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Alterations in Androgen Receptor, Estrogen Receptors and their Coregulatory Genes in Prostate Cancer

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere, on September 25th, 2003, at 12 o'clock.

Acta Universitatis Tamperensis 953
University of Tampere
Tampere 2003

ACADEMIC DISSERTATION
University of Tampere, Institute of Medical Technology
Finland

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Finland

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taju@uta.fi
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 953
ISBN 951-44-5747-1
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 277
ISBN 951-44-5748-X
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2003

TABLE OF CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, referred to in the text by their Roman numerals:

I. Wallen MJ, Linja M, Kaartinen K, Schleutker J and Visakorpi T (1999): Androgen receptor gene mutations in hormone-refractory prostate cancer. Journal of Pathology 189:559-563

II. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL and Visakorpi T (2001): Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Research 61: 3550-3555

III. Linja M, Porkka K, Kang Z, Savinainen K, Jänne OA, Tammela T, Vessella R, Palvimo J and Visakorpi T. Expression of androgen receptor coregulators in prostate cancer. Submitted

IV. Linja MJ, Savinainen KJ, Tammela TLJ, Isola JJ and Visakorpi T (2003): Expression of ERα and ERβ in prostate cancer. Prostate 55: 180-186

ABSTRACT

Androgens are known to be essential for the development of prostate cancer. In addition, recent studies have suggested that an androgen receptor (AR) signaling pathway becomes reactivated during the failure of hormonal therapy. The aim of this study was to investigate the alterations in AR and other genes related to steroid hormone action in prostate cancer. Prostate cancer cell lines, xenografts and clinical tumor samples were analyzed for genetic and expression alterations. Single strand conformational polymorphism (SSCP) analysis was used to screen mutations in the AR gene. Fluorescence in situ hybridization (FISH) was performed to detect AR and steroid receptor coactivator-1 (SRC1) gene copy number alterations. The expression of AR, prostate specific antigen (PSA), estrogen receptors (ERα and β), and 16 different AR coregulatory genes was measured by a quantitative real-time RT-PCR.

The mutation analysis of 32 hormone-refractory prostate carcinomas, obtained from patients treated with castration alone, revealed one previously detected missense mutation and a somatic contraction of CAG repeat in the AR gene. Both mutations were found in tumors containing an AR gene amplification. Still, in most of the tumors containing the AR gene amplification, the gene was wild- type. Next, the expression of the AR gene was measured in benign prostate hyperplasias (BPH), untreated and hormone-refractory locally recurrent prostate carcinomas as well as in 10 prostate cancer xenografts. Hormone-refractory tumors showed, on average, 6-fold higher expression of the AR than androgen-dependent tumors or the BPH ($p<0.001$). 4 of the 13 (31%) hormone-refractory tumors contained the AR gene amplification. Tumors with the gene amplification tended to express more AR than the hormone-refractory tumors without the amplification. Two xenografts showed amplification and high-level expression of the AR gene. These xenografts are the first prostate cancer model systems containing the gene amplification.

Next, the expression of 16 AR coactivators and co-repressors (SRC1, β-catenin, TIF2, PIAS1, PIASx, ARIP4, BRCA1, AIB1, AIB3, CBP, STAT1, NCoR1, AES, cyclin D1, p300, and ARA24) was measured. Both the AR positive and negative cell lines and xenografts expressed the coregulators. Most of the coregulators studied were expressed at

equal levels in BPH, untreated and hormone-refractory carcinomas. However, the expression of the PIAS1 and SRC1 was significantly ($p=0.048$, and 0.017, respectively) lower in the hormone-refractory than untreated prostate tumors. Paradoxically, the SRC1 gene was found to be amplified and highly expressed in one of the prostate cancer xenografts.

The expression of both ERs was detected in all specimens studied. Only low-level expression of the ERα was found in tumors and cell lines. The level of expression was similar to that observed in breast carcinomas found to be negative for an ERα protein by immunohistochemistry. All cell lines showed a low-level expression of the ERβ. In the hormone-refractory carcinomas, the mean expression of the ERβ was about half of that in the BPH or androgen-dependent carcinomas. However, the difference was not statistically significant.

In conclusion, our findings emphasize the central role of the AR in the progression of prostate cancer. The AR gene is commonly overexpressed in the hormone-refractory tumors and is also targeted by genetic alterations, mainly amplification. No common overexpression of the AR coregulators, ERα or ERβ was found. However, the amplification of the SRC1, although being rare, indicates that the coregulator genes could be affected by the genetic alterations in prostate cancer.

ABBREVIATIONS

AES	Amino-terminal enhancer of split
AF	Activation function
AIB1	Amplified in breast cancer 1
AIB3	Amplified in breast cancer 3
AR	Androgen receptor
ARA	Androgen receptor associated protein
ARE	Androgen response element
ARIP4	Androgen receptor interacting protein 4
BAC	Bacterial artificial chromosome
bp	Base pair
BPH	Benign prostatic hyperplasia
BRCA1	Breast cancer susceptibility gene 1
CD1	Cyclin D1
CBP	CREB-binding protein
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CREB	cAMP responsive element binding protein
CV	Coefficient of variation
CYP17	17-hydroxylase cytochrome P450
DAPI	4,6-diamino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DBD	DNA binding domain
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen response element
ERBB2	Avian erythroblastic leukemia viral oncogene homolog 2
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate

GR	Glucocorticoid receptor
GSTP1	Glutathione S-transferase 1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPC	Hereditary prostate cancer
HR	Hormone-refractory
HSD3B2	3α-hydroxysteroid dehydrogenase
IGF	Insulin-like growth factor
kb	Kilobase
LBD	Ligand binding domain
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LOH	Loss of heterozygosity
MAB	Maximal androgen blockade
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MSR1	Macrophage scavenger receptor 1
NCoR1	Nuclear receptor co-repressor
OR	Odds ratio
P300	E1A binding protein p300
P53	Tumor protein p53
PAC	P1-derived artificial chromosome
PAP	Prostate acid phosphatase
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SBMA	Spinal and bulbar muscular atrophy
SHBG	Sex hormone binding globulin

SSCP	Single strand conformational polymorphism
SRC1	Steroid receptor coactivator 1
SRD5A	Steroid 5α-reductase type II
STAT1	Signal transducer and activator of transcription 1
SUMO-1	Small ubiquitin-like modifier
TBP	TATA box binding protein
TIF2	Transcriptional intermediary factor 2
TNM	Tumor-node-metastasis
TR	Thyroid hormone receptor
TRAMP	Transgenic adenocarcinoma of the prostate mouse model
TRAP	Thyroid hormone receptor associated protein
TURP	Transurethral resection of prostate

INTRODUCTION

Prostatic adenocarcinoma is the most common malignancy among men in many Western industrialized countries. In 1999, 3,114 new cases were diagnosed in Finland, and it has been estimated that more than 3,400 cases will be detected during the year 2003 (Finnish Cancer Registry 2002). Both environmental and hereditary factors have been implicated in the etiology of prostate cancer. Known risk factors include age, ethnicity and country of origin (Pienta 1993). In addition, positive family history is one of the strongest risk factors (Steinberg et al. 1990). In a recent Scandinavian twin study, it was estimated that even up to 40% of the risk could be due to hereditary factors (Lichtenstein et al. 2000). Several prostate cancer susceptibility loci have already been found, and three putative predisposing genes, HPC2/ELAC2, HPC1/RNASEL and MSR1 have been identified (Tavtigian et al. 2001, Carpten et al. 2002, Xu et al. 2002). However, it seems that the contribution of these genes to the overall genetic predisposition of prostate cancer is rather limited. The environmental risk factors have also been extensively studied but are poorly known (Grönberg 2003).

The malignant potential of prostate cancer varies from the incidentally found latent form of the disease to the highly lethal, poorly differentiated advanced carcinoma (Gittes 1991). Thus, the clinical behavior of prostate cancer is very unpredictable. The 5-year cancer-specific survival rate is about 75% (Finnish Cancer Registry 2002) indicating that majority of the patients do not die of the disease. Still, due to the high incidence of the malignancy, prostate cancer is the second most common cancer death. In 1999, 773 men died of prostate cancer in Finland (Finnish Cancer Registry 2002).

The growth of prostate cancer is highly dependent on the androgens. Indeed, Huggins and Hodges showed already in the early 1940's that castration is an effective treatment for prostate cancer (Huggins and Hodges 1941). Subsequently, hormonal therapy has become the standard therapy for advanced prostate cancer. More than 90% of the patients show a biochemical response to the therapy (Palmberg et al. 2000), and clinical response rates of 80% have been reported (Gittes 1991). However, during the therapy, the hormone-refractory tumor cells eventually emerge leading to clinical progression. Since there are no effective treatments for the hormone-refractory prostate carcinoma, the prognosis after progression is

poor. The average survival time of the patients with hormone-refractory prostate cancer is only about six months (Gittes 1991).

The mechanisms underlying the progression of prostate cancer during the endocrine treatment are incompletely understood. Earlier, it was believed that other than androgen-related signaling pathways become the primary growth stimulatory factors in recurrent prostate cancer (Visakorpi 2000). Therefore, recurrent hormone-refractory tumors are often called androgen-independent prostate carcinomas. However, during the last decade, evidence indicating that the androgen receptor (AR) mediated signaling pathways become reactivated during the progression of the disease has mounted up. Both the gain-of-function mutations and amplification of the AR gene have been reported in the hormone-refractory prostate carcinomas (Feldman and Feldman 2001). It has also been proposed that other growth factor signaling pathways could activate the AR, especially in the presence of only low levels of androgens. In addition, it has been shown that many of the androgen regulated genes become up-regulated during the progression of the disease (Gregory et al. 2001, Kim et al. 2002).

Understanding the underlying molecular mechanisms of the transition from the androgen-dependent tumor growth to the androgen-independent one is crucial for the development of new and better treatment modalities for the highly lethal hormone-refractory prostate cancer. For example, genetic aberrations associated with this transition may well indicate novel treatment targets. Thus, this thesis aimed to elucidate the role of the AR and other genes related to steroid hormone actions in the development and progression of prostate cancer.

REVIEW OF THE LITERATURE

1. ANDROGENS AND PROSTATE GLAND

Androgens are steroid hormones that induce the differentiation and maturation of male reproductive organs and retain the male phenotype. They are major regulators of cell proliferation and cell death in the prostate gland. Formation of the androgens occurs by δ4- and δ5-biosynthetic pathways in the endocrine glands, primarily in the testes and adrenal gland (Coffey and Isaacs 1981). In healthy men, adrenal androgens, however, contribute only little to prostatic function (Geller et al.1984). Testosterone is the major circulating androgen, the production of which by Leydig cells in the testes is stimulated by luteinizing hormone (LH) produced by the pituitary gland. In some of the end organs, such as the prostate, testosterone is converted to 5α-dihydrotestosterone (DHT) by 5α-reductase activity. The DHT is known to be a more potent ligand for the AR. Furthermore, the concentration of the DHT is higher than testosterone in prostatic tissue, suggesting that the DHT is more important in prostate development and possible also in tumorigenesis (Deslypere et al.1992).

The androgens have been shown to regulate the expression of hundreds of target genes (Nelson 2002). For example, the expression of prostate specific antigen (PSA) and prostate acid phosphatase (PAP) are androgen regulated (Young et al.1992, Perry et al. 1996). The connection between androgen action and cell growth control may be explained by the fact that the androgens also regulate several genes involved in cell cycle control, such as cyclin dependent kinase inhibitors p16 and p21 and cyclin dependent kinases 2 and 4 (Lu et al. 1997, 1999). It is still not known, which of these androgen regulated genes are truly crucial for the development of the prostate gland and cancer.

1.1 Hormone metabolism and prostate cancer

Because of the hormonal dependence of prostate cancer, the levels of the circulating androgens as well as polymorphisms in the genes involved in androgen metabolism have been evaluated as possible risk factors of prostate cancer. Although one study has reported a statistically significant association between serum levels of testosterone and prostate cancer (Gann et al.1996), the majority of the researches have failed to show such an association (Hsing et al. 2002). It has been suggested that in addition to the hormone levels other related factors should be taken into account as well. For example, the serum concentrations of sex hormone binding globulin (SHBG), which transports androgens and estrogens in the circulation, have been evaluated as a prostate cancer risk factor (Hsing et al. 2002). Significant association between low levels of the SHBG and prostate cancer has been reported in one study (Gann et al.1996). However, the finding has not been confirmed by other studies (Dorgan et al. 1998, Mohr et al 2001).

In addition to determining the levels of different circulating molecules, the role of the polymorphisms as risk factors has been studied. For example, several polymorphisms in genes such as SRD5A2, CYP17 and HSD3B2, have been implicated in the etiology of prostate cancer. The SRD5A2 gene encodes type II 5α-reductase which converts testosterone to dihydrotestosterone (DHT) in the prostate gland. The CYP17 gene encodes cytocrome P450 17α-hydroxylase which acts in androgen biosynthesis, and 3α-hydroxysteroid dehydrogenase encoded by the HSD3B2 participates in inactivation of the DHT to a less potent androgen. It has been shown that prevalence of high/low activity alleles in specific ethnic or racial groups may in part explain some of the observed differences in the prostate cancer susceptibilities between the populations (Bosland 2000). More than 22 polymorphisms have been reported for the SRD5A2, and four of these have been examined in 12 studies for their association with prostate cancer, with mixed results (Hsing et al. 2002). Two studies reported a statistically significant association between the A49T mutation and prostate cancer (Makridakis et al. 1999, Margiotti et al. 2000) and one reported that the A49T genotype associates with more aggressive prostate cancer (Jaffe et al. 2000). More recent studies have failed to find any association (Mononen et al. 2001, Lamharzi et al. 2003). Similarly, the relationship between the CYP17 and prostate cancer is inconclusive. Of the nine studies that have investigated the role of the CYP17, four found a positive association with the A2 allele, which has a single base pair change (T to C) in the 5'

untranslated region of the CYP17 gene (Lunn et al. 1998, Gsur et al. 2000, Yamada et al. 2001, Kittles et al. 2001). Recently, it was suggested that a variant genotype in the two genes of the HSD3Bs would have a joint effect associating with prostate cancer (Chang et al. 2002). Additional studies are warranted to confirm this finding.

2. NUCLEAR RECEPTORS

Nuclear receptor superfamily is the largest known family of eukaryotic transcription factors. The nuclear receptors mediate the actions of lipid-soluble steroid hormones and non-steroidal lipophilic hormones. There are also several nuclear "orphan receptors" whose regulatory ligands have not yet been identified. The ligands of different nuclear receptors are diverse, but the receptors are structurally quite similar to each other (Mangelsdorf et al. 1995). The nuclear receptors are cytosolic or nuclear proteins, in contrast to many peptide hormone receptors which are associated in the cell membrane and alarm a second messenger to mediate a regulatory signal. The activation of a steroid receptor from an inactive, chaperone-protein bound state requires the binding of a ligand, which induces a conformational change in the receptor structure. This leads to the dissociation of chaperone proteins, receptor dimerization and localization into the nucleus (Moras and Gronemeyer 1998). In the nucleus, a dimerized receptor complex regulates the transcription of the target genes by binding to its response element in DNA. In addition, it has been suggested that the nuclear receptors can mediate the so-called non-genomic, DNA binding independent effects of steroid compounds in the cells.

The nuclear receptor family can be divided into four subgroups according to the pattern by which they bind to the ligand, to DNA and to each other. Class I, the steroid receptor subfamily, consists of androgen (AR), estrogen (ER), progesterone (PR), glucocorticoid (GR) and mineralocorticoid (MR) receptors. They differ from class II nuclear receptors in two features. First, they can only bind to DNA when liganded, and second, they recognize a palindromic response element sequence. The primary structure of the steroid receptors is composed of three different functional domains: the amino-terminal most variable domain, which mediates the transactivation function of a receptor, the central well-conserved DNA-binding domain, and the moderately conserved carboxy-terminal ligand binding domain, separated from each other by hinge regions (MacLean et al.1997).

Regardless of whether the transcriptional activity is controlled by the binding of a ligand, each of the different steroid receptors is capable of binding to specific DNA sequences that identify particular genes as targets for regulation (Glass 1994). Class I receptors achieve specificity in the target gene activation by several means, such as target sequence

recognition, nucleotide divergence in the DNA-binding half sites, tissue specific expression of receptors and cofactors, and local metabolism of ligands. For example, although the androgen, progesterone and glucocorticoid receptors recognize the same DNA response element there is hormone specific action in the target tissues. For instance, the probasin gene is induced by the androgens and not by the glucocorticoids. The high affinity for the AR to the response element of the probasin is determined by the three amino acid residues in the DNA binding domain of the AR molecule (Funder et al. 1988, Strahle et al. 1989, Schoenmakers et al. 2000).

2.1 Androgen receptor

The human androgen receptor (AR) gene, located in the chromosome Xq11-12, contains 8 exons (Lubahn et al. 1988). Transcription of the gene can occur from two different initiation sites, producing two AR transcripts (10 and 7 kb) (Tilley et al. 1990, Faber et al. 1993). The AR gene is almost universally expressed in different tissues (Faber et al. 1991). The transactivation domain of the AR is encoded by the large first exon, which is about 1,590 base pairs in size, depending on the lengths of polymorphic regions (Faber et al. 1989). This domain harbors the ligand independent activation domain AF-1, and deletions in this region diminish the transactivation power of the receptor (Jenster et al. 1995). The transactivation domain also contains a few homopolymeric amino acid repeats typical for many transcription factors. The most amino-terminal repeat is the polyglutamine (Q) repeat, coded by CAG triplets. Like other genes with the CAG repeats, the repeat length is very polymorphic, ranging from 14 to 35 (Sartor et al. 1999). Lengthening the repeat to 40–62 results in an inherited neuromuscular degenerative disease, Kennedy's disease or spinal and bulbar muscular atrophy, SBMA (LaSpada et al. 1991). The other amino acid repeat encoded by the exon 1 is the polyglycine (GGN) repeat, the function of which has remained unclear. The most common glycine repeat allele is 16 repeats (Irvine et al. 1995).

The exons 2 and 3 in the AR gene encode for the DNA binding domain. The amino acid sequence of this domain is a most highly conserved region among members of the nuclear receptor superfamily. It includes two structures, referred as zinc fingers, in which four cysteine residues bind one zinc ion in each of the motifs. The zinc fingers have been shown to be fundamental to the binding of the response element in DNA (O'Malley 1990). The first

zinc finger harbors the information for the specific recognition of DNA, and the second finger stabilizes the DNA-receptor interaction in contact with the DNA backbone (Glass 1994).

The third domain structure in the AR is the carboxy-terminal ligand binding domain, which is encoded by the exons 4-8. It contains the ligand-dependent transactivation function AF-2. Both the N-terminal transactivation domain and the LBD are responsible for the ligand-dependent transactivation function of the NRs (Tora et al. 1989). The N-terminal activation function (AF-1) is constitutively active on its own, while the AF-2 function in the LBD is induced upon ligand binding. Because the transactivation function is normally androgen dependent, the LBD domain prevents the action of the receptor without the ligand (Kuil and Brinkmann 1996). Deletions in this domain abolish the binding of an androgen, which results as constitutive activity of the AR (Jenster et al. 1991). The activities of both activation functions are dependent on the cell type and promoter content (Beato et al. 1995). The modular structure of the AR is illustrated in figure 1.

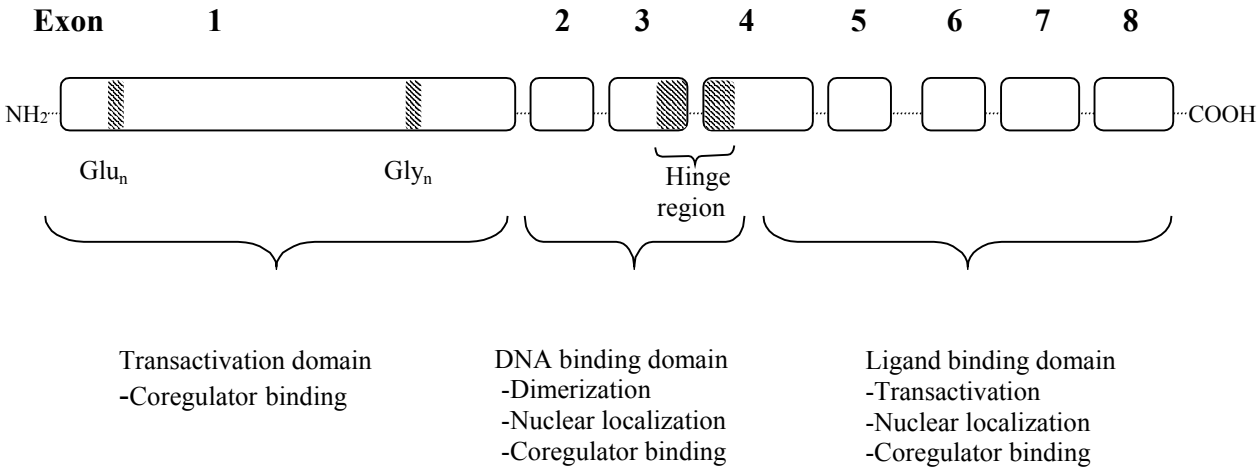

Figure 1. General domain structures and their main functions in the androgen receptor. The NR superfamily members are structurally highly related. The zinc finger type DNA binding domain is highly conserved among the different NRs. The most variable region is the N-terminal transactivation domain which spans the first exon and contains the polymorphic glutamine and glycine repeats.

2.2 Non-genomic actions of androgens and AR

The androgens (like progesterone and estrogen) can exert effects, which are considered to be non-genomic because they can occur in the presence of transcription inhibitors or they are occurring too fast to involve changes in gene transcription. To date, the reported non-genomic effects of the steroids at physiological concentrations appear to be receptor mediated (Heinlein and Chang 2002). The non-genomic actions include stimulation of MAPK (mitogen activated protein kinase) pathway via c-Src tyrosine kinase and induction of cAMP second messenger and PKA (protein kinase A) occurs because the SHBG binds to its receptor and testosterone. Both of these mechanisms also potentially influence the transcriptional activation of the nuclear AR. In addition, the existence of a novel, cell membrane bound androgen receptor has been suggested (Benten et al. 1999) but remains to be identified. One of the effects mediated by this putative receptor is the increase of intracellular calcium levels, which in turn could be able to activate signal transduction cascades such as the PKA, PKC (protein kinase C) and MAPK or to modulate the activity of the transcription factors. Androgen-mediated modulation of the ion channel activity and intracellular calcium levels has been observed in several cell types (Heinlein and Chang 2002) including LNCaP cells (Steinshapir et al.1991). However, it has not yet been determined whether these non-genomic effects are mediated through a membrane androgen receptor or by SHBG or c-Src kinase–AR complex.

2.3 Coregulatory proteins of AR

The AR activates the expression of the target genes and gene networks by facilitating transcriptional initiation. In general, the androgen receptor mediated transcription requires several auxiliary protein complexes (Hermanson et al. 2002) that can interact sequentially, in combination or in parallel. Transcriptional regulation requires the participation of at least three classes of proteins: 1) proteins that recognize specific DNA sequences, 2) proteins that are recruited as promoters by protein-protein interactions and act as transcriptional coactivators or co-repressors, and 3) proteins that alter the architecture of chromatin ATP-dependently or with their histone acetyl transferase (HAT) activity. Histone acetylation

neutralizes the positive charge of the histone N-terminal tails and weakens the interaction between histones and negatively charged DNA. Thus, the recruitment of chromatin remodeling proteins and acetyltransferases is essential to make the target sequences accessible for the liganded receptor. Conversely, deacetylation of the histone tails is achieved by histone deacetylases, HDACs, resulting in transcriptional repression (Hu and Lazar 2000).

Other proteins are needed to bridge the receptor complex to basal transcription machinery. In addition, the formation of a multiprotein complex is also influenced by the enhancers and promoter of the gene in question (McKenna et al.1998). Thus, in addition to the coregulatory proteins that function as acetyltransferases, ATPases and mediators, many other coactivators are likely to be described in the future. The coregulatory proteins are generally categorized into two groups: coactivators and co-repressors. Figure 2 depicts the mode of the androgen receptor action in the cell.

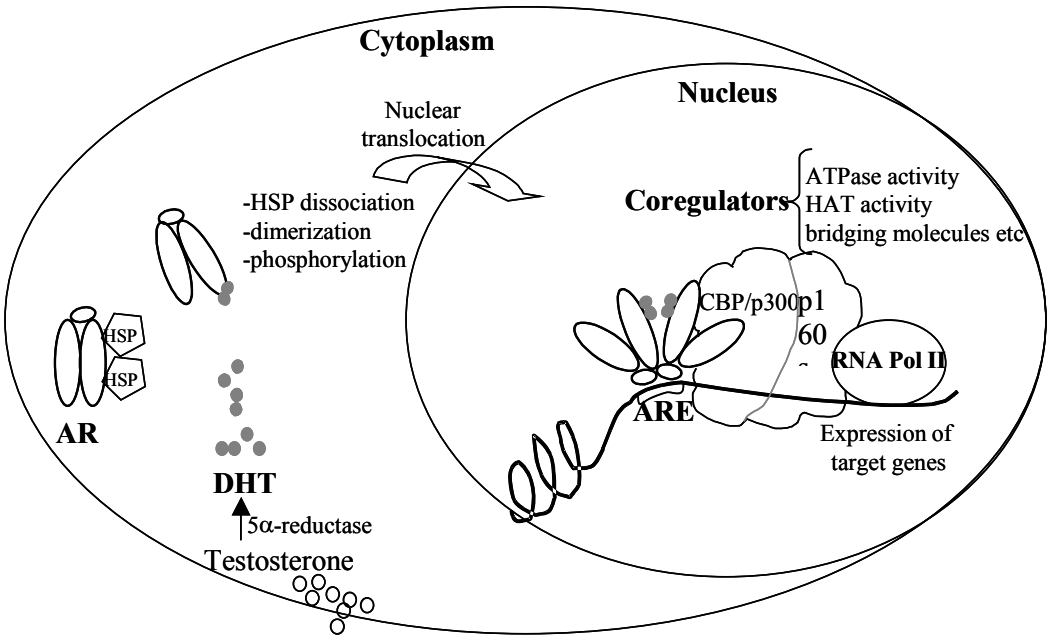

Figure 2.
Mode of the androgen receptor action in the cell: binding of a ligand induces a conformational change in the receptor structure, which leads to dissociation of heat shock chaperone proteins (HSP) and allows the receptor to dimerize. In the nucleus, the receptor dimer binds to its response element (ARE) and thereby regulates the androgen receptor dependent transcription. Coregulators form distinct complexes with different functional properties: ATP-dependent chromatin remodeling modifies chromatin domains. Binding of, e.g. CBP/p300 and p160 family members results in acetyltransferase activity, disrupting the local nucleosomal structure. Mediator molecules contact the components of the basal transcription machinery to effect the transcriptional initiation.

2.3.1 Coactivators

The coactivator proteins act in enhancement of the AR transactivation in a ligand-dependent manner. A specific signature sequence present in many auxiliary proteins has been shown to mediate the binding of these proteins to the AR and to other nuclear receptors (Heery et al. 1997). Part of this activation might also involve the stabilization of the interaction between the N-terminal and C-terminal regions of the AR (Aarnisalo et al. 1998). Some of the AR coactivators identified to date are discussed briefly here.

p160 family and DRIP/TRAP/SMCC complex

The most intensively investigated coactivator group is the p160/SRC protein family. It is composed of three distinct, but structurally and functionally related members, SRC1 (NcoA1), TIF2 (SRC2/GRIP1/NcoA2) and AIB1 (SRC3/RAC3/ACTR) (McKenna et al. 1999). These proteins directly associate with the steroid receptors AR, PR and GR, and enhance their transcriptional activation in a ligand-dependent manner. The p160 proteins have shown to possess the histone acetyltransferase (HAT) activity and they are able to recruit the transcription factor CBP/p300. The resulting p160/CBP complex is thus supposed to modulate a chromatin structure in terms of their intrinsic HAT activities and by the action of the associated chromatin-remodeling proteins (Hermanson et al 2002). An assembly model of the p160 coactivators with the CBP/p300 in a synergistic protein folding has been recently reported (Demarest et al. 2002).

Studies with SRC1 knock-out mice showed that the lack of functional SRC1 results in partial hormone resistance in the target tissues (Xu et al. 1998). The mice were otherwise normal and fertile. However, the expression of the TIF2 was reported to have increased in the SRC1 null mice. This suggests that the p160 coactivator proteins can substitute the functions of each other, at least to some extent.

The other class of the coactivator complexes that is directly associated with the receptor is a non-HAT coactivator complex, DRIP/TRAP/SMCC. The thyroid hormone receptor (TR) associated protein (TRAP) complex, was isolated on the basis of an intracellular ligand-

dependent association with the TR (Fondell 1996). An apparently identical complex (DRIP) was subsequently isolated through interaction with the vitamin D receptor (Rachez et al. 1999). Overexpression of several TRAP subunits (TRAP220, TRAP170 and TRAP100) in the transient transfections enhanced the AR mediated transcription (Wang et al. 2002). The DRIP/TRAP/SMCC complex interacts with the general transcription machinery, bridging the nuclear receptors to it (Fondell et al. 1999).

CBP/p300

The CREB-binding protein (CBP) was initially identified as a coactivator affecting the proper transactivation of the cAMP response element binding protein. The p300 was characterized on the basis of its interaction with the adenoviral-transforming protein E1A. These two proteins have highly conserved sequences (Goodman and Smolik 2000). They share many functional properties and possess the HAT activity, which allow them to acetylate all of the histones in the nucleosomes (Heinlein and Chang 2002). The CBP/p300 may also acetylate non-histone proteins. For example, the acetylation of the AIB1 by the CBP disrupts the AIB1-ER interaction, resulting in a reduced hormone-mediated ER-dependent transcription (Chen et al. 1999).

The CBP/p300 is a common coactivator of the transcription, and knockout models have demonstrated that the proper action of the CBP/p300 complex is critical: the CBP and p300 null mutants are lethal in the uterus. Moreover, p300 heterozygotes also manifest a significant embryonic lethality: compound mutants heterozygous for the p300 and CBP die in the uterus (Tanaka et al 1997, Yao et al. 1998). Mutation of the CBP causes the human disorder Rubinstein-Taybi syndrome (RTS) (Miller and Rubinstein 1995). Both the CBP and p300 are also targeted by viral oncoproteins, mutations and translocations in certain forms of cancer, such as leukemias (Goodman and Smolik 2000).

ARA coactivator family

The ARAs, androgen receptor-associated proteins, is a group of factors that can bind to the AR and modulate its transcriptional activity. Based on their molecular weights, these factors have been named ARA70 (RFG/ELE1), ARA160, ARA54, ARA55, ARA267 and ARA24. (Yeh et al.1996, Fujimoto et al. 1999, Hsiao et al. 1999, Kang et al. 1999, Wang et al. 2001). The ARA24/Ran is the first coactivator which has been shown to interact physically with the

polyglutamine (Q) region of the AR and to enhance the AR-dependent transcription. The interaction of the ARA24 with the poly-Q repeat is decreased by an expanding repeat length (Hsiao et al. 1999), which is concordant with the diminished AR transactivation power with increasing poly-Q-length of the receptor. The ARA 24 is also involved in nuclear transport of the proteins and RNA (Rush 1996).

The PIAS protein family

The PIAS protein (PIAS=protein inhibitor of activated STAT) family consists of four members identified to date: PIAS1, PIAS3, PIASx (two splicing variants PIASxα /ARIP3 and PIASxβ) and PIASy (Shuai 2000, Heinlein and Chang 2002). The PIAS proteins modulate the transcription mediated by several nuclear receptors including the AR (Kotaja et al. 2000). The PIAS1 and PIASxα can act as SUMO-1 (small ubiquitin-related modifier) ligases in sumoylation of the AR repressing the AR-dependent transcription (Nishida and Yasuda 2002). In turn, mutations in the acceptor sites for the SUMO-1 enhanced the transcriptional activity of the AR. (Poukka et al. 2000). It is possible that the sumoylation of the AR by the PIAS proteins may alter the association of the receptor with other coregulators. Although the effects of the PIAS1 and PIASxα on the AR-dependent transcription are dependent on their SUMO-binding motif and the ligase domain all the effects of the PIAS proteins on the AR-dependent transcription cannot be explained through events based solely on the sumoylation modification (Kotaja et al. 2002a). For example, it has been shown that the enhancement of the AR-dependent transcription by the PIAS proteins occurred without the sumoylation of the AR (Nishida and Yasuda 2002). The PIASxα has also been shown to inhibit the AR activity by binding to the AR-DBD. This repression is antagonized by DJ-1, an infertility-related protein and proposed oncogene (Takahashi et al. 2001).

The PIAS proteins have been demonstrated to interact with the p160 coactivator TIF-2 in the steroid receptor mediated transcription. The ability of the TIF-2 to coactivate and colocalize with the AR in the nucleus is also associated with the sumoylation of the TIF-2 by the PIAS proteins (Kotaja et al. 2002b). The PIAS proteins have been suggested to display distinct effects on the AR-mediated transcription activation in prostate cancer cells. The PIAS1 and

PIAS3 have been reported to enhance the transcriptional activity of the AR (Gross et al. 2001), whereas the PIASy acts in a transrepressive manner.

2.3.2 Co-repressors

Nuclear receptor co-repressors were originally identified as proteins associated with unliganded type II nuclear receptors which, unlike type I nuclear receptors, can bind to DNA in the absence of a ligand and mediate transcriptional repression (Horlein et al. 1995). Retinoid acid receptor and thyroid hormone receptor are capable of gene repression by interacting with the co-repressors and recruiting the HDAC activities, whereas the steroid receptors, including the AR, do not repress transcription in the absence of a ligand (Hu and Lazar 2000). For the ER, the switch from gene activation to gene repression by an antagonist is accomplished by association of the co-repressors and HDACs (Shang et al. 2000). Due to the structural and functional similarities between the ER and AR, it has been proposed that the corepression complex may be similarly recruited by antagonist-bound AR (Shang et al. 2002).

Two best characterized co-repressors, NCoR1 and SMRT (silencing mediator of retinoid and thyroid hormone receptor) do not interact with the ER, GR or PR in the absence of a ligand (Wagner et al.1998). The interaction between the AR and NCoR1 has been studied in the presence of the antiandrogen RU486 which is also known to possess partial agonist function with the AR (Berrevoets et al. 2002). It was indicated that the co-repressor N-CoR1 represses the RU486-induced AR activity, most likely as a consequence of a conformational change of the AR-LBD. The NCoR1 is also suggested to mediate the transcriptional repression activity of the nuclear receptors by promoting chromatin condensation, thus preventing the access of the basal transcription machinery (Berrevoets et al. 2002).

Several other co-repressors of the androgen-bound AR, including cyclin D1 and AES (amino-terminal enhancer of split) have also been identified. The D-type cyclins are known to function in the cell cycle progression by binding and activating cyclin-dependent kinases, which is required for the entering to an S-phase (Knudsen et al.1999). However, the transcriptional repression of the AR by the cyclins D1 and D3 is distinct from their action in

the cell cycle signal transduction. The cyclin Ds have been shown to be able to inhibit, e.g. the AR-mediated activation of the PSA gene. The overexpression of the cyclin D1 has been observed in many malignancies (Gumbiner et al.1999).

Although the functions of the co-repressors are often mediated by the HDAC-containing complexes, the AES actively inhibits the AR-dependent transcription in the presence of a deacetylase inhibitor (Yu et al. 2001). This indicates that the AES targets the basal transcriptional machinery rather than chromatin modifications. It has also been suggested that in addition to the AES, other members of Groucho/TLE (transducin-like enhancer of split) family may modulate the transcriptional activity of the AR. Yet another mechanism to prevent the AR action is to influence its cellular localization. For example, the recently identified nuclear receptor family member DAX-1 represses both the estrogen receptors and AR by affecting localization of the receptors in the cell (Holter et al. 2002).

3. PROSTATE CANCER

3.1 Androgen-dependent prostate cancer

Almost all prostate cancers arise from the epithelial compartment of the prostate gland. There are three distinguishable cell types in two defined compartments in the prostate gland epithelia (Garraway et al. 2003). The basal compartment contains basal cells possessing a high proliferative activity and telomerase expression. They are thought to be responsible for the renewal of the epithelia. The basal cells are also androgen independent, and lack the expression of the AR. The second cell type in the basal compartment is neuroendocrine cells, the functions of which are poorly known. The luminal compartment harbors the terminally differentiated secretory cells, which in contrast to the basal cells are androgen dependent and express the AR. It has remained unclear how the prostatic epithelia differentiates to basal, neuroendocrine and luminal secretory cells. Additionally, it is not known which cell type gives rise for prostate cancer. Models describing the epithelial differentiation include the hypotheses (Garraway et al. 2003) of 1) a pluripotent stem cell origin of all the cell types, secretory cells differentiating from intermediate, androgen sensitive transient amplifying basal cells and 2) the discrete lineages for the basal and secretory cells, so that both the basal and luminal compartments have their own discrete repopulating stem cells. The expression of the AR, PSA and PAP in adenocarcinoma cells proposes that the cancer originates from the luminal secretory cell compartment (Isaacs and Coffey 1989, Arnold and Isaacs 2002). However, the cancer cells also bear properties of the basal cells, such as high proliferation rate and telomerase expression (Sommerfeld et al. 1996). Thus, it has been suggested that the cancer arises from the intermediate transient-amplifying cells rather than from clonal expansion of the secretory cells re-entering the cell cycle (Arnold and Isaacs 2002).

The molecular mechanisms of the development of prostate cancer are incompletely understood. As mentioned previously, the crucial role of the androgens in the development of prostate cancer has been recognized. For example, men castrated during the puberty do not develop prostate cancer (Isaacs 1994). During the last 10 years, the genetic alterations in prostate cancer have extensively been studied. According to a comparative genomic hybridization (CGH), the vast majority of primary prostate cancers showed copy number

changes affecting at least one chromosomal region (Visakorpi et al. 1995). A characteristic feature to early prostate cancer is that losses are found about 5 times more often than chromosomal gains or amplifications. This suggests that tumor suppressor genes might be more important than amplified oncogenes in the early development of prostate cancer. The most common losses affect chromosomal regions 6q, 8p, 10q, 13q, 16q and 18q (Visakorpi et al. 1995, Cher et al. 1996, Nupponen et al. 1998).

So far, only a few genes have been implicated in the tumorigenesis of prostate cancer. The most common epigenetic alteration in prostate cancer is hypermethylation of a GSTP1 gene promoter leading to a loss of the gene expression (Lee et al. 1994). The loss of the GSTP1 expression is found practically in all prostate carcinomas and often already in premalignant lesions, such as prostate intraepithelial neoplasia (PIN). The GSTP1 encodes for π-class glutathione S-transferase that participate in detoxification of many environmental carcinogens.

Among the well known tumor suppressor genes, only p53 and PTEN have been implicated in a substantial fraction of the prostate cancers. Inactivation of the tumor suppressor p53, known as guardian of the genome, has been reported to occur in 10–20% of the localized prostate cancers (Visakorpi et al. 1995, Brooks et al. 1996). However, in metastases, mutations have been found in as many as over half of the cases (Meyers et al. 1998). It has also recently been suggested that the androgen-independent growth of prostate cancer would be mediated by a mutant p53 (Nesslinger et al. 2003). The PTEN gene, located in 10q23, has been found to be mutated in 5–27% of the localized, and up to 60% of the advanced prostate cancers (Elo and Visakorpi 2001). The PTEN acts through a PI3K/Akt-pathway in regulating cell proliferation and apoptosis (Di Cristofano and Pandolfi 2000). Third putative tumor suppressor gene that is often inactivated in late-stage prostate cancer is E-cadherin. Its reduced expression is associated not only with the advanced stage but also with poor prognosis (Umbas et al. 1994). Although a loss of one copy of the E-cadherin gene, located at 16q22, is a common finding in prostate cancer, no mutations in the remaining allele have been described (Suzuki et al.1996). It has been suggested that the loss of the expression is due to the hypermethylation of the remaining allele (Kallakury et al. 2001).

The above mentioned tumor suppressor genes seem to be inactivated in the advanced stage of the disease. The two most often deleted chromosomal regions in early prostate cancers as

well as in the PIN are 8p and 13q (Elo and Visakorpi 2001). The target genes for those losses are not known. However, one candidate is a homeobox gene NKX3.1 at 8p21, which is becoming the primary suspect of the target for the 8p loss. It has been reported that the expression of the NKX3.1 is lost in 20% of the PIN and 80% of the metastases (Bowen et al. 2000). No mutations in the gene have been found. However, NKX3.1 heterozygous knockout mice display prostatic epithelial dysplasia indicating haploinsufficiency (Bhatia-Gaur et al. 1999).

3.2. Hormonal therapy

Androgen ablation has been the main therapeutic intervention for treatment of hormone-sensitive prostate cancer during the last half century. The therapy aims at eliminating the androgen activity from the circulation as well as from the prostate tissue (Labrie et al. 1993). 70–80% of the patients respond to the androgen withdrawal therapy (Greyhack et al. 1987). The removal of the androgens or blocking of their effects induces programmed cell death and reduces cell proliferation in both the normal and cancerous prostate epithelium (Kyprianou et al. 1990, Westin et al.1995).

Although surgical castration, i.e. orchiectomy, is an effective means to deplete the androgens, pharmacological methods, especially luteinizing-hormone releasing hormone (LHRH) analogues, have often replaced the orchiectomy. High concentrations of the LHRH agonists down-regulate the LHRH receptors in the pituitary gland, leading to the suppression of the LH release and inhibition of testosterone secretion from the testis (Crawford 1990). Although castration causes over ninety percent reduction in the circulating testosterone levels, considerably high DHT concentrations may still remain in the prostate tissue due to the increased conversion of the adrenal androgens to the DHT (Labrie et al. 1993). Thus, it has been suggested that blocking the effects of the remaining androgen could be therapeutically beneficial. This is achieved by maximal androgen blockade (MAB) therapy combining androgen receptor antagonists (anti-androgens) with castration. The anti-androgens are steroidal (cyproterone acetate) or non-steroidal (flutamide, bicalutamide and nilutamide) compounds which inhibit the androgen action at the androgen receptor level (Schultz et al. 1988). However, an additional benefit of the MAB over castration has not been proven (Prostate Cancer Trialists' Collaborative Group 2000).

3.3 Hormone-refractory prostate cancer

If a patient lives long enough during the hormonal treatment the disease will eventually progress to what is called the hormone-refractory prostate cancer. There are no effective therapies available for such hormone-refractory prostate cancer, and uncontrolled progression of the disease is inevitable. The average survival time of the patients with hormone-refractory diseases is only six months, indicating the aggressiveness of these kinds of tumors (Gittes 1991).

Two theories explaining the progression of the androgen dependent prostate cancer cells to the hormone refractory state have been suggested. First, a clonal outgrowth of the hormone-refractory cancer cells from a heterogeneous cancer cell population due to the selection pressure by the androgen withdrawal. For example, Craft and co-workers (Craft et al. 1999) recently showed that the androgen-independent cells are present in the androgen-dependent prostate cancer xenograft LAPC-9. Castration of the mice resulted in outgrowth of the androgen-independent cells due to the selection. Second, sequential progression or adaptation due to the high genetic instability, resulted in improved survival of the cancer cells (Bruchovsky et al 1990)

Due to the difficulties to obtain samples from the hormone-refractory prostate cancer, the genetic aberrations in these types of tumors have not been studied as much as in the androgen dependent tumors. However, it has already been shown that the mean number of chromosomal aberrations in recurrent prostate tumors is three times higher than in untreated tumors (Visakorpi et al. 1995, Koivisto et al. 1995). The chromosomal changes that are often found in the recurrent prostate tumors include the gain of 7p, 8q and Xq, and losses of 10q and 16q. However, all these, except the gain of the Xq, are also found in the untreated localized tumors and metastases (Visakorpi et al. 1995, Nupponen et al. 1998a)

It was commonly believed earlier that the transition of prostate cancer growth from androgen- dependence to -independence is caused by alternative growth signaling pathways taking over the growth promoting function of the androgen signaling (Visakorpi 2000). Abnormal activation of several growth factor pathways by autocrine signaling has been

suggested to be involved in the AR-independent progression (Djakiew 2000). For example, the autocrine loop of the epidermal growth factor (EGF) system has been implicated. The androgen independent cells express higher levels of EGF receptor (EGFR) ligands such as the EGF and transforming growth factor (TGF) alpha, at least in vitro (Torring et al. 2000). Although the expression of the EGFR is generally decreased in prostate cancer, the aggressive and hormone-refractory types of the tumors seem to retain the EGFR expression (DiLorenzo et al. 2002, Visakorpi et al. 1992). In addition, fibroblast growth factors (FGFs) have been implicated in the development of the androgen independent prostate cancer (Dorkin et al.1999).

3.4 Androgen receptor in prostate cancer

Several alterations take place in the AR signaling pathway during the development and progression of prostate cancer (Gao et al. 2001). First, the action of the AR in a normal and malignant prostate uses distinct pathways. In a normal prostate gland, androgen stimulated proliferation of epithelium requires paracrine involvement of stromal cells expressing the AR. In malignant cells the androgen mediated signaling has been converted to autocrine mode and no interaction with the stroma is needed. In addition, the emergence of the hormone-refractory tumors during the endocrine treatment is associated by restoration of the expression of the genes regulated by the AR (Gregory et al. 2001, Kim et al. 2002). These changes are now believed to be caused at least partly by genetic changes in the AR gene.

3.4.1 Germ-line alterations

The androgen receptor gene has been evaluated extensively as a prostate cancer susceptibility gene (Table 1). It has been suggested that a short CAG repeat may result in an increased risk of prostate cancer and that the length of the repeat could also be partly responsible for the difference in prostate cancer risk in different racial groups (Edvards et al 1992, Irvine et al. 1995). For example, Giovannucci et al. (1997) observed that the shorter repeat was associated with an increased risk for metastatic and fatal prostate cancer. The short CAG repeat length has been reported to correlate with young age at diagnosis (Bratt et al. 1999). However, these observations have not been confirmed by several recent studies (Correa-Cerro et al. 1999; Edwards et al. 1999, Lange et al. 2000, Latil et al. 2001, Miller et al. 2001, Chen et al. 2002, Chang et al. 2002, Suzuki et al. 2002, Gsur et al. 2002).

The biological significance of polyglycine repeat (GGN) in the exon 1 is less clear (Hsing et al. 2002). Nevertheless, some studies have proposed that the size of the glycine repeat might increase the risk of prostate cancer (Irvine et al. 1995, Giovannucci et al. 1997, Stanford et al. 1997). A recent study by Chang et al (2002) suggested that alleles of ≤ 16 GGC repeats are associated with risk of prostate cancer. However, several studies have not found such an association (Platz et al. 1998, Miller et al. 2001, Chen et al. 2002).

Germ-line point mutations in the AR gene are not commonly associated with prostate cancer, but they are occasionally found. In the Finnish population, an Arg726Leu substitution has been reported to increase the risk of prostate cancer (Mononen et al. 2000) but this observation was not confirmed by the study done with the North American population (Gruber et al. 2003).

Table 1. Reported association studies between germ-line alterations of the AR and prostate cancer

Publication	*CAG repeat length*	*GGN repeat length*	*Cases + controls*	*Comments*
Irvine *et al.,* 1995	CAG<22/GGC not-16: RR 2.1 (p=0.08)		57+37	repeat lengths correlate with racial risk groups
Hardy *et al.,* 1996			109	short CAG repeat associated with younger age at diagnosis
Giovannucci *et al.,* 1997	≤18 CAG: RR=1.52(0.92-2.49)		587+588	associated with advanced stage
Stanford *et al.,* 1997	<22 CAG, ≤16 GGC: RR= 2.05(1.09-3.84)	≤16 GGC: OR= 1.60(1.07-2.41)	301+277	
Ingles *et al.,* 1997	<20 CAG: OR=2.10(1.11-3.99)		57+169	
Hakimi *et al.,* 1997	≤17 CAG: OR=3.7(1.31-10.5)	≤16 GGC: OR=4.6 (1.3-16.1)	59+370 54+110	
Platz *et al.,* 1998		GGN 23 OR= 1.2 (0.97-1.49)	582+794	
Correa-Cerro *et al.,* 1999	no association	no association	105+132	
Bratt *et al.,* 1999	no association		190+186	short CAG repeat associated with younger age at diagnosis
Edwards *et al.,* 1999	no association	no association	178+195	long GGC associated with poor prognosis
Lange *et al.,* 2000	no association		226+305	familial cases included
Hsing *et al.,* 2000	<23 repeat: OR= 1.65(1.14-2.39)	<23 repeat: OR= 1.12(0.71-1.78)	190+304	Chinese population
Latil *et al.,* 2001	no association		256+156	
Miller *et al.,* 2001	no association	no association	140+70	familial cases
Mononen *et al.,* 2002	≤18 CAG: OR=1.47(1.00-2.16)		461+574	no association in familial cases
Chen *et al.,* 2002	no association	no association	300+300	
Chang *et al.,* 2002	no association	≤16 GGC: OR= 1.58(1.08-2.32)	327+174	included 129 familial cases
Suzuki *et al.,* 2002	no association		88+53	Japanese population
Gsur *et al.,* 2002	no association		190+190	
	R726L missense alteration			
Mononen *et al.,* 2000	in sporadic cancer OR= 5.8(1.5-22.1) in familial cancer: OR= 5.8(0.95-34.8)		418+900 106+900	mutation frequency 1.91% among Finnish cancer patients
Gruber *et al.,* 2003	no association		548	no R726L mutation found

RR = relative risk, OR = odds ratio, both followed by 95% confidence intervals in brackets

3.4.2 Somatic aberrations of AR gene in androgen-dependent prostate cancer

Most studies (Table 2) have found only a few somatic mutations of the AR in untreated prostate cancer (Newmark et al. 1992, Suzuki et al. 1993, Culig et al. 1993, Ruizeweld de Winter et al. 1994). However, two researches suggesting that mutations are present in a substantial fraction of prostate cancers have been published. First, Gaddipati and co-authors (1994) reported that a codon 877 mutation (known as the LNCaP mutation) was found in 25% of the transurethral resection of prostate (TURP) specimens of the patients with untreated metastatic prostate cancer. Second, Tilley and associates (1996) reported that about 50% of the cancers including early stages of the disease contain a mutated AR. It has been suggested that the reason why most of the researches have failed to find mutations is the methodological problems related to normal cell contamination. However, it has been shown by Marcelli et al. (2000) that there were no differences in mutation detection between microdissected and non-microdissected primary carcinoma samples. No mutations were found using either method in 99 prostate cancer samples. Therefore, it is now generally accepted that the AR mutations are rare in untreated prostate cancer.

Only few of the prostate cancer models have been established on the basis of untreated prostate carcinomas. CWR22 is an androgen-dependent prostate cancer xenograft derived from an untreated tumor (Nagabhushan et al. 1996). It contains a H874Y (histide to tyrosine) mutation in the ligand binding domain of the AR enabling the receptor to bind adrenal androgen dehydroepiandrosterone in addition to several other steroid hormones and hydroxyflutamide (Tan et al. 1997).

3.4.3 Somatic aberrations of AR gene in hormone-refractory prostate cancer

AR gene amplification

While investigating the putative target genes for commonly amplified chromosomal region Xq11-q13, Visakorpi et al. (1995) found a high-level AR amplification in 30% of the hormone-refractory tumors but in none of the specimens taken from the same patients prior to therapy. The finding has subsequently been confirmed by several other studies (Table 2). For example, Bubendorf et al. (1999), found the AR gene amplification in 23% of the 54 locally recurrent and 22% of the 62 metastases of hormone-refractory disease. The findings

suggest that the amplification of the AR gene may be one of the mechanisms by which the prostate tumors acquire growth advantage in an androgen depleted environment. The amplification of the AR may sensitize the prostate cancer cells to minimal amounts of the androgens (Visakorpi et al.1995, Culig et al. 1997). It has now also been shown that the patients with the AR gene amplification respond more often to the second-line MAB treatment than the patients whose tumors do not contain the amplification (Palmberg et al. 2000). The finding demonstrates that the amplified AR is truly functional and that tumors with the amplification are hypersensitive to the androgens. Unfortunately, the treatment response-time for the second-line MAB is short and benefit of the therapy is marginal.

AR gene mutations

The first thoroughly studied mutation in hormone-refractory prostate cancer was the one discovered in the LNCaP prostate cancer cell line (Veldscholte et al. 1992). The LNCaP cell line was originally established from lymph node metastases of a patient treated with hormonal therapy. In the cell line the mutation T877A in the ligand binding domain of the AR enables the receptor to be activated by other steroid hormones such as estradiol and progesterone, and even by antiandrogen flutamide. Recently, it was shown that the cell lines MDA-Pca 2a and 2b, which were established from a prostate cancer bone metastasis developed after orchiectomy, also harbor mutations in the androgen receptor gene. The AR gene of the cell lines contains two mutations, the T877A (threonine to alanine) and L701H (leucine to histidine), also located in the ligand binding domain (Zhao et al. 1999, 2000). These mutations reduce affinity for the androgens, but enhance binding of adrenal corticosteroids. The two mutations have a high synergistic effect in promoting promiscuous ligand binding. Together they increase the affinity of the AR for glucocorticoids by 300% more than the L701H mutation alone (Zhao et al. 1999). It has also been shown that AR point mutations occur spontaneously in transgenic adenocarcinomas of the prostate mouse model (TRAMP), and certain mutations are selected for by the changes (castration) in the androgen environment (Han et al. 2001).

Several reports have suggested that the use of the AR antagonist flutamide is associated with the frequency of the mutations in the AR (Taplin et al 1995, 1999; Balk 2002). For example, Taplin and co-workers (1995,1999) found the mutated AR in 31% (5 out of 16) of the patients receiving the MAB with flutamide compared to only 6% of the patients (1 out of 17) treated with monotherapy. The mutated ARs found from the flutamide-treated patients

were also shown to be stimulated by flutamide. The most frequently found mutation among the flutamide-treated patients was identical to the mutation in the LNCaP (T877A).

The recent studies by Haapala et al. (2001) and Hyytinen et al. (2002) further suggest the influence of treatment to emergence of the mutations in the AR. Haapala et al (2001) analyzed tumors from patients treated with orchiectomy and bicalutamide. Mutations were found in 36% (4 out of 11) of the tumors. It has now been shown that one of the mutations leads to paradoxical activation of the AR by bicalutamide (Hara et al. 2003). Hyytinen and co-workers found mutations in 33% (7 out of 21) of the tumors treated with orchiectomy, estrogens or with a combination of orchiectomy and estramustine phosphate (EMP). The mutations were especially common (4 out of 5) in the tumors treated with orchiectomy and EMP combination. In addition to the missense mutations, it has been demonstrated that silent mutations in the AR gene may influence the mRNA stability or transcriptional regulation (Han et al. 2001).

In conclusion, it is likely that the mutations in the AR gene are selected by hormonal treatment in subset of the recurrent tumors. The mutations then provide growth advantage in the altered hormonal environment.

Table 2. Somatic genetic alterations of the AR in prostate cancer

Gene amplification	*Frequency*	*Comments*
Visakorpi *et al.,* 1995	7 / 23 (30%) in HR	
Koivisto *et al.,* 1997	15 /54 (28%) in HR	
Bubendorf *et al.,* 1999	11/47 (23%) in locally recurrent and in 12/59 (20%) in metastatic HR	
Miyoshi *et al.,* 2000	1/ 5 (20%) in HR	
Hernes *et al.,* 2000	10/18 (56%) in HR	
Palmberg *et al.,* 2000	10/77 (13%) in HR	associated with response to MAB
Edwards *et al.,* 2001	3/20 (15%) in HR	
Haapala *et al.,* 2001	0/11 (0%) in HR	patients treated with combination of orchiectomy and bicalutamide
Hyytinen *et al.,* 2002	4/16 (25%) in HR	
Brown *et al.,* 2002	9/18 (50%) in HR	
AR mutation	*Frequency*	*Comments*
Newmark *et al.,* 1992	1/26 (4%) in AD	
Suzuki *et al.,* 1993	0/7 (0%) in AD, and 1/8 (13%) in HR	
Culig *et al.,* 1993	1/7 (14) in metastatic HR	
Gaddipati, *et al.,* 1994	6/24 (25%) in AD	all T877A mutations in advanced tumors
Schoenberg *et al.,* 1994	1/40 (3%) in AD	CAG repeat contraction 24→18
Ruizeweld de Winter *et al.,* 1994	0/18 (0%) in HR	
Visakorpi *et al.,* 1995	0/23 in HR	only T877A mutation analyzed
Taplin *et al.,* 1995	5/10 (50%) in metastatic HR	patients treated with flutamide
Elo *et al.,* 1995	1/23 (4%) in AD, and 0/6 (0%) in HR	germ-line mutation
Suzuki *et al.,* 1996	0/30 (0%) in AD, and 3/22 (14%) in HR	
Evans *et al.,* 1996	1/31 (3%) in AD, and 0/13 in HR	exon 1 included
Tilley *et al.,* 1996	11/25 (44%) in AD	exon 1 included
Koivisto *et al.,* 1997	1/13 (8%) in HR	all samples AR amplified
Watanabe *et al.,* 1997	5/36 (14%) in AD	exon 1 included
Taplin *et al.,* 1999	5/16 (31%) in HR	exons 7-8 analyzed, patients treated with flutamide
Marcelli *et al.,* 2000	11/137 (8%) in AD	all mutations found in stage D1disease
Haapala *et al.,* 2001	4/11 (36%) in HR	exon 1 included, patients treated with flutamide
Hyytinen *et al.,* 2002	7 /21 (33%) in HR	exon 1 included
Segawa *et al.,* 2002	3/45 (7%) in AD	all mutations silent
Lamb *et al.,* 2003	1/10 (10%) in HR	exon 1 included, both AD and HR tumors analyzed from each patient

AD = androgen dependent prostate cancer, HR = hormone-refractory prostate cancer
MAB = maximal androgen blockade

Interaction of AR with other signaling pathways

It has been proposed that in the absence of a ligand, the AR activation could take place by cross-talk with various growth factors, protein kinase pathways. For example, it has been demonstrated that an epidermal growth factor (EGF), epidermal growth factor receptor-2 (ERBB2/Her-2), keratinocyte growth factor (KGF/FGF-7), insulin-like growth factor–1 (IGF-1), protein kinase A (PKA), mitogen-activated protein kinase (MAPK), as well as IL-6 could activate the AR signaling (Culig et al. 1994, Abreu-Martin et al.1999, Jenster et al. 2000, Craft et al.1999, Sadar 1999). An additional mechanism underlying ligand dependent activation of the AR by these alternative pathways may involve phosphorylation of either the AR or its associated proteins (Sadar 1999, Ueda et al. 2002)

The IGF-1 has been implicated in the progression to androgen-independence at least in LNCaP and LAPC-9 xenograft models, in which both the IGF-1 and its receptor IGF-1R are overexpressed (Nickerson et al. 2001). In addition, the inhibition of the IGF-R1 has been shown to result in suppression of tumor growth and invasiveness in rat prostate cancer cells (Burfeind et al. 1996).

Cross-talk between the EGFR2/ ERBB2 receptor and the AR has also been suggested (Craft et al.1999). It has been shown that the ERBB2 enhances signaling activity of the AR in the absence or in low levels of the androgens in two xenograft models, the LAPC-4 and LNCaP (Craft 1999 et al, Yeh et al. 1999). Forced overexpression of the ERBB2 in the androgen-dependent prostate cancer cells was demonstrated to cause androgen-independent growth in castrated animals. The ERBB2 gene (HER-2/neu) encodes for a transmembrane glycoprotein that contains tyrosine kinase activity and belongs to the epidermal growth factor receptor family (Akiyama et al. 1986). An amplification of the ERBB2 gene, leading to overexpression, has been found e.g. in 25–30% of human breast and ovarian cancers and the amplification is associated with poor prognosis in these malignancies (Slamon et al.1989, Press et al. 1997). A gene copy number and expression of the ERBB2 in prostate cancer have been analyzed in several studies. Most studies have not found the high-level amplification of the ERBB2 gene in prostate cancer (Bubendorf et al.1999, Savinainen et al. 2002). In addition, no overexpression of the ERBB2 has been demonstrated (Visakorpi et al. 1992, Savinainen et al. 2002). Thus, it is unlikely that alterations in the expression of the ERBB2 are involved in the progression of prostate cancer in vivo.

It has been shown that the concentration of the IL-6 is elevated in the sera of the patients with metastatic and hormone-refractory prostate cancer (Drachenberg et al.1999). An IL-6 receptor is also expressed in a normal prostate, PIN and cancer. The IL-6 has been shown to activate the AR through the MAPK pathway interacting with the transcriptional coactivator p300 in the prostate cancer cells (Hobisch et al.1998, Ueda et al. 2002, Debes et al. 2002). It has thus been suggested that a network of protein kinase-coactivator-IL-6 is needed to induce the androgen-independent activation of the AR (Ueda et al. 2002).

3.5 AR coregulators in prostate cancer

In vitro reporter assays have demonstrated that the levels of the coactivators and co-repressors might influence the AR activity in malignant cells. However, little is known about the expression of these coregulators in prostate cancer in vivo.

One of the most studied coactivators in prostate cancer is the SRC1. Li and co-workers (2002) failed to detect any changes in the SRC1 expression in 45 primary prostate cancers by the mRNA in situ hybridization; whereas Fujimoto (2001) reported that the expression of the SRC1, measured by the conventional RT-PCR, was higher in poorly differentiated and unresponsive cancers. On the other hand, expression of the SRC1 has been reported to be decreased in the androgen-independent LNCaP-abl cell line compared to the parental, androgen-sensitive LNCaP (Nessler-Menardi et al. 2000).

Recently, Gregory et al. (2001) found that the expression of both the SRC1 and TIF2, measured by Western analyses, was increased in hormone-refractory prostate cancer. The overexpression of those coactivators affected the ability of low-affinity ligands or minimal concentrations of the adrenal androgens to activate the AR in the castrated environment. Transactivation by the AR was further increased by phosphorylation of the p160 coactivators suggesting a link to growth factor signaling pathways. Interestingly, it has also been shown that the ligand–independent activation of the AR does not occur alone through the overexpression of the SRC1; it requires a functional MAPK pathway (Ueda et al. 2002). In the absence of the androgens, the protein–protein interaction of the SRC1 and AR also seems to require the IL-6 (Ueda et al. 2002).

A potentially important role for a non-HAT coactivator complex subunit, TRAP220 (thyroid hormone receptor-associated protein), in malignancies has been implicated by the finding of a TRAP220/PBP gene amplification and overexpression in breast cancer (Zhu et al.1999). However, no overexpression of the TRAP220 has been reported in prostate cancer (Li et al. 2002).

The expression of the CBP has been detected in different prostate specimens, but no alterations in the level of expression have been reported (Comuzzi et al. 2003). It has also been shown that antagonist/agonist balance of antiandrogens flutamide and bicalutamide is influenced by the CBP. In co-transfection assays the expression of the CBP increased flutamide-induced AR activity up to 50% of the activation induced by the synthetic androgen R1881.

In vitro studies have implicated several members of the ARA coactivator family in prostate cancer, but their significance in vivo has not yet been fully determined. An increased expression of the ARA24 has been reported in primary prostate tumor specimens (Li et al. 2002). In addition, the ARA55 has been shown to be expressed more in hormone-refractory than androgen-dependent cancer. The ARA55 enhances the AR affinity to weak ligands. The dominant negative form of the ARA55 has been shown to suppress AR-mediated transcription, which suggests that the action of the AR in prostate cancer could be prevented by targeting the ARA55 (Rahman et al. 2003).

3.6 Estrogen receptors in prostate cancer

The estrogens are necessary for the regulation of male sexual behavior, maintenance of the skeleton and cardiovascular system and for normal function of the testis and prostate (Pettersson and Gustafsson 2001). In adult men, the estrogens are produced by the adrenals, testes and adipose tissue. The hormonal balance in males changes with age. After 40–50 years of age, the concentration of testosterone and especially the amount of free testosterone starts to decrease. On the other hand, the amounts of the estrogens increase at the same time leading to a change in the androgen-estrogen balance towards a more dominant estrogen action. Rising estrogens appear to increase sensitivity of the prostate tissue to the androgens

(Shirai et al. 1994). Estrogenic stimulation with decreasing androgen levels has been suggested to contribute to the genesis of prostatic dysplasia and subsequent prostate cancer (Habenicht et al. 1988, Raghow et al. 2002). Estrogen exposure during development of the prostate has also been suggested to initiate cellular processes resulting in neoplasia later in life (Santti et al. 1994). In addition, some reports suggest that the offspring of diethylstilbestrol (DES)-exposed mothers have a higher risk of prostate cancer (Strohsnitter et al. 2001).

In 1986, cloning of the first estrogen receptor (ERα) was reported by two groups (Green et al. 1986, Greene et al. 1986). In 1995, a second estrogen receptor (ERβ) was cloned from a rat prostate cDNA library (Kuiper et al. 1996) and, subsequently, also the human ERβ was identified (Mosselman et al. 1996). The ERα and ERβ are highly homologous and regulate transcription through interaction with specific binding sites on the DNA (estrogen response elements, EREs) in the promoter regions of the target genes in response to the 17β-estradiol. In addition, the estrogen receptors are transcriptional regulators at AP-1 and SP-1 binding sites. Both receptors have the same transactivation profile with different ligands at the ERE site, whereas at the AP-1 site the receptors signal in opposite ways (Paech et al.1997). The relationship of these two ERs in mediating the estrogen action seems controversial. For example, the ERβ is able to inhibit the ERα-mediated transcription (Lindberg et al. 2003). On the other hand, in the absence of the ERα, the ERβ can partially replace its functions. The ERβ has also been demonstrated to have an antiproliferative function in rodents due to the modulation of the effects of the ERα in the uterus (Weihua et al. 2000) and prostate (Weihua et al. 2001). The physiological role of the ERβ also seems to consist of adjusting the ERα action, at least in mice.

Unlike the ERα, the expression of which is almost predominantly associated with hormone-sensitive tissues, the expression of the ERβ is more widespread (Taylor et al. 2000). The role of the ERα in the prostate is not fully understood. The developmental estrogenization of the prostate is mediated by the ERα, as has been demonstrated by using ERα- and ERβ-knockout mice (Prins et al. 2001). However, during the development of the human fetal prostate, only the ERβ has been detected using both the immunohistochemistry and RT-PCR (Adams 2002). Most studies have reported the lack of the ERα expression in adult prostate epithelial cells, whereas it is expressed in the stromal cells (Schulze et al. 1990, Kruithof-

Dekker et al.1996, Leav et al. 2001). ERα promoter methylation has been observed in several human cancers, including breast (Ottaviano et al. 1994), lung, colorectal and prostate cancers (Issa et al.1994, 1996, Li et al. 2000, Sasaki et al. 2002). In concordance with the hypermethylation, most of the studies have not found the ERα expression in the prostate cancer cells by immunostaining. An exception is a study by Bonkhoff et al. (1999) that suggests an increased expression of the ERα in hormone-refractory and metastatic prostate cancers as measured by the immunohistochemistry and mRNA in situ hybridization.

The expression of the ERβ has been demonstrated in both the stroma and epithelial compartment of the human prostate (Horvath et al. 2001). The ERβ knockout (BERKO) mice develop prostatic hyperplasia spontaneously and have an increase in the expression of the AR and proliferation markers in the prostate epithelium (Weihua et al. 2001). It has thus been proposed that the ERβ is a negative regulator of the AR in the rodent prostate. Several studies have suggested that the expression of the ERβ is decreased in prostate cancer compared to the normal tissue (Leav et al. 2001, Latil et al. 2001, Horvath et al. 2001, Fixemer et al. 2003) although also an increased expression has been reported (Ito et al. 2001, Royuela et al. 2001). Paradoxically to the decreased expression of the ERβ in cancer, it has been reported that a retained expression of the ERβ is associated with poor prognosis (Horvath et al. 2001). Loss of the ERβ expression due to the promoter methylation has been demonstrated in both the prostate cancer cell lines and tumor samples (Sasaki et al. 2002).

AIMS OF THE STUDY

The overall aim of the study was to elucidate the role of the AR and other genes related to the steroid hormone action in prostate cancer. The main emphasis was to find the changes underlying the transition of the growth from androgen-dependence to -independence. The specific aims were:

1. to study the frequency of the AR gene mutations and amplification in hormone-refractory prostate cancer
2. to investigate whether the AR gene amplification is associated with the expression of the AR and the down-stream gene PSA
3. to find out whether the expressions of the 16 AR coregulators (SRC1, β-catenin, TIF2, PIAS1, PIASx, ARIP4, BRCA1, AIB1, AIB3, CBP, STAT1, NCoR1, AES, cyclin D1, p300 and ARA24) are altered in prostate cancer.
4. to find out whether the increased expression of the AR coregulators could be due to the gene amplification
5. to study whether the expression of the ERα and ER-β is altered in prostate cancer.

MATERIALS AND METHODS

1. Cancer cell lines and xenografts

The prostate cancer cell lines PC-3, DU-145, LNCaP, NCI-H660, 22Rv1 and breast cancer cell lines (Study IV) (MCF-7, ZR-75-1, T-47D, BT-474, MDA-134, CAMA-1, UACC893, MDA-436, SK-Br-3) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured under the recommended conditions. Specimens of 10 human prostate cancer LuCaP xenografts were kindly provided by Dr. Robert L. Vessella (Department of Urology, University of Washington, Seattle, WA, USA).

2. Clinical tumor samples

For study I, 32 formalin-fixed, paraffin-embedded hormone-refractory prostate carcinomas were obtained from Tampere University Hospital. 30 of the samples were transurethral resection (TURP) specimens and two were lymph node metastases obtained at the time of progression during the endocrine therapy. The endocrine therapy included either orchiectomy (22 cases), LHRH analogue (1 case), estrogens (4 cases), orchiectomy and estrogens (2 cases), bicalutamide (1 case) or unspecified hormonal therapy (2 cases). The median time from diagnosis to tumor recurrence and obtaining the sample was 32 months (range 7–124). The DNAs were isolated from the formalin-fixed paraffin-embedded (n=23) freshly frozen (n=9) tumor blocks using routine techniques (Isola et al. 1994). DNA from healthy male lymphocytes was used as a normal control.

For studies II, III and IV, freshly frozen specimens from 9 benign prostate hyperplasias (BPH), 33 androgen-dependent untreated primary prostate carcinomas (32 prostatectomy specimens and 1 TURP specimen) and 13 locally recurrent, hormone-refractory prostate carcinomas (TURP specimens) were obtained from Tampere University Hospital. Of the untreated primary carcinomas, 12 were grade I, 15 grade II and 6 grade III according to the WHO classification (Mostofi 1980). The pTNM distribution of the cases was pT1N0M0 2, pT2N0M0 12, pT2N1M0 2, pT3N0M0 13, pT4N0M0 2, T4NXM0 2. The hormone-refractory tumor specimens were obtained from patients experiencing urethral obstruction during the hormonal monotherapy. The endocrine therapy forms were either orchiectomy

(11 cases), luteinizing hormone-releasing hormone analogue (1 case) or estrogens (1 case). The time from the beginning of the therapy to a TURP operation varied from 21 to 72 months. 36 untreated primary breast cancer tissue samples (Study IV) were obtained from Tampere University Hospital. All specimens were histologically examined for the presence of more than 60 percent of tumor or epithelial (in the BPH cases) cells using hematoxylin and eosin-stained slides.

3. Single strand conformational polymorphism (SSCP) analysis and sequencing

The target sequences were amplified using the PCR with 16 previously published primer pairs (Tilley et al.1996) and AmpliTag Gold polymerase (PerkinElmer, Foster City, CA, USA) followed by 7% polyacrylamide gel electrophoresis to evaluate the size and quality of PCR-products. Subsequently, the reaction products were purified using a Qiagen Qiaquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA). In order to create a single stranded DNA, the PCR products were denatured with methylmercury hydroxide (MMH) in 95° C for 4 minutes. The products were run for 3.5 hours using 250 V voltage in a 4–20% gradient polyacrylamide gel in three different temperatures (15°, 22° and 30° C) using an Xcell II™ gel run unit and Thermoflow™ Electrophoresis Temperature Control Unit (Novex, San Diego, CA, USA). Subsequently, the gel was stained with a SYBR Green II (Molecular Probes, Eugene, OR, USA) dye. Aberrantly moving bands were cut from the gel, re-amplified and purified for sequencing reactions. The cycle sequencing reaction was performed using the corresponding PCR primers and an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Foster City, CA, USA). The samples were then run in an ABI310 Genetic Analyzer (PerkinElmer).

The repeat length analyses of the CAG/GGN were done as follows. Nested PCR reactions for both the CAG and GGC repeats were performed using the primer sequences published by Irvine et al 1995. The labeling of the PCR products was performed in the second PCR with inner primers, of which one primer was labeled with 5'-prime-6-FAM. The samples were then run in the ABI310 Genetic Analyzer and results were analyzed with the Gene Scan software program (PerkinElmer).

4. Fluorescence in situ hybridization (FISH)

The human P1-probe for the AR gene (LCG-P1AR) (Visakorpi et al. 1995) and BAC probe for the SRC1 (GenBank Acc.no. AC013459) were labeled with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany), and the BAC probe for pHyde (GenBank Acc.no. AC016673) and a pericentromeric alphoid repeat probe for chromosome X (DXZ1) were labeled with FITC-dUTP (NEN, Boston, MA, USA) by nick translation. The dual-color hybridization was done principally as previously described (Visakorpi et al. 1995). Briefly, 5μm freshly frozen tissue sections were fixed with a series of 50%, 75% and 100% 3:1 methanol-acetic acid (Carnoy's fixative). Subsequently, the slides were denatured in a 70%formamide/2×SSC solution (pH 7.0) at 70°C for 3 minutes and dehydrated in an ascending ethanol series. Hybridization was carried out overnight at 37°C. After stringent washes, the slides were stained with anti-digoxigenin-rhodamine (Roche Diagnostics) and counterstained with an antifade solution (Vectashied, Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino 2-phenylindole (DAPI). The FISH signals were scored to determine the copy number of the probe targets from the non-overlapping and intact nuclei of the epithelial cells using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). The amplification of the gene was defined as a >2-fold higher copy number of a target than reference probe, or more than 5 copies of the target gene or tight clusters of the target gene.

5. Immunohistochemistry

Expression of the ERα was studied immunohistochemically using a monoclonal antibody 1D5 (0.5 μg/ml, Novocastra Laboratories, Newcastle, UK) and high-temperature antigen retrieval (in 1mM Tris-EDTA, pH 9). Intense nuclear immunostaining present in >10% of the cells was scored as positive.

6. Quantitative real-time RT-PCR

Total RNA from the cell lines was isolated using a Trizol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. For an RNA extraction from the tumor material, cancerous tissue was either cut by cryotome (Study II) or scratched from the frozen tumor blocks using a pre-cooled, sterile scalpel (Studies III,

IV). Subsequently, tissue powder or the slices from cryotome were added directly into the Qiagen RNeasy MiniKit tissue lysis buffer (Qiagen Inc, Valencia, CA, USA), and the total RNA was isolated according to the manufacturer's instructions. The first-strand cDNA was synthesized with a Superscript™ II reverse transcriptase and oligo $d(T)_{12-18}$ primer according to the manufacturer's protocol (Life Technologies Inc.). For preparing standard curves, the total RNA from the LNCaP was used. After the first strand cDNA synthesis, serial dilutions were made to prepare the standard curves.

The primers (Table 3) were designed using The Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3) to different exons in order to avoid amplification from the genomic DNA. The probes (for the AR, PSA, ERα, ERβ, and TBP) were designed and synthesized by TIBMolBiol (Berlin, Germany) (Table 4). It was also carefully checked that the primers could not detect pseudogenes or retropseudogenes. The PCR reactions were performed in the LightCycler™ apparatus (Wittwer et al. 1997) using either the LC DNA Master Hybridization probes Kit (Study II), LC Fast Start DNA SYBR Green I Kit (Study III) or LC Fast Start DNA Master Hybridization Probes Kit (study III-IV) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's guidelines. The elongation time was calculated by dividing the size of the amplicon by 25. While using the hybridization probe detection, the fluorescence emitted by the probes was measured in every cycle at the end of the annealing step. After proportional background adjustment, the fit point method was used to determine a crossing-point value. Whereas, while using the SYBR Green I detection, the fluorescence was measured in every cycle at the end of the elongation step or, in order to avoid any fluorescence from unspecific products, in higher temperatures. After arithmetic background adjustment, the fit point method was once again used to determine the crossing-point value. Software produced the standard curve by measuring the crossing-point of each standard and plotting them against the logarithmic value of the concentrations. The concentrations of unknown samples were then calculated by setting their crossing-points to the standard curve.

Table 3. The primers used in the real-time RT-PCR

Gene	*Primer sequences* (*5'-3'*)
AR	CTCACCAAGCTCCTGGACTC
	CAGGCAGAAGACATCTGAAAG
PSA	GCAGCATTGAACCAGAGGAG
	AGAACTGGGGAGGCTTGAGT
ER α	CCACCAACCAGTGCACCATT
	GGTCTTTTCGTATCCCACCTTTC
ER β	AAGAAGATTCCCGGCTTTGT
	CTTCTACGCATTTCCCCTCA
TBP	GAATATAATCCCAAGCGGTTTG
	ACTTCACATCACAGCTCCCC
SRC1	ATGGTGAGCAGAGGCATGACA
	AAACGGTGATGCTCATGTTG
TIF2	TAATGCACAGATGCTGGCC
	TCTGTGTATGTGCCATTCGG
PIAS1	CCACATGACACCCATGCCTT
	CCAAAGATGGATGCCGGGTC
PIASx*	TCTTCTGACGAAGAGGAAGACC
	TCAGAAGATGTTCCAAGCTTCA
ARIP4	ATAGCAAGTTCCTACAGGGC
	CAGATTCACACCCAAGCATC
BRCA1	TTCAGGGGGCTAGAAATCTG
	CTACACTGTCCAACACCCACTCTC
β-Catenin	AATACCATTCCATTGTTTGTGCAG
	AGCTCAACTGAAAGCCGTTT
AIB3	TCCAGAACTTCTACCCAGCA
	ATCAAGTCGCAGTCCTGCTT
AIB1	CGTCCTCCATATAACCGAGC
	TCATAGGTTCCATTCTGCCG
CBP	CAGAGCGGATCATCCATGACTA
	GCTTCTCCATGGTGGCATAC
STAT1	TATAGAGCATGAAATCAAGAGCC
	GGGCATTCTGGGTAAGTTCA
NCoR1	GGCCCTCTTCAGTCTCCTCT
	GGCAGGTTTTTGACCTGCTA
AES	CGCGATTGACATGATGTTTC
	CTCTCAATGGCTCCCAAGAC
Cyclin D1	CCCTCGGTGGGTCCTACTTCAA
	TGGCATTTTGGAGAGGAAGT
p300	CCTGAGTAGGGGCAACAAGA
	GTGTCTCCACATGGTGCTTG
ARA24	CCACCAGAAGTTGTCATGGAC
	ACAAGGGATGAGTTCACTTGC

*The primers amplify a region that is common to the PIASxα and PIASxβ isoforms.

Table 4. The probes used in the real-time RT-PCR.

Gene	*Hybridization probe sequences* (5'-3')
AR	TCCACACCCAGTGAAGCATTGGAA- fluorescein
	Red640-CTATTTCCCCACCCCAGCTCATG
PSA	GGTGGTGCATTACCGGAAGTGGATCA- fluorescein
	Red640-ACACCATCGTGGCCAACCCCTG
ER α	CACTTCGTAGCATTTGCGGAGCC-fluorescein
	Red640-GCCTGGCAGCTCTTCCTCCTGTTT
ER β	GCGCCACATCAGCCCCATCATTAA-fluorescein
	Red640-ACCTCCATCCAACAGCTCTCCAAGAG
TBP	TTTCCCAGAACTGAAAATCAGTGCC-fluorescein
	Red640-TGGTTCGTGGCTCTCTTATCCTCATG

The intra-assay coefficient of variation (CV) for the genes was determined by repeating analyses of 2 samples 10 times. The CV values were 5.7% for SRC1, 6.8% for TIF2, 6.9% for BRCA1, 17.3% for NCoR1, 11% for AES, 13.2% for STAT1, 20% for cyclin D1, 10.6% for AIB1, 12.6% for PIAS1, 4% for β-catenin, 19% for PIASx, 26% for ARIP4, 11.5% for ARA24, 8.7% for AIB3, 13% for CBP, 28% for p300, 8.3% for ERα, and 6.6% for ERβ. The relative expression was illustrated by dividing values from the target genes with the TBP value. The TBP was chosen to be the reference gene because there are no known retropseudogenes for it and the expression of the TBP is lower than in many commonly used abundantly expressed reference genes.

In addition to melting the curve information (in SYBR Green I detection) obtained from the LightCycler, the PCR products were run in a 1.5% agarose gel electrophoresis to ensure that a right size but not other products were amplified in the reaction. The genomic DNA was also used as a negative control to ensure that no amplification could result from a residual DNA remaining in the RNA preparation.

7. Statistical analyses

The associations of the tumor type, histological grade and clinical stage with the expression level were calculated with non-parametric Kruskal-Wallis and Mann-Whitney U-tests. The Spearman rank test was used to study correlation of the AR and coregulator expressions. The Grubb´s test was used to detect the outlier values.

RESULTS

1. SSCP method validation (Study I)

The non-radioactive SSCP method was validated using 5 different mutation controls: two cell lines (LNCaP and DU145), the previously detected Gly674Ala mutation (Koivisto et al 1997) and 2 additional mutation controls, which were generated by site-directed PCR mutagenesis. The SSCP method consistently identified all mutation controls. Since the tumor specimens usually contain non-tumor cells, a normal DNA is likely to be present in the sample DNA. To test the sensitivity of the non-radioactive SSCP gel run to detect mutations in heterogeneous samples, we prepared experimental samples by mixing varying amounts of a known mutant and the normal DNA together. According to the experiment, a sample containing at least 3% of the mutant DNA could be identified in the SSCP gel.

2. AR gene mutations in hormone-refractory prostate cancer (Study I)

30 locally recurrent, hormone-refractory and two metastatic prostate cancers were analyzed by the SSCP. 20 samples were analyzed for all eight exons of the AR gene, whereas 12 samples had previously been analyzed from the exon 2 to the exon 8, and thus only the exon 1 was analyzed in this study. In one of the 12 samples, a point mutation (Gly674Ala) had been previously detected (Koivisto et al.1997). However, this mutation did not change the transactivation properties of the receptor. A total of 48 fragments from 20 samples, which showed band shifts, were sequenced. However, all these sequences were determined to be wild-type.

The lengths of the CAG repeat and GGN repeat were also determined. The lengths were similar to what has been observed in normal populations (Sartor et al.1993). In addition, the repeat lengths were similar in tumors with and without the gene amplification. However, in one sample, two discrete peaks were observed, corresponding 18 and 20 CAG repeats. The normal DNA obtained from a formalin-fixed paraffin-embedded tissue block of the gall bladder from the same patient, showed only one peak of 20 repeats. Subsequently, the product corresponding 18 repeats was observed in a carefully microdissected tumor sample.

Finally, the somatic contraction of the CAG repeat in the tumor tissue was confirmed by sequencing.

3. Amplification and expression of AR in prostate cancer (Study II)

A total of 54 prostate tissue and 10 xenograft samples were analyzed for the AR gene copy number by the FISH. 4 of the 13 (31%) hormone refractory prostate cancers showed the amplification of the AR gene. As expected, the AR gene amplification was not detected in any of the 33 primary tumors or 8 benign prostate hyperplasias studied. A high-level amplification of the AR gene was also found in two xenografts (LuCaP 35 and 69). Additionally, two copies of an X centromere as well as the AR were found in four other xenografts (LuCaP 23.1, 23.8, 23.12, and 70).

The expression levels of the AR and PSA were measured from the same sample material using the real-time RT-PCR. The median expression of the AR was 6-fold higher in the hormone-refractory tumors than in the primary tumors or the BPH ($p<0.001$) The median value of the AR expression in the hormone-refractory tumors with the gene amplification was over 2 times higher than in the refractory tumors without the amplification. The expression level of the PSA was about the same in all tumor types. There was no association between the AR gene amplification and PSA expression.

Of the xenografts, the LuCaP 49, a small cell carcinoma of the prostate (Helpap et al. 1999), did not express the AR at all. There was also a clear tendency that the expression of the AR increased with the gene copy number. In addition, the highest expression of the AR was observed in the xenograft LuCaP 69 containing the highest level of the AR amplification. The expression of the PSA was detected in every xenograft, except the LuCaP 49.

4. Androgen receptor coregulators in prostate cancer (Study III)

Expression of AR coregulators

The expression levels were analyzed by using the real-time RT-PCR. On average, most of the AR coregulatory genes (SRC1, β-catenin, TIF2, PIAS1, PIASx, ARIP4, BRCA1, AIB1, AIB3, CBP, STAT1, NCoR1, AES, cyclin D1, p300, and ARA24) were equally expressed

in the BPH (n=9), untreated (n=30) and hormone-refractory (n=12) prostate carcinomas, even though the expression levels of the genes varied considerably between individual samples. Two exceptions were the PIAS1 and SRC1 whose expressions were, on average, 2-fold lower in the hormone-refractory than in the untreated prostate carcinomas (p=0.048, and 0.017, respectively). However, no differences in the levels of the expression were found between the primary tumors and BPH. In the hormone-refractory tumors, the expression of the SRC1 varied considerably: although the median expression of the SRC1 was lower in the hormone-refractory tumors than in the BPH or untreated tumors, the highest individual relative expression value was found in a hormone-refractory tumor.

Statistically significant associations between expression of some of the coregulators and histological grade or clinical stage of the primary untreated tumors were also found. The decreased expression of the p300 and AIB3 were associated with a high histological grade (p=0.034 and p=0.033, respectively). And, the increased expression of the ARIP4 was associated (p= 0.006) with an advanced stage of the disease (T3-4, or N+ or M+ against T1-2N0M0). The expression of any of the genes did not correlate with the expression of the AR.

In the cell lines and xenografts, a measurable expression level of every AR coregulatory gene was detected. Interestingly, there was no association in the expression levels of the AR coregulatory genes and hormone-dependence or AR positivity of the samples.

Gene copy number of SRC1

Finally, the expression data from the RT-PCR was combined with the data on the chromosomal alterations of the cell lines and xenografts previously obtained by the CGH (Nupponen et al.1998b Laitinen et al. 2002) in order to identify the putative amplifications of the coregulator genes. The xenograft LuCaP 70 containing a high-level gain at a chromosome 2p21-pter, where the SRC1 gene is located, expressed more SRC1 than the other cell lines or xenografts. The FISH analysis confirmed that the SRC1 is indeed amplified in the LuCaP 70. However, no SRC1 amplification was found in other xenografts or 9 tumor samples (3 hormone-refractory and 6 untreated) studied.

5. ERα and ERβ in prostate cancer (Study IV)

ERα

The mRNA expression of the ERα was detected in all prostate cancer cell lines (LNCaP, PC-3, DU145, 22Rv1) by the real-time RT-PCR. To evaluate the level of expression, the breast cancer cell lines with a known ERα expression status were also analyzed. The ERα-positive breast cancer cell lines showed levels of expression several hundred times higher than the ERα-negative cell lines ($p = 0.024$). The expression of the ERα in the prostate cancer cell lines was similar to that in the ERα-negative breast cancer cell lines.

There were no significant differences in the levels of expression of the ERα between the BPH (n=6) and untreated (n=30) or hormone-refractory (n=13) prostate carcinomas. However, in every sample studied, the expression of the ERα exceeded the smallest standard. Also 36 breast carcinomas were analyzed for the ERα expression using both the immunohistochemistry and real-time RT-PCR. The immunostaining positive breast carcinomas showed, on average, about 60 times higher expression of the ERα than the ERα-immunonegative breast tumors by the real-time RT-PCR ($p = 0.003$). Again, the prostate tumors expressed approximately equal levels of the ERα than the ERα-immunonegative breast carcinomas.

ERβ

In normal tissues, measurable levels of the ERβ expression were detected by the real-time RT-PCR. The kidneys, brain, mammary gland, heart, lungs, uterus, testes and prostate expressed ERbeta in approximately equal levels. The highest expression (about 2-fold higher than in the other tissues) was found in the trachea, whereas the skeletal muscle and liver showed the lowest levels of expression.

In the prostate cancer cell lines, there was a detectable and approximately equal ERβ expression. In the prostate tumors, the mean (± S.D.) relative ERβ expression value was 4.4

± 4.6 (median 2.9) in the hormone-refractory carcinomas, 10.1 ± 12.6 (median 5.0) in the untreated carcinomas, and 7.7 ± 9.8 (median 4.6) in the BPH. The differences were not statistically significant ($p = 0.419$). There was not, either, an association between the histological grade and ERβ expression in the androgen-dependent tumors.

DISCUSSION

1. Methodological considerations

1.1 SSCP

There are several methodological concerns related to the screening of mutations in tumor material. First of all, the representativeness of the samples must be ensured because the tumor tissue specimens also contain non-malignant cells. In our study, only the tumor blocks containing more than 60 percent of cancer cells were used. In addition, the fact that the genetic aberrations in these samples had previously been identified by the CGH (Nupponen et al. 1998b) indicates that the DNAs were mostly from malignant cells. Thus, it is unlikely that the mutant DNA would have been totally masked under the normal DNA contamination. Secondly, the method has to be chosen in a way that it can detect the sequence variations even if only a small proportion of the sample contains a mutation. The SSCP has generally been considered to be a highly sensitive mutation screening technique (Hongyo et al.1993, Evans et al.1996). The major disadvantage of the SSCP is that in order to have good sensitivity gel runs have to be performed in several different temperatures (Hongyo et al. 1993). We decided to choose a non-radioactive application of the SSCP because it is safer and faster than the conventional radioactive SSCP. In addition, the gel run apparatus used in the present study allowed adjustments to the gel run temperature. Thus, every sample was run in three different temperatures to maximize the mutation detection. To validate the method, we used five different mutation controls which the SSCP was able to detect consistently. Our concern was whether the SYBR Green staining is equally sensitive for the detection of an aberrant band in the gel than autoradiography. Therefore, we tested the minimum amount of a mutant DNA required in order for it to be detected in the gel. The experiment showed that as little as only 3% of the mutant DNA could be detected by using the SYBR Green staining of the gel. Thus, we were convinced that possible mutations in the coding region of the AR gene would be detected.

1.2 Quantitative real-time RT-PCR

The clinical tumor samples are typically small restricting the number of techniques for measuring the gene expression. For example, Northern blot analysis requires tens of

micrograms of the total RNA, which cannot be extracted from the usually small specimens. In addition, Northern analysis is, at its best, semi-quantitative. On the other hand, in situ methods, such as the immunohistochemistry and mRNA in situ hybridization, suffer from the lack of good reagents, such as antibodies, and they are usually even less quantitative than the filter hybridization techniques. The RT-PCR has been widely used for the expression analysis especially when only small tissue specimens are available. The RT-PCR is a relatively simple, fast, sensitive and specific method. However, the disadvantage of the conventional RT-PCR is the qualitative nature of the method.

During the recent years, the development of quantitative methods based on real-time PCR techniques has made the quantitative methods popular. In the real-time PCR, fluorescence dyes are utilized to detect the advancement of the PCR reaction in a real-time manner. The quantification of the template is based on the detection of the cycle in which the reaction enters the exponential phase and a fluorescence signal can be distinguished from the background, instead of measuring the end product as in the conventional PCR. Since the quantification takes place during the early PCR cycles, none of the reagents becomes a limiting factor. Furthermore, the gradual inactivation of the polymerase and accumulation of substances inhibiting the PCR reaction does not affect the amplification efficiency during the early cycles. There are two strategies to measure the fluorescence in the reaction: dyes, such as the SYBR Green I, binding to the double stranded DNA, and sequence-specific fluorochrome labeled probes. The advantage of the probe-based detection is the specificity of the assay since the target sequence must be recognized by two primers and two probes, whereas, the advantage of the SYBR Green based detection is that the expensive probes do not need to be designed and obtained (Bustin 2002).

In the end, the expression of 21 genes was measured by the real-time RT-PCR. Both the probe (for 5 genes) and SYBR Green (for 16 genes) strategies were used. The standard curves obtained by serial dilution of the total RNA from the LNCaP showed strong linearity ($\sim r^2=1$). To evaluate the reliability of the assays, the intra-assay CVs were calculated. The CV values varied from the low value of 5.7% to the unsatisfactory high of 28%. However, even in the worst performance, several-fold differences in the expression should have been detected.

Since there is no reliable method to quantitate the amount of the total RNA in the sample the expression of the test genes was normalized against the expression level of a house keeping gene, TBP. However, the use of a panel, with two or three housekeeping genes, has been recommended for normalizations (Bustin 2002). To investigate the effect of the housekeeping gene, the expression levels were, in study II, normalized also with a β-actin. The results were similar with both control genes. Due to the potential problems with β-actin retropseudogenes, the TBP was used for the normalization in further analyses.

2. Frequency of AR mutations in hormone-refractory prostate cancer

Most of the published studies have shown that mutations are rare in the untreated, primary prostate cancers (Culig et al. 2001). However, in many previous studies, the exon 1 of the AR gene, comprising almost half of the coding region, has been excluded from the analyses. Furthermore, only a small number of the hormone-refractory tumors has previously been screened for mutations.

In the present study, we aimed to evaluate the frequency of the mutations in the whole coding region of the AR gene in the hormone-refractory prostate carcinomas. Only one patient had received antiandrogen (bicalutamide), whereas the rest of the patients were treated with castration. Only two mutations were found, one point mutation and a contraction of the CAG repeat in the exon 1. The point mutation had previously been shown not to alter the transactivation properties of the receptor (Koivisto et al. 1997). The somatic contraction of the CAG repeat has also been previously reported in a prostate cancer sample (Schoenberg et al. 1994). Since the CAG repeat length affect the transactivational properties of the AR the contraction could theoretically have functional significance. Twelve of the samples in our study were also known to have the AR gene amplification. Both tumors that were found to be mutated also contained the AR amplification. However, the majority of the amplified ARs were wild-type.

Finding a low mutation frequency in the hormone-refractory tumors is contradictory to what has been found in patients treated with antiandrogens. It has been reported that up to 42% of the flutamide treated patients have a mutated AR (Balk 2002). Most of these mutations are located in the ligand-binding domain (LBD). The most frequent mutation found in these patients is identical to the mutation in the LNCaP cell line. The mutations have been shown

to lead to a paradoxical activation of the AR by flutamide (Taplin et al. 1995, 1999, Balk 2002). Recent studies have shown that also tumors from patients treated with another antiandrogen, bicalutamide, often contain AR mutations. Haapala and co-workers (2002) found a mutated AR in 36 % (4 out of 11) of the patients treated with combination of orchiectomy and bicalutamide. It has been suggested that some of these mutations could lead to the stimulation of the AR by bicalutamide, in the similar fashion as the AR containing the LNCaP-mutation is transactivated by flutamide (Hara et al. 2003). Thus, it seems likely that the frequency and the localization of the point mutations in the AR are influenced by the type of treatment.

No functional studies have been performed on all the mutations detected which makes it difficult to evaluate the significance of the mutations. Only a subset of all the mutant AR forms identified demonstrates an expanded ligand spectrum or increased activities in the absence of a ligand. Recently, Shi et al. (2002) investigated the functional status of 44 missense mutations previously identified in prostate cancers. 20 out of the 44 mutations (45%) studied had gains-of-function and five of them (11%) showed a promiscuous activity, transactivation by non-androgens. Combination of estradiol and progesterone at physiological concentrations weakly activated seven additional mutated receptors. Thus, it clearly seems to be that in some circumstances the AR mutations allow the tumors to adapt to low androgen conditions enabling them to progress despite the androgen withdrawal. Still, the low frequency of the AR mutations in the patients not treated with antiandrogens does not support mutations as the major mechanism of the AR re-activation in the androgen-independent prostate cancer (Marcelli et al. 2000, Culig et al. 2001).

3. Amplification and overexpression of AR in prostate cancer

One of the main debates concerning the fail of hormonal treatment has been whether the hormone-refractory prostate carcinomas are truly androgen-independent or are they maybe hypersensitive to the androgens (Feldman and Feldman 2001). In breast cancer, the loss of the estrogen receptor α expression is clearly associated with a hormone-independent phenotype of the tumor (Pettersson and Gustafsson 2001). Based on the observations on Shionogi mouse mammary tumor cells about the loss of the androgen sensitivity after the androgen withdrawal (Darbre and King 1987), the same mechanism was also incorrectly

proposed for prostate cancers. In addition, two extensively studied androgen-independent human prostate cancer cell lines, DU145 and PC-3, lack the expression of the AR.

It is now generally accepted that the expression of the AR does not reflect the response of the tumor cells to the androgen ablation in prostate cancer. Immunohistochemical studies have shown that all the stages of prostate cancers, including the metastatic lesions and hormone-refractory prostate carcinomas express the AR in vivo (Ruizeweld de Winter et al. 1994, Visakorpi et al.1995). In this study, we showed that the hormone-refractory tumors actually express significantly more AR than the untreated prostate carcinomas or the BPH by using the quantitative real-time RT-PCR. It has been reported that a small subset of the hormone-refractory prostate tumors loses the expression of the AR due to the hypermethylation of the gene promoter (Kinoshita et al. 2000). Since we did not find any such case, the frequency of the AR-negative hormone refractory tumors must be low. The finding of an increased expression of the AR in the hormone-refractory tumors is in good agreement with a previous small study suggesting the overexpression of the AR measured by semi-quantitative mRNA in situ hybridization (Koivisto et al. 1997). In addition, a more recently published study, also utilizing the quantitative RT-PCR, showed the increased expression of the AR in the hormone-refractory tumors confirming our findings (Latil et al. 2001).

We also studied the association of the AR gene amplification and overexpression. 4 out of the 13 hormone-refractory tumors showed the amplification of the AR gene by the FISH. These cases tended to express more AR than the tumors without the amplification, although the difference was not very significant statistically ($p=0.057$). In addition, the prostate cancer xenograft showing the highest level of the AR gene amplification expressed more AR than the other xenografts. These findings demonstrate that the AR gene amplification is one mechanism for the overexpression. Interestingly, the xenografts containing two copies of the AR gene showed the increased expression compared to the xenografts with only one copy of the AR suggesting that one additional copy of the AR may have biological significance. Gain of the chromosome X has been previously shown to occur in 20–65% of the recurrent carcinomas (Koivisto et al. 1997, Miyoshi et al. 2000, Edwards et al. 2001).

To study the functionality of the AR, we measured the expression of the PSA, which is a well characterized androgen regulated gene. All tumors expressed the PSA, which suggests

that the AR signaling pathway was active. However, it has now been shown that the expression of the PSA is also regulated by other factors suggesting that the expression of the PSA does not reflect only AR signaling (Oettgen et al. 2000).

It has previously (Koivisto et al. 1997) been shown, and confirmed in this study that the AR amplification is not found in tumors from the patients who have not received the androgen deprivation therapy. This suggests that the amplification of the AR gene is selected by the therapy. Similar types of gene amplifications related to a treatment relapse have now been reported also in other malignancies. For example, one mechanism for the failure of the treatment with the STI571 in leukemia seems to be the amplification of the BCR-ABL fusion gene encoding the target of the drug (Gorre et al. 2001). The amplified and overexpressed BCR-ABL gene reactivates the ABL signaling despite the ST571 therapy. Our findings suggest that a hormone-refractory tumor cell, instead of being androgen-dependent might actually be androgen hypersensitive. The increased expression of the AR during the androgen ablation was first described by Kokontis et al (1994) who showed that the androgen responsive prostate cancer cell line (LNCaP), while grown without an androgen, expressed more AR than the cells grown with the androgens. However, the phenomenon was transient. The findings of the present study clearly indicate that a persistent AR overexpression, one mechanism of which is the gene amplification, is common in the hormone-refractory tumors in vivo.

There is a lot of data showing that some of the prostate cancer cells do not die, instead, they remain in the growth arrest, at onset of the androgen deprivation (Isaacs and Coffey 1981, Craft et al.1999). Thus, even though the androgen ablation results in an increased apoptosis and decreased proliferation, at least a portion of the cancer cells present in the primary tumor stay in a non-proliferative state during the ablation, and eventually achieve the ability to grow androgen independently. Coincidentally, the expression of certain genes, including the AR and growth promoting pathways, such as the Akt pathway (Murillo et al. 2001), start to function. This suggests that the treatments used today do not prevent AR-mediated mechanisms of the progression that are developed by the recurrent cancers overexpressing the AR.

This phenomenon has recently been targeted by experimental approaches that down-regulate the AR. It has been shown that the inhibition of the AR expression is associated with a

retarded prostate cancer cell proliferation (Zegarra-Moro et al. 2002). The disruption of the AR by a hammerhead ribozyme or antisense oligonucleotides seems to inhibit the proliferation of both the androgen sensitive and hormone-refractory cell lines (Zegarra-Moro et al. 2002, Eder et al. 2000, 2002). The critical question is whether better drugs can be developed to suppress the AR activity also in the hormone-refractory prostate cancer. The currently available antiandrogens are clearly not good enough. In order to develop better drugs, good model systems are needed. Unfortunately, there are only a few prostate cancer models available. Most of the commercially available prostate cancer cell lines are androgen receptor negative, and thus not representing prostate cancer very well. In addition, the cell lines expressing the AR, such as the LNCaP and 22Rv1, do not express the wild-type receptor. Furthermore, certain xenografts, such as the CWR22 that has been extensively used in preclinical drug studies, express the mutated AR. Interestingly, in the present study the first prostate cancer model systems, xenografts LuCaP 35 and 69, are characterized with the amplification of the wild-type AR gene. Thus, these models can be utilized for examining the consequences of the AR amplification as well as hopefully for drug design against the androgen hypersensitive prostate cancers.

4. Changes in the AR coregulators in prostate cancer

The steroid receptors mainly activate their target gene transcription in response to the hormonal stimulus. Their transactivation properties are known to be modulated by several auxiliary proteins, which exert their actions through different mechanisms (Heinlen and Chang 2002). The involvement of the coregulators in the development and progression of cancer has been very unclear, although according to in vitro studies it seems likely that the overexpression of at least some of the coactivators may significantly modify the transactivation properties of the AR in the prostate cancer cells (Gregory et al. 2001).

We examined the expression of 16 coregulators, SRC1, β-catenin, TIF2, PIAS1, PIASx, ARIP4, BRCA1, AIB1, AIB3, CBP, STAT1, NCoR1, AES, cyclin D1, p300 and ARA24 in the BPH, primary untreated and locally-recurrent hormone-refractory prostate tumors. The coregulators have not previously been studied in this extent in prostate cancer specimens. In general, there were no major differences between the tumors in the expression of the coregulators studied. Our findings indicated that the overexpression of the coregulators is not common in prostate cancer. The findings are concordant with the studies by Nessler-Menadi et al (2000) and Li et al (2002) who did not detect the overexpression of the few coregulators they studied in the LNCaP and hormone-refractory LNCaP-abl cell lines and in 43 primary prostate cancers.

The only statistically significant alterations related to the progression of the disease we found were decreased expressions of the PIAS1 and SRC1 in the hormone-refractory prostate cancer. The finding that the SRC1 expression was decreased in the hormone-refractory prostate cancer is contradictory to the findings by Gregory et al. (2001) who showed an increased SRC1 protein expression in such tumors. Also Fujimoto et al. (2001) reported the increased expression of the SRC1 in high-grade or poorly therapy responsive cancers. Although the average SRC1 expression was lower in our samples, the highest individual expression value was nevertheless found in a hormone-refractory tumor indicating a high variability in the level of expression. This may explain, in part, the discrepancy between our results and the study by Gregory et al. (2001). In addition, the discrepancy could be due to the fact that Gregory et al. (2001) studied protein levels, whereas we analyzed the mRNA expression. Interestingly, the decreased expression of the SRC1 measured by the RT-PCR has been detected in tumors from the breast cancer patients who did not respond to tamoxifen which suggests that the SRC1 might influence the tamoxifen treatment response (Berns et al. 1998). Consistent with our findings, the median expression of the SRC1 was also detected to be lower in the primary tumors and cell lines than in the normal breast tissue (Berns et al. 1998).

In contrast to the findings in the clinical tumor samples, an amplification associated with the high expression of the SRC1 gene was detected in this study in the prostate cancer xenograft LuCaP 70 established from liver metastasis. The xenograft had previously been shown to contain gain at 2p23 by the CGH, and in the present study we demonstrated by the FISH analysis that the AR gene was amplified in the xenograft. Further studies are needed to

evaluate the frequency of the amplification. However, based on our analyses, it seems that the amplification is not common in prostate cancer.

Also the decreased expression of the PIAS1, a member of the PIAS (Protein Inhibitor of Activated STAT) protein family, was detected in the hormone-refractory compared to the untreated cancers in the present analyses. One previous study has shown an increased expression of the PIAS1 in 33% of the primary prostate cancers as compared to the normal prostates, determined by the mRNA in situ hybridization (Li et al. 2002). The reliability of the mRNA in the in situ hybridization in quantification can, however, be questioned. Interestingly, the PIAS1 has been reported to both activate and repress the AR dependent transcription (Gross et al. 2001, Nishida and Yasuda 2002), and thus the importance of the finding warrants additional studies.

We also analyzed the expression of the coregulators in 5 prostate cancer cell lines and 10 xenografts. The expression of the coregulators was found in all cell lines and xenografts. The xenografts and cell lines could be divided into two groups, androgen-sensitive and -independent ones. Of the models, the xenograft LuCaP 49 and cell line NCI-H660 represent small cell carcinomas, which do not express the AR, and are therefore considered truly androgen-independent. In addition, the cell lines PC-3 and DU145 are AR negative. The cell line 22Rv1 expresses a low amount of the AR containing a double mutation. It also behaves in vitro as an androgen-independent cell line. The rest of the xenografts and the cell line LNCaP, although originating from patients treated with hormonal therapy (except LuCaP 58), are androgen-sensitive as demonstrated, e.g. by the fact that the growth of the xenografts in mice is inhibited by castration (Kim et al. 2002).

The finding that the AR negative models expressed the coregulators may be explained by the fact that only a few of the coregulators are truly AR specific, most of them possess interactions with other steroid hormone receptors as well. In addition, many, if not all of them, have functions discrete from the steroid hormone receptors. For some of the suggested AR coregulators studied in the present research, such as the BRCA1, Cyclin D1 or β-catenin, the AR-related functions probably represent minor functions of the proteins. For example, the tumor suppressor functions of the BRCA1 seem to be distinct from its relation to the AR action. It is also known that the cyclin D1 function in the transactivational repression of the AR is distinct from its action in the cell cycle (Knudsen et al. 1999).

In conclusion, the suggested role for the AR coregulators in prostate tumorigenesis has been obtained by using in vitro models (McKenna et al. 1999). In such reporter assays the cellular environment of endogenous coregulators might, for instance, influence the ability of an exogenous coactivator to enhance the transcription. Therefore, it is important to study these coregulators in the cancer tissue in vivo. The results of the present study mainly suggest that the alterations in the expression of the AR coregulators are not likely to be major mechanisms underlying the progression of prostate cancer. However, the finding that the SRC1 gene was amplified in one case indicates that genetic alterations do occur in the AR coregulator genes. Thus, it will be important to study whether genetic aberrations, including mutations, of the other coregulators are found in prostate cancer.

5. Expression of estrogen receptors in prostate cancer

Studies on the knockout mice have suggested that the estrogen receptors could be important in the tumorigenesis of prostate cancer. For example, it has been shown that the developmental estrogenization is mediated by the ERα. On the other hand, the ERβ knockout mice (BERKO) have abnormalities and even pre-malignant features in the prostate epithelium. This is coincident with the increased proliferation and elevated AR levels, which suggests (Weihua et al. 2001) that the ERβ is a negative regulator of growth and the AR expression in a rodent prostate. However, because of the low level of expression it has been difficult to analyze the expression pattern of the ERβ in a normal or malignant human prostate and in prostate cancer cell lines. Previous immunohistochemical or immunoblot studies have shown very inconsistent results (Bonkhof et al. 1999, Royuela et al. 2001, Horvath et al. 2001). It is likely that in some immunohistochemical studies the antibodies for the ERβ have not worked properly. For instance, Bonkhoff et al (1999) reported the lack of the ERβ expression in prostate cancer using immunohistochemistry, whereas others have reported the increased expression (Ito et al. 2001, Royuela et al. 2001). In a recent study, Fixemer et al. (2003) studied 132 samples by immunohistochemistry from patients with prostate cancer. They reported a variable but significant level of the ERβ expression in all informative cases (60 primary tumors, 7 lymph node and 5 bone metastases) studied, which indicates that the untreated primary and metastatic prostate cancers retain the ERβ

expression. No correlation between the ERβ status and histological grade (Gleason score) or TNM distribution was found. However, significant correlations ($P<0.001$) were found between the decreased expression in a high-grade PIN (N=40) compared to a normal epithelium, as well as between the untreated tumors and hormone-refractory TURP (n= 40) specimens. In addition, Horvath et al (2001) have reported a significantly decreased survival rate of the patients who retained the ERβ expression. However, this has not yet been confirmed by other studies.

First, we tried several antibodies for the immunohistochemistry of the ERβ. However, none of them gave reliable staining. In addition, even at its best, immunostaining is a semi-quantitative method. Thus, we decided to use the real-time RT-PCR to measure the expression levels of the ERβ. We found a detectable, but low-level ERβ expression in all the prostate tumor samples studied. The expression of the ERβ was approximately equal in the BPH and untreated tumors. On average, the hormone-refractory tumors expressed half of the ERβ than the untreated tumors. Despite of the tendency, the difference was not statistically significant.

One other study on the ERβ expression, measured by the quantitative real-time RT-PCR, has been published (Latil et al. 2001). In that research, a highly variable expression of the ERβ was found, especially in the hormone-refractory prostate carcinomas. However, the expression was found to be significantly lower in the hormone-refractory than untreated tumors. The discrepancy to our study could, at least partly, be explained by a high sample to sample variation in the detected expression levels and by small sample numbers.

We measured the level of the ERα in the prostate samples. To evaluate the ERα levels in relation to ER immunopositive and -negative breast carcinoma cell lines and tumors, the immunohistochemistry was used to examine the ERα status in 9 breast cancer cell lines and 36 tissue samples. The expression data showed that the ERα is present in prostate cancer in the levels comparable to the ERα negative breast cancers. The very low level of the ERα was present approximately equally in different prostate samples studied. Majority of the published studies have shown that the ERα is expressed in stromal and not epithelial cells in the prostate gland (Kruithof-Dekker et al. 1996, Lau et al. 1998, Prins et al. 2001). Our unpublished immunostaining data also confirms the stromal expression of the ERα. The

finding that the prostate cancer cell lines originating from the epithelial cells express some levels of the ERα suggests that the gene might be expressed in very low levels also in the prostate epithelial cells.

Finally, it has recently been shown that anti-estrogen toremifene prevents the development of prostate carcinoma in the mouse TRAMP prostate cancer model (Raghow et al. 2002). It was also demonstrated that the effect was not likely to be mediated through the AR, because the blocking of testosterone action by the toremifene in the tissues was not found. The main target of the toremifene is the ER even though it may also have an influence to unknown targets. However, it should be kept in mind that the expression levels of the estrogen receptors in the prostate seem to be quite different in humans and rodents, for example, the ERβ, which is prominently expressed in the rat prostate, is expressed less in the human prostate as we have also shown. Our data clearly indicates that the expression alterations of the estrogen receptors are unlikely to be involved in the progression of prostate cancer. In addition, the estrogens are probably not directly functionally significant in prostate cancer.

CONCLUSIONS

The main findings and conclusions of the study were:

1. The AR gene mutations were rare in the hormone-refractory prostate carcinomas, whereas the AR gene amplification was found in one-third of the hormone-refractory tumors. Thus, mutations are not likely to be the major mechanism underlying the androgen withdrawal therapy failure. The amplification of the wild-type AR gene seems to be the most prevalent form of the genetic alterations affecting the AR gene.

2. The AR is commonly overexpressed in the hormone-refractory prostate cancer. One mechanism leading to the overexpression of the AR is the amplification of the gene. The expression of the PSA indicates the functionality of the AR signaling pathway in the hormone-refractory tumors. Two xenograft models, derived from the hormone-refractory prostate cancer metastases, were found to contain a high-level amplification of the AR gene, which indicates that these models may be useful in studying, e.g. the mechanistic consequences of the gene amplification.

3. The overexpression of any of the 16 analyzed AR coregulatory genes was not found in the clinical prostate carcinomas. The result suggests that the overexpression of these coregulators is not likely to have a major role in the progression of prostate cancer. However, the decreased expression of the PIAS1 and SRC1 detected in the hormone-refractory compared to untreated cancers suggests that these genes may contribute to the progression of prostate cancer. The expression of the AR coregulators even in the AR negative samples, such as small cell carcinomas of the prostate, suggests that the coregulatory genes are likely to have other functions which are distinct from the AR signaling pathway.

4. Of the coregulator genes, the SRC1 was found to be amplified and overexpressed in one of the xenografts. Although the amplification seems to be rare, it proposes that genetic the alterations of the SRC1 may have significance in a small subset of the hormone-refractory tumors.

5. Both estrogen receptors were expressed at the detectable level in all stages of prostate cancer. The expression level of the ERα in the BPH and all prostate cancers was approximately the same than in the estrogen receptor negative breast cancers, which strongly supports the view that the ERα is not likely to have a role in the progression of prostate cancer. In addition, the ERβ was expressed in all human prostate cancers and no major changes in the level of expression were found. Thus, it seems unlikely that the alterations in the expression of the ERβ, at least at the mRNA level, are involved in the prostate tumorigenesis.

Altogether, the observations support the idea that the functional AR signaling pathway is the progression mechanism of prostate cancer during the hormonal treatment. This is consistent with the view that the hormone-refractory prostate cancers might even be androgen hypersensitive instead of androgen-independent. The AR gene is targeted by genetic alterations, such as amplification and mutations, in the hormone-refractory prostate tumors suggesting that the AR should be considered as a target for novel therapeutic approaches.

ACKNOWLEDGEMENTS

This study was carried out in the Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital during the years 1997-2003.

I would like to express sincere thanks to Professor Tapio Visakorpi, M.D., Ph.D., the supervisor of this work, for giving me the opportunity to join his research group. Without his extensive knowledge on cancer research and his skillful guidance, this thesis would have been impossible for me to carry out. I also want to express my deepest gratitude to Mika Wallén, Ph.D, for all the guidance during this project.

Professor Jorma Isola, M.D., Ph.D. is thanked for his advice and collaboration.
Professor Olli Silvennoinen, M.D., Ph.D., the Head of Institute of Medical Technology, University of Tampere, is thanked for providing the research facilities. I also want to thank Professor Kalle Saksela, M.D., Ph.D., the Head of Tampere Graduate School in Biomedicine and Biotechnology, University of Tampere, for offering the graduate school position.
I wish to thank Professor Matti Poutanen, Ph.D., and Docent Ari Ristimäki, M.D., Ph.D., for carefully reviewing the manuscript.
Ms. Sini Kuukka, M.A., is acknowledged for revising the language of the thesis.

I am grateful to all my co-authors and collaborators for their important contribution.

I also wish to express my sincere thanks to friends and colleagues at the Laboratory of Cancer Genetics for all the help and guidance during the years. The past and present members of Tapsalab, thank you! Especially, I wish to thank Kati Porkka, M.Sc., for her friendship as well as for her practical advice. In addition, Päivikki Kauraniemi, M.Sc., Outi Saramäki, M.Sc., and Kimmo Savinainen, M.Sc., are thanked for both friendship and help. Merja Helenius, Ph.D., is warmly thanked for the guidance in cell culture.

I greatly appreciate the skillful technical assistance of Mrs. Mariitta Vakkuri and Ms. Maarit Ohranen.

I also express my gratitude to my parents Sirkka and Heikki for the support which has covered both good times and hard times in life.

The publishers of the original communications are thanked for their permission to reproduce the papers in this thesis.

This study was financially supported by the Pirkanmaa Cancer Society, the Pirkanmaa Cultural Foundation, the Emil Aaltonen Foundation, the Cancer Society of Finland, the Reino Lahtikari Foundation and the Scientific Foundation of the city of Tampere.

Tampere August 2003

Marika Linja

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Original communications