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Androgen receptor abnormalities in castration-recurrent prostate cancer

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Abstract

The androgen receptor (AR) plays a critical role in prostate cancer (PCa) development and progression. Despite the success of androgen-deprivation therapy, remission occurs in almost all cases. This stage of the disease is called castration-recurrent PCa (CRPC). CRPC cells adapt to low circulating levels of androgens, and active AR is maintained by numerous cellular mechanisms. Some mutations in the AR make it more responsive to lower androgen levels or other steroids. Furthermore, in this advance stage of the disease, PCa cells express the enzymes necessary for *de novo* synthesis of androgens. AR is also activated in a ligand-independent manner. Therefore, it is important to understand the mechanisms of AR activation and its deregulation during CRPC. The purpose of this article is to discuss mechanisms that are involved in modulation of AR activity and specificity.

Keywords

androgen receptor; AR variants; castration-recurrent; gene expression; post-modification; prostate cancer

Nuclear receptors (NRs) are intracellular proteins activated by small molecules, such as steroid hormones, fatty acids or retinoids [1]. NRs mediate cell-fate events, including cell metabolism, organ development, cell growth and proliferation [2,3]. According to their ligand and cell function, NRs can be divided into three groups: steroid hormone receptors, thyroid/retinoid receptors and orphan receptors [1,3].

The androgen receptor (AR) is a 110-kDa protein that belongs to the steroid hormone receptor subclass of NRs [4]. Overall, the AR is composed of: a variable N-terminal ligand-independent

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transactivation domain (NTD) containing activating function (AF)-1; a central DNA-binding domain (DBD), composed of two highly conserved zinc-finger motifs that interact with specific hexanucleotide-responsive elements; a short amino acid sequence called the hinge region, which confers flexibility for proper rearrangement, dimerization and specific DNA interaction; and a C-terminal region composed of the ligand-binding domain (LBD), the ligand-dependent transactivation/AF-2, which is necessary for recruitment of coregulators, and a bipartite nuclear localization signal (NLS) [1,4].

AR & prostate cancer

Prostate cancer (PCa) is the most frequently diagnosed cancer and second leading cause of cancer-related deaths among men in the Western hemisphere [5]. Correlation between PCa and hormones was first described by Charles Huggins and Clarence V Hodges in 1941 [6], who observed that patients with PCa have higher phosphatase activity than normal men, and that this activity decreases following bilateral castration. Moreover, injection of testosterone, but not estrogen, led to a rapid recovery of phosphatase activity in castrated patients [6]. These studies demonstrated, for the first time, a relationship between androgens and PCa, and established the basis for the current treatment of androgen ablation in advanced PCa [7].

Androgens mediate their effect by binding to and activating the AR, thereby promoting proliferation and differentiation of the prostate during normal development, as well as during initiation and progression of PCa [8–10]. Deregulation of the androgen–AR signaling axis may occur in PCa following long-term androgen-deprivation therapy. In advanced stages of PCa, the most common treatment is androgen-deprivation therapy. This therapeutic approach consists of suppressing androgen levels by surgical castration or by chemically inhibiting its synthesis with leuprolide, in combination with an AR inhibitor, such as Casodex[®], to block AR-dependent transcription [11–13]. However, despite the effectiveness of such therapies, PCa eventually becomes unresponsive to hormonal manipulation, a stage of the disease known as castration-recurrent PCa (CRPC) in which AR levels and activity remain elevated [9,14]. Although AR suppression can result in decreased cell survival and proliferation in PCa, AR activation may be independent of hormone binding in CRPC cells [14,15].

Prostate cancer cells may utilize different mechanisms to compensate for low androgen levels [16,17]. In CRPC, this compensation may be accomplished by gain-of-function mutations in the AR [18,19], increased AR expression [20], overexpression of AR coregulators [16], intracrine synthesis of androgens by PCa cells [21,22] and, perhaps, a ligand-independent transactivation of AR by other signaling pathways [14,17]. Although androgen ablation reduces circulating testosterone to levels similar to that resulting from castration, the intraprostatic levels remain elevated [23]. This suggests that standard medical or chemical methods of castration leave significant levels of androgens that may be responsible for AR stimulation during CRPC. Elevated intraprostatic androgen levels could be the consequence of alternative synthesis pathways that do not require precursors, such as dehydroepiandrosterone, dehydroepiandrosterone sulfate and androstenediol [23,24]. Several steroid enzymes that synthesize androgen from cholesterol are elevated in CRPC compared with androgen-dependent PCa [23,25,26]. These findings are leading to new therapeutical approaches combining castration, AR inhibitors and inhibition of the enzymes responsible for androgen synthesis [24,27–29]. CYP17 is a key enzyme in the process of androgen synthesis. Screening for small molecules, which are capable of inhibiting CYP17, led to the discovery of abiraterone, a potent, specific and irreversible inhibitor of CYP17 [30]. Currently, abiraterone is being tested in clinical trials for CRPC patients. Results show a significant anti-tumor effect in up to 70% of the patients [31].

However, there is also evidence of ligand-independent activation of AR in PCa cells [14,18]. It was recently found that AR can also be abnormally processed at the RNA level in CRPC, generating C-terminal truncated variants of AR that lack the LBD, but remain constitutively active (discussed later). Thus, understanding the signaling pathways regulating AR, and how such modification may affect its activity, location and stability is crucial for the development of new drugs and to improve therapeutic strategies.

Activation of AR

Androgenic steroids are responsible for male-specific organ differentiation and maintenance, as well as secondary sex characteristics. These 19-carbon steroids, of which testosterone is the prototype, are primarily produced by the testis. Testosterone is the most abundant steroid in circulation, and by action of the 5-reductase enzyme is converted to dihydrotestosterone (DHT) predominantly within the prostate and genital tissues [32,33]. Both, testosterone and DHT can bind to and activate AR under physiological conditions. However, DHT has a higher affinity for AR, thereby activating target genes at lower concentrations than testosterone, which makes DHT a more potent AR agonist [29,34,35]. In resting, unstimulated cells, AR is stabilized in the cytosol by heat-shock proteins (HSPs) HSP-90, -70 and -56 [36,37]. HSPs maintain an AR conformation required for high affinity binding to androgens, which leads to proper AR maturation, interaction with cellular trafficking systems and translocation into the nucleus [37,38]. Once in the nucleus, AR forms homodimers and binds to specific DNA sequences termed androgen response elements (AREs) within 5'-flanking regions of target genes. The transcription machinery is completed by recruitment of coregulators, which ultimately results in modulation of gene expression [39].

To date, over 200 coregulators have been identified [39,40], which have specific and distinct functions, either enhancing (coactivators) or repressing (corepressors) AR activity, depending on the targeted gene [40]. Several coregulators are enzymes responsible for post-translational modifications of AR itself or the DNA microenvironment, eventually resulting in modulation of gene expression [40]. Identification of the coregulators that constitute specific transcription complexes is important for understanding the effectiveness of a therapeutic drug and for the development of new approaches to treating the disease [27]. Synthetic antagonists or partial agonists may induce differential conformational changes in the AR, thereby affecting the ratio of enhancer–repressor coregulators, resulting in suppression (or stimulation) of transcription of survival and proliferative genes, while stimulating (or suppressing) transcription of apoptotic genes [27].

Modulation of AR activity

Androgen receptor coregulators can modulate expression of different genes by affecting either the intrinsic activity of AR or the structure of its surrounding chromatin. The intrinsic activity of the AR is also affected by a broad range of post-translational modifications [41]. Several phosphorylation sites have been identified in the AR. Recent reports show AR-activating tyrosine phosphorylation [42,43]. This modification is triggered by growth factors and is independent of androgens; however, the specific amino acid on the AR responsible for this activity remains controversial [43]. Furthermore, at least one of the phosphotyrosine kinases responsible for these modifications is Ack1, which is inappropriately activated in PCa [42]. AR tyrosine phosphorylation correlates with high Gleason grade and may be, at least in part, responsible for androgen-independent activation of AR in CRPC [42,43]. By contrast, phosphorylation of other amino acids on the AR lead to its degradation, thus opposing the activating effect of tyrosine phosphorylation.

E3 ubiquitin ligases are multiprotein complexes that mono-, di- or polyubiquitinate protein substrates, thus changing the function, location and/or stability of the protein substrate [44,

45]. A key step in this process is recognition of the specific substrate. One of the most common recognition signals is phosphorylation of specific amino acids within the substrate [44]. Akt, a serine/threonine protein kinase, phosphorylates AR at two serines residues (independent reports mapped either serines 210 and 790 or serines 213 and 791; differences in sites may depend on the natural variant analyzed) [46–48]. Phosphorylation of these sites serve as recognition motifs for the Mdm2 E3 ubiquitin ligase, which polyubiquitinates a still unknown lysine residue, thus targeting AR for proteasomal degradation [49]. However, the fate of a polyubiquitinated protein is not always proteasomal degradation. RNF6 is an E3 ubiquitin ligase that interacts with and polyubiquitinates AR when bound to an ARE [50]. Unlike Mdm2, RNF6-dependent ubiquitination modulates AR activity and specificity but does not affect its stability [50]. RNF6 modifies two lysine residues in the AR (K845 and K847). RNF6 forms polyubiquitin chains linking the sequential ubiquitin to K6 or K27 residues of the antecedent ubiquitin, instead of the K48 or K63 residues, which target substrates to the proteasome [45, 50]. Although the detailed mechanism remains uncertain, RNF6-dependent modifications may induce structural changes in the AR, thereby increasing its activity on specific genes by selectively recruiting cofactors to AREs of a subset of AR target genes [50,51]. RNF6 is highly expressed in PCa and correlates with progression of the disease [50].

In addition to ubiquitin, other ubiquitin-like modifiers, such as small ubiquitin-like modifier (SUMO), can also modulate AR activity [52,53]. Similarly to ubiquitination, SUMOylation requires enzymes for activation (E1), conjugation (E2) and ligation (E3) of the SUMO modifier to one or multiple lysine residues of the target protein. Although the mechanisms are alike, the enzymes catalyzing SUMOylation are distinct from those of ubiquitination. SUMOylation is a dynamic modification that is rapidly reversed by a family SUMO-protease enzymes called sentrine-specific proteases (SENP) [54,55]. AR interacts with the E2 SUMO-conjugating enzyme UBC9 [56]. UBC9 targets AR residues K386 and K520 for SUMOylation, resulting in downregulation of AR activity and modulation of its specificity for a subset of genes [57, 58]. Removal of SUMO from AR is catalyzed by SENP1 [59]. Overexpression of SENP1 leads to increased AR activity, consistent with the findings that SUMOylation abrogates AR activity [59]. SENP1 expression was found to be elevated in PCa [52], which may overcome the inhibitory mechanism of AR by SUMOylation.

In summary, multiple and diverse post-translational modifications of AR have been described [3]. These modifications are coordinated in a spatial and temporal manner, and suggest that the AR transcriptional machinery is a dynamic complex where the interplay between AR and coregulators regulate gene expression [39]. Deregulation of such modifications could be responsible for or contribute to the pathogenesis and progression of PCa. Therefore, elucidating the pathways and enzymes responsible for AR modification might provide novel therapeutics for PCa.

Androgen-independent AR variants

Despite the success of androgen-deprivation therapy for PCa, the recurrence of androgen-independent tumors is inevitable. Therefore, much research has focused on understanding the mechanism of AR activation, particularly in the castration-recurrent scenario [60]. Several point mutations and insertions within the NTD, hinge region and LBD of AR lead to a gain of function in transcriptional activity, owing to a higher affinity for DHT or responsiveness to other steroid ligands [15,18]. The mechanisms for such enhanced AR activity include changes in affinity for the ligand, stabilization of AR levels and/or cellular localization, which may explain, at least in part, how cells can proliferate at low androgen levels during the castration-recurrent stage of the disease [61–65].

In contrast to AR mutations imparting a hypersensitivity to ligand, truncation of the LBD leads to a constitutively active AR [61]. The human *AR* gene contains eight exons: exon 1 encodes the NTD and constitutes approximately 60% of the protein, exons 2 and 3 encode the DBD and exons 4–8 encode the C-terminal domain (CTD), which includes the LBD and a NLS. Tepper and colleagues identified two *AR* variants in the CWR22Rv1 human cell line, a model of CRPC [61]. One variant contains a duplicated exon 3 and three zinc finger motifs within the DBD. The other variant consists of an 80-kDa AR protein, which lacks the CTD [61]. Although functionally active in the absence of ligand binding, the origin and physiological relevance remained uncertain. It was first proposed that the truncated forms were products of proteolytic cleavage of AR [62]. However, three independent groups recently identified several *AR* variants in castration-recurrent cell line models and in PCa tissues [63–65]. The *AR* variants result from the insertion of a premature stop codon as a result of duplication and insertion of exon 2 and exon 3, rather than proteolytic cleavage of the AR protein as was originally proposed [63–65]. The first variants identified in CWR22Rv1 cells contain a duplicated 17-bp portion of exon 2 after either exon 2 or exon 3, generating two different AR variants, AR^{Ex1,2,2b} and AR^{Ex1,2,3,2b}, respectively [63]. AR contains two zinc finger motifs: one encoded by exon 2 and the second by exon 3. Surprisingly, both variants are active independently of the presence of ligand. This observation implies two major conundrums. First, the partially duplicated exon 2b does not code for a zinc finger motif [63]. Second, the AR^{Ex1,2,2b} variant contains only one zinc finger domain encoded by the full exon 2 [63]. Therefore, these findings suggest that only one zinc finger domain is necessary for AR transcriptional activity [63]. These results were confirmed by other groups [64,65]. Antibodies that specifically recognize the truncated, but not the full-length forms of AR, were developed to analyze the presence of these variants in human samples. Two independent groups revealed the presence of the newly identified variants in tissues corresponding to PCa patients [64,65]. Although expression levels are lower than full-length AR [64], some of the variants are constitutively active, and suppression of the variants results in decreased proliferation of PCa cell lines [65]. Furthermore, gene-expression profile analysis shows a difference between genes regulated by the full-length AR compared with one of the variants [64]. Therefore, the variants may regulate different biological processes by selectively targeting genes [65]. These differences could be due to affinity changes between full-length AR versus truncated variants, which may favor specific AREs and/or differential recruitment of coregulators to the transcriptional machinery [66].

It is worth noting that all the variants are CTD-truncated. As mentioned previously, AR contains two independent transcription activating domains: a unique AF-1 within the N-terminal, and an AF-2 within the C-terminal region, which is structurally conserved among other members of the steroid receptor family [1,4]. In CRPC cell models, AR activity depends on the NTD rather than CTD [67,68]. Thus, we speculate that androgen-deprivation therapy results in a selection of cells containing constitutively active NTD-AR variants. Altogether, the presence of these newly identified variants contributes to our understanding of androgen-independent growth and proliferation during CRPC.

Expert commentary & five-year view

Ligand-independent active AR is a hallmark of CRPC. To understand such deregulation, research has focused on elucidating the mechanisms regulating AR activation and their role in development and progression of PCa. Formation of active AR transcriptional machinery requires proper maturation of AR to assure translocation into the nucleus, dimerization and binding to AREs where cofactors are recruited and determine whether the AR is transcriptionally activated or inhibited. The newly identified constitutively active truncated forms of AR challenge this canonical mechanism of AR activation since they lack LBD and NLS.

Multiple and diverse post-translational modifications coordinated in space and time have been ascribed to AR. Therefore, a future key issue is to elucidate the mechanism of activation of the AR variants. Such knowledge should facilitate the design of new drugs and improve therapeutic approaches for PCa. The first step is to understand how some of the newly described AR variants that lack the NLS [63–65] can translocate into the nucleus. Several proteins interact with AR in the cytoplasm and may serve as transporters. Furthermore, it should also be determined whether nuclear translocation is a spontaneous event or an event that is triggered by signaling pathways.

In addition, full-length AR contains two zinc finger domains, which facilitate binding to DNA. By contrast, some AR variants contain only one zinc finger motif and all are truncated at the C-terminal end of the protein [63]. A possible explanation is that only one zinc finger domain is sufficient for AR activity. Furthermore, AR interacts with DNA as a dimer for which an N-terminal–C-terminal interaction is required for stabilization of the transcriptional complex. However, the truncated forms of AR do not possess the C-terminal portion of the protein; therefore, their transcriptional complexes may not have the same structural components of the full length AR. One can speculate that coregulators may help to stabilize the complexes formed with AR variants. Another possibility is that the variants interact with different coregulators or transcription factors leading to a noncanonical activation of AR.

Finally, the turnover of AR is not fully understood. The E3 Ubiquitin ligase Mdm2 recognizes an Akt-dependent phosphorylated serine residue on AR. However, two serines have been identified as targets for Akt: serine 210 within the NTD, and serine 790 within CTD, which are absent on truncated variants of AR. Therefore, these variants may be more stable than full length AR, which would make their contribution to tumor progression even more significant.

Key issues

- After an initial response to androgen deprivation therapy, prostate cancer relapses and growth of prostate cancer is independent of androgens: this constitutes castration-resistant prostate cancer (CRPC).
- An active androgen receptor (AR) is required in CRPC.
- Post-translational modification plays an important role in AR activation and, ultimately, in the subset of genes targeted for transcription.
- AR contains point mutations, insertions and deletions in CRPC cell models and patient tissues.
- AR-truncated variants lacking ligand-binding domains are constitutively active, and promote cell growth and proliferation independently of androgen stimulation.
- Spliceosome deregulation leads to formation of constitutively active AR variants.

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- of considerable interest

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