancer Res 2000; 60:944–9.

17. Taplin ME, Rajeshkumar B, Halabi S, et al. Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 2003;21:2673–8.

18. Veldscholte J, Ris-Stalpers C, Kuiper GG, et al. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173:534–40.

19. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23:175–200.

20. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.

21. Kokontis J, Takakura K, Hay N, Liao S. Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. Cancer Res 1994;54:1566–73.

22. Dignam DJ, Lebovitz RM, Roeder RG. Nucleic Acid Res 1983;11:1475–89.

23. Thompson J, Lepikhova T, Teixido-Travesa N, Whitehead MA, Palvimo JJ, Jänne OA. Small carboxyl-terminal domain phosphatase 2 attenuates androgen-dependent transcription. EMBO J 2006;25:2757–67.

24. Johnson WE, Li W, Meyer CA, et al. Model-based analysis of tiling-arrays for ChIP-chip. Proc Natl Acad Sci U S A 2006;103:12457–62.

25. Saramäki OR, Harjula AE, Martikainen PM, Vessella RL, Tammela TL, Visakorpi T. TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. Clin Cancer Res 2008;14:3395–400.

26. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nat Rev Cancer 2008;8:497–511.

27. Makkonen H, Jääskeläinen T, Pitkänen-Arsiola T, et al. Identification of ETS-like transcription factor 4 as a novel androgen receptor target in prostate cancer cells. Oncogene 2008;27:4865–76.

28. Wang Q, Li W, Liu XS, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 2007;27: 380–92.

29. Nelson PS, Clegg N, Arnold H, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99:11890–5.

30. Hendriksen PJ, Dits NF, Kokame K, et al. Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 2006;66:5012–20.

31. Korenchuk S, Lehr JE, MClean L, et al. VCaP, a cell-based model system of human prostate cancer. In Vivo 2000;15:163–8.

32. Chen S, Xu Y, Yuan X, Bubley GJ, Balk SP. Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. Proc Natl Acad Sci U S A 2006;103:15969–74.

33. Swinnen JV, Heemers H, van de Sande T, et al. Androgens, lipogenesis and prostate cancer. J Steroid Biochem Mol Biol 2004;92:273–9.