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MOLECULAR BIOLOGY OF CASTRATE-RESISTANT PROSTATE CANCER: BASIS FOR THE NOVEL THERAPEUTIC TARGETS

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Summary.- Prostate cancer cells express the androgen receptor (AR) and need the presence of androgens to survive. Androgen suppression is the gold standard first-line therapy for metastatic disease. Almost all prostate cancer patients initially respond to hormonal therapy, but most of them gradually develop castration-resistant progression. Recent evidence has shown that progression at the castration resistant prostate cancer (CRPC) stage is often mediated by AR signalling. Importantly, subsequent AR androgen inhibition, by abiraterone acetate or enzalutamide, has shown to improve patients’ survival. Several mechanisms that enhance AR signalling in an androgen-depleted environment have been elucidated: (1) AR mutations that allow activation by low androgen levels or by other endogenous steroids, (2) AR amplification and/or overexpression, (3) increased local intracrine synthesis of androgens, (4) changes in AR cofactors and (5) cross-talk with cytokines and growth factors. Today, there are under development a number of novel agents targeting the AR signaling pathway. This article reviews the postulated mechanisms of AR-driven resistance to androgen suppression that have contributed to the development of new hormonal therapeutic strategies in prostate cancer.

Keywords: Castration-resistant prostate cancer. Androgen receptor. Abiraterone. Enzalutamide. Biología molecular.

Resumen.- Las células del cáncer de próstata expresan receptores de andrógenos (RA) y necesitan la presencia de andrógenos para sobrevivir. La supresión androgénica es el patrón oro en el tratamiento de primera línea de la enfermedad metastásica. Casi todos los pacientes con cáncer de próstata responden inicialmente al tratamiento, pero la mayoría de ellos desarrollan progresivamente progresión resistente a castración. La evidencia reciente ha demostrado que la progresión en el estadio de cáncer resistente a castración está mediada frecuentemente por la señalización del RA. De una manera importante, la inhibición androgénica posterior del RA, por medio de abiraterona o enzalutamida, ha demostrado mejorar la supervivencia de los pacientes. Se han elucidado varios mecanismos que mejoran la señalización del RA en un entorno reducido de andrógenos: (1) mutaciones del RA que permiten la activación por niveles de andrógenos bajos o por otros esteroides endógenos, (2) amplificación y/o sobreexpresión del RA, (3) síntesis de andrógenos local intracrina aument-
Prostate cancer is considered an hormone-dependent tumor, where malignant cells express the androgen receptor (AR) and need the presence of androgens to survive. For that reason, androgen suppression (including orchiectomy or LHRH agonists associated or not to antiandrogens) is the gold standard first-line therapy for metastatic disease. Almost all prostate cancer patients initially respond to hormonal therapy, but most of them develop resistance and disease progresses despite of castrate levels of testosterone, developing a castration resistant status.

Castration resistant prostate cancer (CRPC) was defined by The Prostate Cancer Clinical Trials Working Group 2 (PCWG2) as the presence of PSA and/or clinical progression with serum castration levels of testosterone (<50 ng/dL or <1.7 nmol/L), and progression despite anti-androgen withdrawal for at least 4–6 weeks (1). However, there is evidence that CRPC continue to depend on AR signalling, which may persist activated despite castrate levels of androgens and be responsible for tumor progression. Moreover, targeting AR in CRPC patients has shown to induce significant clinical benefit and increase survival (2-4).

In the present review, we focus on the AR pathway in the biology of CRPC and its relevant therapeutic implications.

**AR STRUCTURE AND FUNCTION**

The AR gene is located in the X chromosome (Xq11-1) and is essential for the growth and differentiation of prostate cells, and for the development and maintenance of male reproductive organs. Loss of function of AR induces lack of development of prostate and prevents prostate cancer.

Actions of androgens are mediated by the AR which is a ligand dependent transcription factor belonging to the superfamily of nuclear receptors. This family includes receptors for steroid hormones, thyroid hormones, all-trans and 9-cis retinoic acid, 1,25 dihydroxy-vitamin D, ecdysone and peroxisome proliferator-activated receptors.

AR contains a DNA-binding domain (DBD) comprised of 2 zinc finger motifs that determine de DNA sequences recognized by the receptors, and a carboxyl terminal hormone (ligand) binding domain. This domain also contains a region, termed activation function 2 (AF-2) which is important for the transcriptional activity of the receptor. The DNA and the ligand domains are linked by a region which contains a nuclear localization signal. The amino-terminal domain contains a region important for transcriptional activity, termed AF-1; which appear to be the major transactivation domain (Figure 1).

It is not well known if alterations in AR and/or AR signalling are present on the primary tumor and clones harbouring them are selected during PC progression; or they appear de novo in later stages of disease. However, the co-existence of both mechanisms may be possible. Among the processes of progression under castration, genetic, epigenetic or microenvironement-dependent factors may be affecting AR signalling. Moreover, the biology of the progressing tumor may be also influenced by specific therapeutic exposure (“therapeutic mediated pressure”), as the reported AR mutations induced by anti-androgens therapy (5-7). In addition to AR signalling, multiple molecular pathway alterations have been identified in CRPC. Some of them have been already tested or are being targeted in phase 3 trials: immunoregulatory pathways (Sipileucel T, ipilimumab, Prostvac-VF-TRICOM), Src (dasatinib), Met (cabozantinib), clusterin (custirsen), and angiogenesis (aflibercept, tasquinimod) (8).
receptor function. A change in the ratio of coactivators and/or corepressors may modify AR sensitivity to hormone binding. Among them, the coactivator p300 bridges the transcriptional machinery of AR, and the coactivator family p160 modifies chromatin structure (Figure 2).

AR can also mediate non-genomic signalling, which does not require nuclear translocation and DNA binding: AR is able to activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways (5-10).

MODEL OF PROGRESSION OF PROSTATE CANCER

A model of progression of prostate cancer has been proposed based on the mechanisms of AR activation and the sources of androgenic ligands. This model proposes four stages of progression and sensitivity to therapy:

1) Endocrine androgen dependent and AR dependent: the AR receptor is activated by testicular androgens and tumors are sensitive to castration and/or antagonists;

2) Intracrine androgen dependent and AR dependent: AR is activated, despite of castration levels of testosterone, by androgens synthesized in the adrenal gland or in the tumor. Tumors do not respond to castration but may respond to inhibitors of adrenal/tumor androgen synthesis.

3) Androgen independent and AR dependent: AR remains active in absence of ligands through crosstalk with other signal transduction pathways (i.e., growth factors, cytokines,...)

4) Androgen and AR independent: Progression occurs despite of the abolition of AR signal (11) (Figure 3).

ANDROGEN RECEPTOR SIGNALLING IN CRPC

Several mechanisms involved in the AR signalling activation in an androgen-depleted environment have been elucidated: 1) AR mutations/alternative splicing; 2) AR gene amplification and/or overexpression; 3) increased local intracrine synthesis of androgens; 4) changes in AR cofactors implicated in ligand-independent activation of AR signalling and 5) cross-talk with cytokines and growth factors (5,6,11-14).

AR gene mutations

AR mutations are present in about 10-20% patients with PC. The percentage of mutations is higher in CRPC compared with untreated early stage prostate cancer; and in metastasis than in primary tumors. Different mutations have been described in patients receiving anti-androgens (flutamide, bicalutamide) or LH-RH agonists. The mutations may change the effect of the anti-androgen, which acquires an agonist
function and stimulates AR. This agonist function of the anti-androgen may explain the response to anti-androgen withdrawal, that can be observed in up to the 30% of patients (15-22).

Mutations in the AR gene were first described in the hormone-dependent human prostate cancer cell line LNCaP, derived from a lymph node metastasis. This cell line harbours a mutation at codon 877 (Thr to Ala). As a consequence of this mutation, AR can be stimulated by hormones other than androgens, such as progesterone, estrogens and several anti-androgens (promiscuous ligand interaction). Today, a number of mutations have been described (http://androgendb.mcgill.ca/map.gif), but only a small proportion have been functionally characterized. Most of the studied mutations affect critical ligand-receptor and protein receptor interactions; and are supposed to lead to a gain of function, showing an enhancement of transactivating capacities compared to wild-type AR. However, loss of function mutations have also been reported. Moreover, AR splice variants have been also described. These variants lacking LBD don’t need androgen binding to be activated and may not be blocked by current antiandrogens (15-22).

**AR amplification/overexpression**

CRPC cells may contain additional copies of the AR gene (AR amplification) or may have AR protein overexpression. This mechanism sensitizes tumor cells to low levels of androgens, allowing them to survive despite castration levels of testosterone (hypersensitivity of AR).

It has been shown that the AR gene is consistently up-regulated during castration resistance, and that AR overexpression is necessary and sufficient to induce castration resistant progression. In in vitro studies, AR inhibition in androgen-independent cell lines inhibited tumor growth (23-25). Moreover, the overexpression of AR sensitizes the receptor binding to chromatin (26).

Amplification of the AR gene is observed in about one-third of patients progressing to androgen

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**Figure 3.** Molecular states of prostate carcinoma progression on the basis of sources of androgenic ligands and the activity of AR.
AR gene amplification results in AR overexpression. However, AR overexpression at the mRNA and protein level has been also observed in absence of AR amplification, which is attributed to transcriptional, translational, and post-translational mechanisms (27). Moreover, AR amplification has been proposed as one of the mechanisms of resistance to classic antiandrogens such as bicalutamide or flutamide (28).

**Synthesis of intra-tumoral androgens**

Androgen suppression therapy reduces the levels of circulating androgens by 95%, but low serum levels of androgens persists and can activate the AR. These androgens may come from an adrenal source (a fact that explain the responses observed with inhibitors of the adrenal androgen synthesis, such as ketoconazole), but may also be synthesized within the tumor. Indeed, in CRPC the concentration of androgens in the prostatic tumor is maintained at levels that can activate the AR. Thus, the androgen levels achieved by standard medical and surgical methods of castration are inadequate to fully suppress the processes regulated by androgens.

Recent studies support the high relevance of the de novo synthesis of androgens within the tumor. A transcriptional profil study of castration-resistant bone metastasis found abundant transcripts for androgen regulated genes in most of the tumor samples (29). In addition, over-expression of enzymes key to androgen synthesis has been observed in castrate-resistant tumors (30,31). In the prostate, conversion of testosterone to its active metabolite, dihydrotestosterone, is catalyzed by the enzymes 5 alpha-reductase types 1 and 2. Prostate cancer cells may increase the synthesis of dihydrotestosterone from testosterone, by increasing the activity of 5 alpha-reductase (32).

One study (33) showed that soft tissue metastasis from castration-resistant cancers exhibit elevated testosterone concentrations compared with untreated primary tumors. To determine

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**Figure 4.** Fluorescence in situ hybridization (FISH) and immunofluorescence (IF) images corresponding to examples of circulating tumor cells with copy alteration of AR gene.
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whether prostate cancer metastasis were capable of synthesizing androgens de novo, the authors quantified transcripts encoding enzymes involved in testosterone biosynthesis from cholesterol precursors. Compared with untreated tumors, castration-resistant metastasis expressed higher levels of enzymes responsible for the synthesis of adrenal androgens from progesterone, and CYP19A1, that mediates aromatization of testosterone to estradiol. They also found high levels of intratumoral androgens in castration-resistant xenografts lacking adrenal CYP17 (enzyme involved in androgen synthesis) expression, a finding that supports the de novo synthesis of androgens in tumor tissue (Figure 5). Targeting adrenal and testicular CYP17 enzymes by abiraterone has proved to be an important therapeutic strategy in CRPC (2).

**Cross-talk with cytokines and growth factors**

AR may be activated in the absence of androgen-AR interaction by ligand-independent mechanisms. The cross-talk with growth factor receptors can transactivate AR, inducing the expression of androgen-response elements in absence of androgens but not AR. AR transactivation by the epidermal growth receptor (EGFR), nuclear factor kappa B (NF-kB) and various cytokines, to cite some examples, have been reported. The HER2 receptor tyrosine kinase, belonging to the EGFR family, is progressively overexpressed in advanced, CRPC. High levels of Her-2/neu were found associated with shortened survival times in prostate cancer patients (34,35).

In experimental systems, the overexpression of HER2 resulted in increased AR activity and stability, while pharmacologic inhibition or knockdown of the protein resulted in growth suppression. In hormone-dependent LNCaP cells, Her-2/neu inhibition led to impairment of AR-mediated functions, such as androgen-stimulated growth (36). Activation of HER2/HER3 by heregulin increased androgen-dependent AR transactivation of reporter genes in hormone-independent CWR-R1 cells, and activated downstream signaling, including mitogen-activated protein kinase and phosphatidylinositol-3 kinase and Akt pathways. Tyrosine phosphorylation of HER2 and HER3, AR transactivation, and cell proliferation induced by heregulin can be inhibited by the EGFR/HER2 dual tyrosine kinase inhibitor lapatinib (37). Reports of lapatinib in castrate-resistant PC have shown a modest clinical activity in these patients (38).

The NF-kB system activation is also implicated in androgen-independent growth of PC by regulating AR action. NF-kB pathway activation results in

![Figure 5: CYP17 in androgen synthesis.](image)

DHEA: Dehydroepiandrosterone; DHT: Dihydrotestosterone.
increased levels of AR in LNCaP cells. By blocking NF-κB signalling in vitro, AR activation is inhibited. In addition, the continuous activation of NF-κB signalling in vivo can sustain high levels of nuclear AR, maintaining continued proliferation of the prostatic epithelium (39). The ubiquitin/26S proteasome pathway induces AR expression by activating NF-κB, and promotes AR activity by participating in the assembly of an AR transcription complex (40). Interleukin-6 (IL-6) and -8 (IL-8), cytokines regulated by NF-κB, have also showed to activate the AR. IL-6 is known to stimulate androgen receptor activity and expression of its downstream target genes. IL-6 may cause growth of androgen receptor-positive tumours in vitro and in vivo through activation of the AR, and this growth can be inhibited by bicalutamide (41).

In addition to the mechanisms of AR transactivation discussed above, the interaction with a number of other growth factor receptors and signalling molecules (e.g. EGFR, IGF-1R, IL-6R, BRAF, SRC) can enhance AR signalling and confer castration resistance in preclinical models. These receptors induce downstream activation of critical growth and survival pathways, including the AKT, MAPK, and STAT pathways. AKT activation in PC has been associated with loss of PTEN, that occurs in a high percentage of tumors. Inhibition of different pathways is now being clinically tested as a strategy to treat castration-resistant PC (42).

**Other ways to regulate AR activity**

Prior to ligand binding, AR exists in a complex with heat shock protein 90 (Hsp90) and other co-chaperones. The AR-Hsp90 interaction maintains AR in a high-affinity ligand-binding conformation, which is necessary for efficient response to hormone. Clinical trials with HSP inhibitors are currently ongoing in prostate cancer patients (43, 44).

<table>
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<tr>
<th>Clinical trial</th>
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mCRPC: metastatic CRPC; P: prednisone; OS: overall survival, PFS: progression free survival; rPFS: radiographic progression free survival; HR: hazard ratio.
Histone deacetylases (HDAC) are proteins involved in the regulation of the interplay between transcription factors, such as AR and a chromatin state that supports active gene transcription. Several HDAC inhibitors (depsipeptide, SAHA, and LBH589) have demonstrated promising anti-tumor activity, direct suppression of AR transcription and are under clinical development (45).

AR co-activators may regulate histone modification, proteasomal degradation, chaperones, sumoylation, chromatin remodeling, and cytoskeleton modification. Co-factors regulation results in the alterations of multiple cellular functions and may be valid targets for novel therapies in prostate cancer. (46).

NEW AR-TARGETED THERAPIES

Specific CYP17 inhibitors

Abiraterone acetate is an oral, selective, and irreversible inhibitor of CYP17, a critical enzyme in androgen biosynthesis, which blocks non-gonadal androgen production (Figure 4). Abiraterone in combination with prednisone demonstrated a survival benefit compared with placebo in a randomized phase III trial (COU-AA-301) in patients with metastatic CRPC who had progressed to docetaxel-based therapy (2). Results of a similar placebo-controlled phase III trial (COU-AA-302) evaluated abiraterone in docetaxel-naïve patients progressing after first-line androgen deprivation therapy. This study also showed a survival benefit for abiraterone (Table I) (Figure 5) (47).

TAK-700 (orteronel) is an oral, selective, reversible, non-steroidal androgen synthesis inhibitor of the 17:20 lyase activity, one of two enzymatic reactions catalyzed by CYP17 (Figure 5). (48) Two randomized, phase III, placebo-controlled multicenter studies evaluating the efficacy and safety of TAK-700 in chemotherapy naïve and docetaxel-pre-treated metastatic CRPC patients are ongoing (Table II).

New AR antagonists

First-generation AR antagonists, such as bicalutamide or flutamide, bind reversibly to ARs and may have androgen-agonist properties. New antiandrogens with improved binding properties have been produced. The two lead compounds are MDV3100 (enzalutamide) and ARN-509. They are more potent than bicalutamide, with higher binding affinities to AR. Importantly, both ligands remain antagonists in models of CRPC overexpressing AR, and enzalutamide antagonizes the mutated AR which converts bicalutamide to an agonist. Another mechanism of resistance to bicalutamide is that the bicalutamide-AR complex translocates to the nucleus and interacts with regulatory regions of AR target genes but forms unproductive transcriptional complexes. In the context of CRPC, where AR co-activators are often elevated, target gene activation may occur. New antiandrogens circumvent this problem by limiting nuclear translocation (49).

Enzalutamide has demonstrated a survival benefit in a phase III (AFFIRM) trial in the post-docetaxel setting (50). Another phase III study (PREVAIL) in patients not previously treated with chemotherapy has been recently closed. ARN-509 with a mechanism of action similar to that of MDV3100, is also in clinical development (49).

TUBULIN-TARGETING AGENTS AND AR

The taxanes, docetaxel and cabazitaxel, act as a tubulin-targeting agents and are current standard therapies in CRPC. Several recent studies have reported that tubulin-targeting drugs cause cytoplasmic androgen receptor (AR) sequestration, down-regulation of AR and prostate-specific antigen (PSA) expression, impairing AR activity. These data suggest that taxanes may act in part through AR activity inhibition. Areas the need to be defined in the near future include; if AR-dependent CRPC tumors are more sensitive to chemotherapy, the existence (or lack of) of cross-resistance between new hormonal agents and taxanes, or the role of combination therapy (51-53).

REFERENCES AND RECOMMENDED READINGS

(* of special interest, ** of outstanding interest)


