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Androgen Receptor: A Key Molecule in the Progression of Prostate Cancer to Hormone Independence

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Abstract Despite earlier detection and recent advances in surgery and radiation, prostate cancer is second only to lung cancer in male cancer deaths in the United States. Hormone therapy in the form of medical or surgical castration remains the mainstay of systemic treatment in prostate cancer. Over the last 15 years with the clinical use of prostate specific antigen (PSA), there has been a shift to using hormone therapy earlier in the disease course and for longer duration. Despite initial favorable response to hormone therapy, over a period of time these tumors will develop androgen-independence that results in death. The androgen receptor (AR) is central to the initiation and growth of prostate cancer and to its response to hormone therapy. Analyses have shown that AR continues to be expressed in androgen-independent tumors and AR signaling remains intact as demonstrated by the expression of the AR regulated gene, *PSA*. Androgen-independent prostate cancers have demonstrated a variety of AR alterations that are either not found in hormone naïve tumors or found at lower frequency. These changes include AR amplification, AR point mutation, and changes in expression of AR co-regulatory proteins. These AR changes result in a ''super AR'' that can respond to lower concentrations of androgens or to a wider variety of agonistic ligands. There is also mounting evidence that AR can be activated in a ligand independent fashion by compounds such as growth factors or cytokines working independently or in combination. These growth factors working through receptor tyrosine kinase pathways may promote AR activation and growth in low androgen environments. The clinical significance of these AR alterations in the development and progression of androgen-independent prostate cancer remains to be determined. Understanding the changes in AR signaling in the evolution of androgen-independent prostate cancer will be key to the development of more effective hormone therapy. J. Cell. Biochem. 91: 483–490, 2004.

Key words: androgen receptor; prostate cancer; androgen-independent; hormone refractory; steroid hormone receptor

Prostate cancer continues to be the most common cancer diagnosis and the second leading cause of cancer deaths in American men. Despite earlier diagnosis and refinements in surgery and radiation, it is estimated that 28,900 men will die from prostate cancer in the United States in 2003 [Jemal et al., 2002]. Prostate cancers that have spread beyond the gland are typically treated with hormone therapy aimed at inducing castrate levels of testosterone or blocking testosterone signaling at the androgen receptor (AR). Although surgical castration is effective, the majority of men choose medical castration with a leutenizing hormone releasing hormone (LHRH) agonist that is often combined with an oral anti-androgen. Castration induces apoptosis in the majority of prostate cancer cells, which translates clinically to improvement in cancer related symptoms and lowering of AR regulated genes including prostate specific antigen (*PSA*). Clinically prostate cancer progression has been defined in terms of the patient's response to or failure from hormone therapy.

Hormone therapy remains the mainstay of systemic treatment for prostate cancer and in fact several recent reports describe benefits to early and more prolonged androgen deprivation [Bola et al., 1997; Messing et al., 1999]. The majority of prostate cancers (85%) will have an initial favorable response to hormone therapy, however, over time molecular and cellular changes occur that allow prostate cancer cells

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to grow despite a physiologically low serum testosterone level. At the time of progression, some prostate cancers will respond to sequential hormone therapies such as the addition of anti-androgens, estrogens, or suppression of adrenal hormones [Small and Vogelzang, 1997]. Eventually, unfortunately, these prostate cancers will stop responding to all currently prescribed hormone therapy; at this stage the cancers are described as androgen-independent or hormone refractory. Chemotherapy will have favorable palliative response in 50–75% of men with androgen-independent prostate cancer but most will die from their disease within 1–2 years [Savarese et al., 2001]. We believe that the AR remains an important mediator of growth in androgen-independent prostate cancer and that an improved understanding of the role of the AR will lead to the development of more effective targeted hormone therapy for these patients.

AR GENE STRUCTURE, SIGNALING, AND PROTEIN EXPRESSION IN PROSTATE CANCER

The AR is central to the initiation and growth of prostate cancer and to its response to hormone therapy. The AR is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors [Evans, 1988]. The AR is located on the X chromosome, Xq11-12, and is composed of 8 exons [Brinkmann et al., 1989]. Similar to other steroid receptor proteins, the full length AR protein contains four functional domains: an amino-terminus regulatory domain, a DNA-binding domain, a hinge region, and a ligand-binding domain. The N-terminus, encoded by exon 1, comprises almost half of the AR and contains the important transcriptional activation function (TAF-1) site and homopolymeric polyglutamine (CAG), and polyglycine (GCC) repeats [Faber et al., 1989]. Exons 2 and 3 encode the DNA-binding domain, which contains two zinc fingers essential for androgen-response-element (ARE) recognition. The hinge region is encoded by the proximal part of exon 4 and contains a nuclear localization signal that directs the AR into the nucleus. The ligand-binding domain, encoded by part of exon 4 and exons 5–8, contains a second transcription activation function (TAF-2) site as well as ligand binding activity. Unligated ARs are bound to heat shock proteins (HSPs) 90, 70, and 56 that stabilize their tertiary structure in a conformation that allows androgen binding [Veldscholte et al., 1992]. Androgen binding to ARs leads to dissociation from HSPs, dimerization, phosphorylation, interaction with AREs, recruitment of co-regulators, formation of a pre-initiation complex, and ultimately transcriptional activation of androgen-regulated genes [Prins, 2000]. Co-regulatory proteins form a bridge between AR, the pre-initiation complex, and RNA polmerase; possible alterations in AR co-regulatory proteins favoring growth in prostate cancer are emerging and will be discussed below.

Intact AR signaling is felt to be necessary for the development of prostate cancer. Inherited disease states such as several of the androgen resistance syndromes and spinal and bulbar muscular atrophy which have no or reduced AR signaling result in underdeveloped prostates that do not become cancerous [Caskey et al., 1992; Griffin, 1992]. Results from immunohistochemistry studies reveal that AR is present in primary and metastatic prostate cancer regardless of stage and grade, as well as in androgen-independent cancers [Ruizeveld de winter et al., 1991]. AR immunoreactivity is heterogeneous within tumors and unlike the expression of estrogen receptor (ER) and progesterone receptor (PR) in breast cancer, AR expression bears no apparent correlation with prognosis or with duration of response to hormone therapy [de Vere White et al., 1997]. Some studies, however, have noted that an increase in AR heterogeneity or decrease in AR-positivity particularly in periepithelial stroma is associated with higher grade and poorer prognosis [Henshall et al., 2001]. At this point AR immunohistochemistry patterns are not used to make treatment decisions but with more basic knowledge and the development of more effective anti-androgen therapy AR expression patterns may become important in therapy decisions.

Exon A of the AR contains two regions of repetitive DNA sequence of CAG and GCC triplet repeats; the genomic number of repeats has been implicated in the development of prostate cancer and the biologic aggressiveness of certain cancers [Irvine et al., 1995; Giovannucci et al., 1997]. The CAG repeat length normally ranges between 14 and 35 repeats in individuals and has ethnic distributions [Caskey et al., 1992]. In the laboratory short CAG repeat

lengths correlated with a more transcriptional active receptor that may reflect increased stability [Chamberlain et al., 1994; Choong et al., 1996]. It has been observed that the incidence of prostate cancer in African–Americans, Caucasians, and Asians correlates with the average CAG repeat lengths for these populations [Irvine et al., 1995]. African–Americans who have on average shorter CAG repeats have the highest incidence and Asians who have the longest average repeats have a lower incidence. A possible mechanism for prostate cancer to gain growth advantage in a low testosterone environment would be somatic shortening of tumor repeat lengths. At this point the studies that have compared genomic and tumor repeat lengths have demonstrated only two somatic alterations of repeat lengths in androgen-independent prostate cancer [Schoenberg et al., 1994; Taplin et al., 2003]. Thus, triplet repeat contraction likely not important in the development of androgen resistance.

ANDROGEN RECEPTOR AMPLIFICATION

A variety of AR molecular alterations which result in a hyperactive AR or AR with broadened ligand specificity have been investigated in the laboratory and described from human prostate tumor specimens. Laboratory AR constructs containing deletions in the hormone-binding domain are constitutively active [Jenster et al., 1991], however, these types of AR deletion mutations have not been found in human prostate cancers and will not be discussed further. Amplification of the *AR* gene or increased protein expression by other means in prostate tumors is a potential mechanism to utilize low levels of androgens, which are present in castrated patients. *AR* gene amplification has been reported in 25–30% of androgen-independent prostate cancer [Visakorpi et al., 1995; Koivisto et al., 1997; Miyoshi et al., 2000; Linja et al., 2001]. Of note the AR amplification was not found in any untreated prostate cancer samples suggesting that AR amplification is involved in the development of androgen-independence. AR amplification was associated with increased mRNA expression and clinical correlation demonstrated that patients harboring AR amplified tumors had longer response duration to initial hormone therapy and a longer median survival after recurrence [Koivisto et al., 1997]. Additionally patients with AR amplification had an increased likelihood of responding to second-line hormone therapies than patients without amplification [Koivisto et al., 1997; Palmberg et al., 2000]. AR amplification highlights the strong selective pressure for continued AR signaling as tumors evolve over the course of therapy and provides impetus for development of more effective AR signal blockade.

AR POINT MUTATIONS

The first AR mutation in prostate cancer was described in the well-studied LNCaP cell line [Veldscholte et al., 1990]. LNCaP cells were established from the lymph node of patient who had had long-term hormone treatment with estrogens, as anti-androgens were not commonly prescribed at that time [Horoszewicz et al., 1983]. The AR from LNCaP has a point mutation at codon 877 of the hormone-binding domain [Veldscholte et al., 1990]. Since discovery of the LNCaP mutation, AR sequence analysis has been carried out on numerous clinical samples. The true incidence of AR point mutations in prostate cancer is not known but the literature suggests that they can be found in 20–40%. Factors that make it difficult to determine the in vivo frequency of AR mutations include patient selection, tumor heterogeneity, tissue source (prostate gland vs. metastases), method of tissue preservation, and molecular methods including exon A analysis. A comprehensive list of all reported AR mutations in prostate cancer and androgen insensitivity syndromes can be found in the AR mutation database [Gottlieb et al., 1998]. These data suggest that selection of AR mutations confers growth advantage at least for a subset of prostate cancers during the development of androgen-independence.

AR point mutations have been reported from a subset of hormone naïve prostate cancers and more frequently from androgen-independent tumors [Suzuki et al., 1993; Taplin et al., 1995, 1999; Tilley et al., 1996; Marcelli et al., 2000]. In a comprehensive analysis Marcelli et al. [2000] reported AR point mutations in 8 of 38 (21%) regional lymph nodes from hormone naïve patients; AR mutations were not found in 99 prostatectomy gland samples from men not on hormone therapy. Tilley et al. [1996] reported AR mutations in 11 of 25 (44%) of prostate gland

tumor samples from hormone naïve patients; interestingly 40% of these men progressed rapidly to androgen-independent disease. Evaluating AR sequence from androgen-independent tumors obtained from metatastic bone marrow samples we have reported AR mutants in 15–50% of the tumors [Taplin et al., 1995, 1999, 2003]. The higher frequency of mutations correlated with more advanced disease patients who had had long durations of exposure to anti-androgen therapy. At this time, it can be concluded that AR point mutations are found in a subset of local or locally advanced hormone naïve patients and these tumors may be biologically more aggressive than the average. Additionally, AR mutations are found in advanced androgen-independent cancers particularly if patients have been treated with anti-androgens; it has not been demonstrated that androgen-independent prostate cancer patients harboring tumor with AR mutation have a worse or more favorable prognosis than patients without mutation [Taplin et al., 2003].

Several AR mutations have been isolated with increased frequency from prostate tumors and have been evaluated functionally in the laboratory. These AR mutants include Thr877Ser, Thr877Ala, His874Tyr, Val715Met, Leu701His + Thr877Ala, Tyr741Cys [Culig et al., 1994; Taplin et al., 1995, 1999, 2003; Krishnan et al., 2002; Hara et al., 2003]. Other AR mutations from prostate cancers have been single isolates whose functional significance is uncertain at this time. The functional properties of AR variants have been studied in vitro by transfecting each mutant AR into an AR-negative cell line, in conjunction with an androgen-regulated luciferase reporter gene and measuring the agonist activity of various compounds including anti-androgens. The Thr877Ala mutation found in LNCaP and many clinical tumor samples was activated by progestagens, estradiol, cyproterone acetate, and the anti-androgens, anandron, and hydroxyflutamide [Veldscholte et al., 1990]. AR mutations Val715Met, Thr877Ser, His874Tyr were activated by progesterone; mutations Thr877Ser and His874Tyr were stimulated by estradiol. Adrenal androgens and hydrocortisone were agonists to Val715Met and Leu701-His + Thr877Ala. In addition to activation of Thr877Ala, hydroxyflutamide was able to stimulate Val175Met, Thr877Ser, and His874Tyr. In the laboratory after exposure to bicalutamide, an LNCaP subline bearing either a Tyr741Cys or Tyr741Leu mutation was activated by bicalutamide [Hara et al., 2003]. The Tyr741Cys mutation was recently sequenced from a bone marrow metastasis of a patient who had been treated with bicalutamide [Taplin et al., 2003]. These data imply that androgen signaling remains intact and that selection of cells harboring mutated AR which can act as a "super receptor" and respond to a wider variety of agonists at lower concentration occurs during the development of androgen-independent prostate cancer.

The three dimensional crystallographic structures of the wild-type AR hormone binding-domain and the Thr877Ala mutant have been characterized [Matias et al., 2000; Sack et al., 2001]. This information has further increased our understanding of mechanism by which AR point mutations alter receptor function. The majority of AR point mutations in prostate cancer have clustered in three areas of the hormone-binding domain, codons 670–678, 701–730, and 874–910 [Buchanan et al., 2001; Gelmann, 2002]. Mutations in these areas flank the ligand-binding pocket and alter this pocket relative to the wild-type AR to allow binding of ligands other than testosterone or dihydrotestosterone [Sack et al., 2001]. The mutations that involve codons 874–910 are adjacent to the AF-2 domain that is involved in binding of the p160 coactivatior molecules and also binds with the AR N-terminal domain. The locations of the AR point mutations in prostate cancer which are gain of function mutations differs from the locations of the AR inactivating mutations found in androgen insensitivity syndromes [Gelmann, 2002]. When a mutant AR binds to an agonist such as hydroxyflutamide the conformation of the ligated complex changes relative to wild-type such that the AR helix 12 (in the ligand-binding domain) moves in proximity to helices 3–5 which generates a coactivator binding site. Thus prostate cancer AR mutations have been selected for growth advantage by not only loosening the ligand-binding pocket and allowing a wide variety of molecules to function as agoinsts, but also changing the interaction between various components of the AR transcriptional complex in favor of growth.

The clinical implications of AR mutations are poorly understood because of the difficulty of obtaining samples of metastatic prostate cancer

for large-scale correlative analysis. In an advanced patient population bone marrow biopsy is a good source of harvesting metastatic prostate cancer for AR sequence analysis [Taplin et al., 1995]. We reported AR sequence from 33 bone marrow biopsies of men with androgen-independent prostate cancer, some of these subjects were enrolled on a clinical trial of high dose bicalutamide [Joyce et al., 1998; Taplin et al., 1999]. AR mutations were found in the ligand-binding domain in 5 of 16 patients who had been treated with testicular castration plus flutamide compared to 1 AR mutation in 17 subjects not exposed to flutamide. In the laboratory these AR mutations were stimulated by flutamide. Patients who had been initially treated with hormone therapy that included flutamide were more likely to respond to second-line bicalutamide therapy. The trial suggested that the presence of AR mutation correlated with response to bicalutamide but this conclusion could not be statistically proven. A similar clinical result (no AR sequence analysis preformed) was obtained in a Memorial Sloan Kettering trial of bicalutamide (200 mg) in androgen-independent prostate cancer in which response rates also were correlated with prior flutamide therapy [Scher et al., 1997]. No correlation of patient response to second-line bicalutamide with prior response to anti-androgen withdrawal could be made and survival data was not available from either study.

In a second trial bone marrow biopsies were obtained from 184 subjects enrolled on a cooperative group trial of anti-androgen withdrawal and simultaneous versus sequential adrenal blockade [Taplin et al., 2003]. Forty-eight samples had adequate prostate tumor for AR analysis and mutations were found in 10%. This was the first analysis of patients with long-term bicalutamide exposure and mutations were found in equal numbers in subjects previously treated with bicalutamide or flutamide. The low frequency of mutations may have resulted from a shorter duration of anti-androgen exposure that had become the norm over the course of the trial. Because of the small number of patients with AR mutation, no correlation of anti-androgen withdrawal response and AR mutations could be made. There was no difference in survival of subjects with and without AR mutation.

The molecular mechanism of the anti-androgen withdrawal response seems unlikely to be AR mutation. An alternative explanation could be an abnormality in AR interaction with an AR cofactor [Rahman et al., 2003]. Laboratory studies have suggested possible mechanisms of AR co-regulatory factor involvement in the anti-androgen withdrawal response. Rahman et al. [2003] demonstrated that the AR coactivator ARA70 may contribute to the agonist activity of anti-androgens and that a dominant-negative ARA70 when transfected into LNCaP cells resulted in reduced agonist activity of the anti-androgens. Confirmation of the role of AR co-regulatory proteins with either wild-type or mutant AR awaits further investigation. Finally, as hydroxyflutamide has well documented weak agonist activity even on the wild-type AR, it is possible that multiple mechanisms can enhance this activity.

AR CO-REGULATORY MOLECULES IN PROSTATE CANCER PROGRESSION

The details of the complex positive and negative regulatory elements involved in AR mediated transcription are staring to unfold [Heinlein and Chang, 2002]. Over the last several years as our understanding of the basic mechanisms involved in AR signal transduction has increased, a search for alterations in AR co-regulatory molecules in prostate cancer has begun. Gregory et al. [2001] evaluated the expression of AR and three nuclear receptor coactivators,transcriptional intermediary factor 2 (TIF2), steroid receptor coactivator 1 (SCR1), and nuclear receptor coactivator amplified in breast cancer 1 (AIB1) in specimens containing benign prostatic hyperplasia (BPH), androgen-dependent prostate cancer ($n = 8$), and androgen-independent prostate cancer ($n = 8$). They demonstrated that levels of TIF2 and SRC1 increased with increases in AR expression in androgen-independent prostate cancer. In addition, in vitro co-transfection assays exploring overexpression of TIF2 in the presence of wild-type or mutant AR demonstrated that androstenedione, estradiol, and progesterone at physiologic concentrations became potent activators of AR in the presence of TIF2 [Gregory et al., 2001]. Thus overexpression of AR coactivators may enhance AR response to low levels of androgen or broaden ligand specificity similar to mutation of AR and allow transcription activation and growth by AR in the setting of castration.

Halkidou et al. [2003] examined the role of another AR coactivatior, Tat interactive protein 60 kDa (Tip60), in the development of androgen-independent prostate cancer. Tip60 expression was evaluated in 10 cases of BPH, 43 clinical cases of untreated prostate cancer, and 15 hormone refractory cancer cases. The majority (87%) of the hormone refractory cancer specimens demonstrated nuclear accumulation of Tip60 in contrast to a more diffuse distribution in BPH and primary prostate cancer. Additionally Tip60 expression and nuclear accumulation was up-regulated upon androgen withdrawal in the CWR22 xenograft and the LNCaP cell line. Confirmation of the role of AR coactivators in the progression of prostate cancer to androgen-independence awaits larger scale and more comprehensive functional analysis. At this point corepressor mutation or down-regulation has not been reported from clinical prostate cancer specimens.

LIGAND-INDEPENDENT ACTIVATION OF AR IN PROSTATE CANCER PROGRESSION

Laboratory data reveal that AR in prostate cancer cell lines can be activated in the absence of androgens or at decreased androgen levels by several factors or pathways acting in an independent or combined manor [Grossmann et al., 2001; Navarro et al., 2002]. The clinical significance of this type of AR activation in androgen-independent prostate cancer patients remains to be determined. Several examples of so called ligand independent AR activation are described below.

The AR is a phosphoprotein although how it's transcriptional activity correlates with its phosphorylation status is unclear [Wang et al., 1999]. Forskolin has been shown to activate AR through a protein kinase A (PKA) signaling pathway by increasing levels of cyclic adenosine monophosphate (cAMP) [Grossmann et al., 2001]. In the laboratory the increased levels of cAMP allow unligated AR to bind to the response elements regulating target gene expression [Navarro et al., 2002]. Peptide growth factors such as insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) can also activate AR. These growth factors are ligands for receptor tyrosine kinases and activation of one or several of the tyrosine kinase pathways may promote AR activation and growth in low androgen environments. As an example, IGF-1 was able to activate AR signaling in prostate cancer cell lines and the anti-androgen bicalutamide was able to inhibit activation suggesting direct interaction between IGF-1 and AR [Culig et al., 1994].

Her2/Neu (erbB2) is another in vitro example of how receptor tyrosine kinase activation of AR may be involved in ligand-independent activation of AR [Grossmann et al., 2001]. A subset of clinical prostate cancer specimens has demonstrated Her2/Neu overexpression but the clinical activity (20% PSA response) of an antibody treatment directed against Her2/Neu (trastuzumab) as a single agent has been disappointing [Morris et al., 2002]. Clinical trials are now focusing on adding trastuzumab to conventional chemotherapy, an approach that has been successful in breast cancer [Small et al., 2001]. The cytokine, IL-6, has also been shown to increase the expression of AR regulated genes in the absence of androgens [Ueda et al., 2002b]. IL-6 activation of AR has been reported to be via p300, erbB2 or STAT3, or mitogen activated kinsae (MAPK) [Debes et al., 2002; Ueda et al., 2002a]. Clinically elevated plasma levels of IL-6 and its soluble receptor have been associated with prostate cancer progression and metastasis [Shariat et al., 2001]. Other proteins that have been demonstrated to interact with AR include beta-catenin, caveolin-1, cyclin E, tumor susceptibility gene *101*, cyclin D1, Rb, p53, c-jun, and the Smad3 pathway [Grossmann et al., 2001]. Further studies are necessary to determine the physiologic significance of these interactions.

In summary, patients with metastatic prostate cancer will experience a predictable progression of their disease from an androgen responsive state to relentless androgen-independent tumor. The AR is central to growth signaling in prostate cancer cells and compiled data suggest that the AR remains active in progressive androgen-independent prostate cancer through a variety of mechanisms aimed at increasing the growth response to lower levels and a wider variety of compounds. In the castrate environment, prostate cancer cells develop growth advantage by amplifying or mutating AR, altering AR co-regulatory molecules and developing ligand-independent AR activation pathways. Our present mandate is to apply our knowledge of AR signaling in prostate cancer progression to the development of

more effective therapeutics for prostate cancer patients.

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