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Clinical relevance of androgen receptor alterations in prostate cancer

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Abstract

Prostate cancer (PC) remains a leading cause of cancer-related deaths among men worldwide, despite continuously improved treatment strategies. Patients with metastatic disease are treated by androgen deprivation therapy (ADT) that with time results in the development of castration-resistant prostate cancer (CRPC) usually established as metastases within bone tissue. The androgen receptor (AR) transcription factor is the main driver of CRPC development and of acquired resistance to drugs given for treatment of CRPC, while a minority of patients have CRPC that is non-AR driven. Molecular mechanisms behind epithelial AR reactivation in CRPC include AR gene amplification and overexpression, AR mutations, expression of constitutively active AR variants, intra-tumoural and adrenal androgen synthesis and promiscuous AR activation by other factors. This review will summarize AR alterations of clinical relevance for patients with CRPC, with focus on constitutively active AR variants, their possible association with AR amplification and structural rearrangements as well as their ability to predict patient resistance to AR targeting drugs. The review will also discuss AR signalling in the tumour microenvironment and its possible relevance for metastatic growth and therapy.

Key Words

- ▶ prostate cancer
- ▶ castration resistance
- ▶ androgen receptor

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Introduction

Prostate cancer (PC) is one of the most common forms of cancer and a leading cause of cancer-related deaths in men worldwide (1). Androgens regulate normal and malignant prostate tissue growth via activation of androgen receptor (AR) signalling in epithelial and stroma cells. Androgen deprivation therapy (ADT) is the standard therapy for locally advanced and metastatic PC, including surgical and chemical castration that deprives tumour cells of testicular androgens and thereby reduces tumour growth. Although ADT is initially effective in most patients, the disease progresses within a few years into an incurable and lethal stage of castration resistance. Castration-resistant prostate cancer (CRPC) is usually established as bone metastases, but also as soft tissue metastases and through local recurrence. The AR in tumour epithelial

cells is active in nearly all CRPC tumours despite castrate levels of circulating testosterone (2, 3), while a minority of CRPC metastases seem to be non-AR driven (4, 5). Mechanisms leading to epithelial AR reactivation in CRPC include, AR amplification and overexpression, AR mutations, expression of constitutively active AR variants, intra-tumoural androgen synthesis and promiscuous AR activation by other factors, as extensively reviewed previously (6, 7). Thus, the AR has for a long time been the main target in the treatment of advanced PC, and the combination of castration therapy with 1st-generation antiandrogens (flutamide, nilutamide and bicalutamide) reduces the risk of PC death by 10–30% compared to castration alone (8). Through the development of the 2nd-generation AR antagonists such as enzalutamide and



apalutamide and the steroidogenesis inhibitor abiraterone acetate, CRPC patients show further improved survival (9, 10, 11). However, not all CRPC patients respond to those AR-targeting drugs, and drug resistance also develops with use. This review will summarize AR alterations of clinical relevance for patients with CRPC, with focus on constitutively active AR variants, their possible association with AR amplification and structural rearrangements as well as their ability to predict patient resistance to AR-targeting drugs. It will also discuss AR signalling in the tumour microenvironment and its possible relevance for metastatic growth and therapy.

The AR structure, activation and function

The AR gene is located at chromosome X (Xq11–12) and contains eight exons encoding a 110kDa protein. The AR protein consists of a NH₂ terminal transactivation domain (NTD, encoded by exon 1), a DNA-binding domain (DBD, encoded by exons 2 and 3), a hinge region (H, encoded by the 5' portion of exon 4) that contains the nuclear localization signal (NLS) and a ligand-binding domain (LBD/CTD, encoded by the remaining exon 4 through exon 8) (12). Testosterone and dihydrotestosterone (DHT) bind to the LBD of the AR and induce a conformational change of the protein that leads to dissociation of chaperone proteins and exposes the NLS in the hinge region. The AR dimerizes and translocates to the nucleus where it interacts with transcriptional co-regulators, binds to androgen response elements (ARE) and regulates the transcriptional output of hundreds to thousands of androgen-regulated genes depending on cell type. In prostate epithelial cells the AR regulates the expression of *NKX3.1* and *FOX* family transcription factors, *IGF1R*, *UBE2C*, *UGT2B15*, *KLK3*, *TMPRSS2*, *FKBP5* and other genes controlling cell growth, differentiation and function in the normal prostate and during PC growth and progression (13).

Overexpression of the AR in CRPC

Increased AR expression is consistently seen in tumour epithelial cells during development of CRPC (2), whereas a loss of AR signalling is generally observed in the primary tumour and metastasis stroma (14). Overexpression of the AR in tumour epithelial cells could be a result of AR gene amplification as described below, but is probably also an instant response to castration as androgens normally

suppress AR transcription in prostate epithelial cells (15, 16, 17). AR amplification is the most frequent genetic alteration reported for CRPC tumours, as observed in up to 50% of the cases (18, 19, 20, 21). This stands in contrast to untreated primary prostate tumours (22) where AR amplification is rarely detected, suggesting that AR amplification is an adaptive response to ADT. AR amplification has been linked to AR overexpression in clinical samples and experimental systems and thereby to sensitising tumour epithelial cells to low androgen levels (23, 24). Detection of AR amplification in circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) isolated from patients with CRPC has been associated with therapy resistance to the AR antagonist enzalutamide and the CYP17 blocker of steroidogenesis, abiraterone acetate (25, 26).

Activating AR mutations in CRPC

AR signalling in CRPC tumour epithelial cells could also be caused by activating AR point mutations. Such mutations are very rare in untreated PC, but detected in 15–20% of CRPC patients (19, 20, 27) and in up to 40% of CRPC patients treated with AR antagonists (28). Activating AR point mutations generally affect the c-terminal LBD, while about one-third occur in the transactivating NTD (29, 30) resulting in broaden ligand specificity and AR activation by weak adrenal androgens and other steroid hormones, including DHEA, progesterone, oestrogen and glucocorticoids as well as in turning antagonists into agonists, as recently reviewed in (31) and summarized in this article in Table 1. The first and most frequently identified AR point mutation is the flutamide-driven T878A mutation (32, 33, 34, 35), while W742C and H875Y also have been reported after treatment with first-generation AR antagonists (36, 37, 38, 39, 40, 41). Also, the use of 2nd-generation AR antagonists and the CYP17 inhibitor abiraterone acetate seems to select for activating AR mutations. The F877L-mutated AR has been detected in cfDNA from CRPC patients progressing on enzalutamide or apalutamide (25, 42) and is, accordingly, activated by flutamide, enzalutamide and apalutamide in experimental models systems for PC (38, 42, 43, 44). The F877L mutation seems to be a rare event as it was not detected in a study of 150 CRPC metastases, of which about a half of the patients were pre-treated with enzalutamide (20). Enzalutamide and ARN509 (apalutamide) have agonist effects also on the H875Y, T878A and T878S mutations, all detected in CRPC patients (38), but acquired resistance to those

Table 1 Activating androgen receptor mutations recurrently identified in CRPC.

Mutation	Aberrant effect	References
T878A	Activated by progesterone, estrogen, flutamide, bicalutamide, enzalutamide and apalutamide	(25, 32, 33, 34, 35, 38, 47)
W742C	Activated by bicalutamide, flutamide	(36, 37, 38, 39)
H875Y	Activated by estrogen, progesterone, glucocorticoids, adrenal androgens, bicalutamide, flutamide, enzalutamide and apalutamide	(25, 35, 36, 37, 38, 40, 41)
F877L	Activated by flutamide, apalutamide and enzalutamide	(25, 38, 42, 43, 44)
L702H	Activated by glucocorticoids	(26, 38, 50, 51, 52)

drugs may primarily depend on increased expression of the glucocorticoid receptor and AR bypass (45, 46) or the induction of constitutively active AR variants, as discussed below. The *T878A*, *H875Y* and the *L702H* mutations have been observed in CRPC patients progressing during abiraterone treatment and associated with either the increased progesterone levels obtained after CYP17 inhibition (25, 47, 48, 49) or with the co-administration of exogenous glucocorticoids given to compensate for significantly reduced cortisol levels (26, 50, 51, 52). The activating AR point mutations *T877A*, *L702H* and *H875Y* have been detected in cfDNA from patients with CRPC and shown to be associated with resistance to abiraterone and enzalutamide (25, 26, 42, 49).

Structural AR gene alterations in CRPC

In addition to *AR* amplification and point mutations in tumour epithelial cells, diverse structural *AR* alterations including deletion, duplication, inversion and translocation events have been reported; first for experimental model systems of CRPC and then for a substantial fraction of clinical CRPC samples (53, 54, 55, 56, 57). By targeted paired-end DNA sequencing of the coding and non-coding *AR* region in 30 soft tissue metastases collected at rapid autopsy of 15 CRPC patients, structural *AR* alterations were demonstrated in 10/30 cases (6/15 patients), while no alterations were observed in the 21 hormone-naïve primary prostate tumours analysed (56). In another study, cell-free DNA was analysed from 30 CRPC patients and 50% were found to have structural *AR* alterations (57). Together, these studies show that the *AR* gene structure is frequently altered in CRPC and, moreover, that sub-clonal heterogeneities may exist within and in-between patients. Possible associations between structural *AR* alterations and the generation of constitutively active, truncated AR variants as well as their prognostic and therapy-predictive value will be discussed in more detail below.

Constitutively active AR variants in CRPC

The first truncated AR variants (AR-Vs) with subsequently proved gain of function were identified in 22Rv1 cells due to the presence of 75–80 kDa AR immunoreactive species that were initially thought to be proteolytic fragments of wild-type AR (ARwt) (58). However, later work demonstrated that RNA interference (RNAi) targeting the LBD of the AR reduced expression of the full-length AR, but not of the shorter isoforms (59). This suggested that the truncated AR-Vs were not products of ARwt, but instead derived from unique RNAs. To date, over 20 truncated AR-Vs have been identified in human PC cell lines, xenografts and clinical specimens and some have proven to be constitutively active, i.e. they are able to translocate into the nucleus and initiate transcription without the need for ligand binding (20, 60, 61).

In clinical samples, truncated AR-Vs has been detected in the normal prostate, in primary prostate tumours and in non-treated PC metastases, but highly increased levels have been seen only in CRPC (20, 62, 63, 64, 65, 66, 67) and mainly in association with *AR* amplification (21, 57).

Nuclear translocation and constitutive activity of AR variants

Gain-of-function AR-Vs lack portions of the LBD and instead have divergent COOH-terminal extensions encoded by unique transcripts. Some AR variants are recurrently found in CRPC and have been described as constitutively active, such as the AR-V7 (also termed AR3) (62, 63), AR-V567es (also termed AR-V12) (63, 64) and AR-V3 (59, 63), whereas others (i.e. AR-V1 and AR-V9) seem to be conditionally active, depending on cellular context (68, 69) (Table 2). The majority of the AR-Vs identified today harbour the same NTD and DBD as the ARwt. Between the DBD and the LBD is the hinge region, which harbours the canonical NLS required for the nuclear localization of the ARwt following ligand binding. In contrast to the canonical AR signalling pathway, the

Table 2 Gain-of-function AR variants recurrently identified in CRPC.

Variant	Transcriptional activity	Clinical relevance	References
AR-V7 (AR3)	Constitutive	<ul style="list-style-type: none"> Resistance to ADT, enzalutamide and abiraterone Short time to disease relapse after radical prostatectomy and more rapid progression to CRPC Poor PSA response, short progression-free survival, short overall survival and short cancer-specific survival of CRPC patients 	(48, 62, 63, 65, 66, 69, 103, 104, 105, 107)
AR-V567es (AR-V12)	Constitutive	<ul style="list-style-type: none"> Resistance to ADT Enriched in CRPC and metastases 	(64, 65, 68, 106)
AR-V3	Constitutive	<ul style="list-style-type: none"> Resistance to ADT and abiraterone Short progression-free survival of CRPC patients 	(57, 59, 63, 69)
AR-V1	Dependent on cell context	<ul style="list-style-type: none"> Enriched in CRPC and metastases 	(63, 65, 68, 71)
AR-V9	Dependent on cell context	<ul style="list-style-type: none"> Resistance to ADT and abiraterone Short progression-free survival of CRPC patients 	(68, 69)

mechanisms enabling AR variants to enter the nucleus are less clear. Depending on the presence/absence of the hinge region, some constitutively active AR variants (i.e. AR-V567es) express a NLS, whereas others (i.e. AR-V3, AR-V7, AR-V9) do not (59, 62, 63, 64, 68). Studies have suggested that a NLS-like signal located in the unique COOH-terminal extension allows the NLS-negative AR variants to enter the nucleus (63, 68). However, Chan and coworkers reported that a truncated AR molecule consisting only of the AR NTD/DBD core exhibits a basal level of nuclear localization sufficient for ligand/androgen-independent transcriptional activity regardless of whether they harbour the exon 4-encoded NLS or NLS-like COOH-terminal extensions (70).

Two of the most well characterized AR-Vs, AR-V7 and AR-V567es, have demonstrated constitutive activity in different cell systems. The AR-V7 was originally discovered and functionally tested in the androgen-independent 22Rv1 and CWR-R1 cell lines (59, 62, 63). Specific depletion of endogenous AR-Vs in 22Rv1 cells and overexpression of AR-V7 in LNCaP cells resulted in decreased and increased growth, respectively, under androgen-depleted *in vitro* and *in vivo* conditions (59, 62, 71). AR-V567es was first identified in the LuCaP 86.2 and 136 PC xenografts, and found to increase proliferation of LNCaP cells in the absence of androgen as well as to enhance proliferation in response to very low levels of androgen (64). In preclinical *in vivo* models, expression of AR-V567es and AR-V7 has been shown to increase after castration and to confer both primary and acquired resistance to abiraterone and enzalutamide (64, 72, 73, 74, 75, 76, 77, 78). A recent paper furthermore displays constitutive activity of AR-V9 when transfected into the AR-positive LNCaP and the AR-negative DU145 cell lines (69), while AR-V9 was previously reported as conditionally

active due to transfected activity into LNCaP but not PC-3 cells (68). Conditionally active AR-Vs may depend on the expression of certain co-regulators, as discussed below in relation to the AR-V transcriptome.

The transcriptome of constitutively active AR variants

The wild-type AR is well known to mediate its transcriptional effects after forming homodimers in response to ligand binding. Whether or not AR-Vs also form homodimers or possibly heterodimers with the ARwt needs to be further clarified. Heterodimers of ARwt and AR-V7 have not been detected in the 22Rv1 or the CWR-R1 cell line (58, 62, 79), while AR-V567es has been shown to co-immunoprecipitate with ARwt in lysates from a patient-derived xenograft, indicating that AR-V/ARwt complexes are able to form (64). A recent study by Xu and coworkers characterized protein–protein interactions between AR-V7, AR-V567es and the ARwt in the PC3 cell line (80). They concluded that, in PC3 cells, both AR-V7 and AR-V567es could form heterodimers with ARwt and were also able to form homodimers in the absence of androgens. The hetero-dimerization of AR-Vs and ARwt was mediated by NTD and CTD interactions (NTD of AR-Vs and CTD of ARwt) and by DBD-to-DBD interactions, whereas AR-V homodimerization was mediated by DBD-to-DBD interactions only. PC3 cells with mutant AR-Vs that prevented DBD–DBD homodimerization abolished the ability of AR-Vs to induce transcription and to induce castration-resistant cell growth, suggesting that dimerization was required for AR-V function. In an earlier study where ARwt, AR-V7 and AR-V567es were ectopically expressed in COS-7 cells, AR-Vs could activate ARwt in the absence of androgen and facilitated nuclear translocation and transcriptional activity (75). The same study showed

that in 22Rv1 cells the PSA promoter was co-occupied by AR-V7 and ARwt, whereas the promoter of the *UBE2C* gene was bound only by AR-V7. From these results and with the knowledge that also when expressed at high levels in clinical samples, the AR-V seems to be co-expressed with the ARwt, it can be speculated that AR-V homodimers and AR-V/ARwt heterodimers form in parallel and have the potential to regulate different sets of target genes (75).

AR-Vs may transcribe canonically androgen-regulated genes or a unique subset of genes or possibly both. In clinical bone metastases, we found high AR-V7 levels to be associated with expression of certain canonical AR genes, such as *UBE2C*, *CCNA2*, *UGT2B17* and *C-MYC*, but not with others (*KLK3*, *KLK2*, *NKX3-1*, *TMPRSS2* and *FKBP5*) (65). Many functional studies have been performed to evaluate the AR-V transcriptome by ectopically expressing or knocking-down AR-Vs in different cell lines, but unfortunately without consistency (63, 64, 73, 81). Some studies have suggested sets of AR-V7-regulated genes such as *AKT1* (62) and genes associated with the M-phase cell cycle progression, including above-mentioned *UBE2C* and *CCNA2* (82) and the FOXA1-repressed target genes *EDN2* and *ETS2* (83). In contrast, chromatin immunoprecipitation sequencing (CHIP-seq) of the R1-D567 and R1-AD1 cell lines showed a high concordance between the AR-V567es and ARwt cistromes, with chromatin sites engaged by both AR-V567es and ARwt including *UBE2C*, *CCNA2*, *EDN2* and *ETS2* (84). *UBE2C* and *CCNA2* were also not unique targets for AR-Vs in the 22Rv1 and CWR-R1 cell lines, but also regulated by the ARwt (73, 81). Importantly, a recent paper highlighted frequent parallel synthesis of AR-V7 and AR-V9 in PC cell lines (and in patients) due to a tandem site for the cryptic exons giving rise to alternative splicing of those two variants (69). Without previous knowledge of this tandem site, recent studies performed to specifically quantify or knock-down the AR-V7 transcript may have been targeting the AR-V9 in parallel. The AR-V transcriptome therefore need to be further refined by precise knock-down of AR-V7 without affecting AR-V9 levels and vice versa. Furthermore, with specific antibodies now available for AR-V7, AR-V9 and other AR-Vs more specific immunohistochemistry and CHIP-seq studies can be performed in the future.

It should also be considered that the AR-V transcriptome may depend not only on the specific AR-V, but also conditionally on the cell type and the expression of specific AR co-regulators. The ARwt harbour activating functional domains both in the NTD (AF-1, Tau1 and Tau5/AF5) and the LBD (AF-2) and, while the AF-2 site

is lost in LDB-truncated AR-Vs, co-factors binding to the NTD could theoretically affect the activity of most AR-Vs, as recently reviewed in (85). Also co-factors that in the presence of androgens interact with the LBD of ARwt have been shown to enhance the activity of AR-Vs, probably by interaction through alternative domains. One interesting example is the more potent recruitment of PI3K/AKT phosphorylated MED1 to the enhancer region of the *UBE2C* promoter by AR-V567es compared to ARwt (86, 87, 88), something that may explain the high level of *UBE2C* expression seen in AR-V-driven tumours. The pioneer factors FOXA1 and GATA2 co-localize with both ARwt and AR-Vs on chromatin and increase their activities (88, 89), and FOXA1 was shown to be obligate for the proliferative effect of AR-Vs in CWR22Rv1 cells (90). GATA2 induces AR transcription (and thus potentially also AR-V expression) and as ligand-activated ARwt normally should repress *GATA2* expression, it may be that *GATA2* increases both expression and activity of ARwt and AR-Vs in androgen-deprived CRPC (89, 91, 92, 93). Further studies are needed to decipher how co-regulators, activators and repressors, interact with and facilitate the activity of different AR-Vs.

Origin of truncated AR variants

The molecular mechanisms mediating increased levels of truncated AR-Vs in CRPC tumour epithelial cells still need to be clarified. While most transcripts coding for truncated AR-Vs seem to arise from alternative splicing due to incorporation of cryptic exons in the *AR* (59, 62, 63) some may also be derived from exon skipping or by genetic deletions/rearrangement (55, 64). The enrichment of AR-Vs in CRPC may be related to the increased transcription of *AR*, observed in prostate epithelial cells after castration and in *AR* amplified tumours, or to structural *AR* rearrangements and/or aberrant expression of specific splice factors, as discussed below. Another possible explanation for the observed enrichment of AR-V in CRPC may be a concomitant reduction of micro-RNAs normally downregulating AR and AR-V levels (94, 95).

The abundance of specific splice variants may be controlled both by gene transcription rate and by splice factor recruitment to the pre-mRNA during the process of alternative splicing (96, 97, 98). An early observation was that specific inhibition of the ARwt protein in PC cell lines (via castration, antiandrogen treatment or siRNA) led to increased expression of AR-V7 but also of ARwt, and DHT treatment lead to decreased expression of both AR-V7 and ARwt (77, 82). Enzalutamide treatment was

shown to increase transcript levels of ARwt and AR-V7 and, furthermore, to increase the recruitment of the splicing factors to the binding sites near the 3' terminal cryptic exon of AR-V7. The ASF/SF2 and U2AF65 splicing factors were shown to be critical for the formation of AR-V7, while the knockdown of the hnRNPA1 splicing factor reduced levels of both the AR-V7 and the full-length AR transcript (99). Another study, however, reported enhanced recruitment of hnRNPA1 to the AR-V7 splicing sites in enzalutamide-resistant cells, while no change in recruitment of hnRNPA1 to the full-length AR mRNA was observed (100). The authors further provided evidence that alternative splicing and generation of AR-V7 might be regulated by hnRNPA1 through NF- κ B2/p52 and c-Myc. Another study implicated a role for the molecular chaperone HSP90 in the splicing of AR-V7, as HSP90 inhibition in PC cells with endogenous expression of AR-V7 lead to the disruption of AR-V7 splicing and reduced AR-V7 mRNA levels (101). Thus, increased transcription rate and subsequent recruitment of certain splice factors might be the link between castration, AR amplification and the enrichment of AR-Vs in relation to ARwt in CRPC cases.

Certain structural AR gene alterations such as a 8.5 kb deletion of the 5, 6 and 7 exons as well as a genomic inversion of the corresponding part have been identified and provided mechanisms for AR-V567es synthesis in the LuCaP 86.2 and LuCaP 136 xenograft models, respectively (54, 55). Diverse structural AR alterations have further been suggested to increase the probability for occurrence of alternative splicing; both in experimental models and clinical samples of CRPC. The 22Rv1 cell line harbours a 35 kb intragenic tandem duplication containing AR exon 3 and several 3' cryptic exons (CEs), including the terminal exon CE3 of AR-V7 and as subsequently shown also the CE5 of AR-V9 (53, 69). Structural AR events involving exon 3 were in parallel observed in clinical samples from CRPC patients by Li and coworkers. The high expression of AR-V7 in the CWR-R1 cell line was later linked to a 48 kb deletion in AR intron 1 (54).

More recent studies have strengthened the associations between AR amplification, structural AR alterations and the expression of truncated AR variants in clinical samples, by targeting DNA analysis of the AR and AR-V7 mRNA levels in metastatic tissue and liquid biopsies from CRPC patients (21, 56, 57). The studies by Henzler and De Laere further refined that AR rearrangements can occur in the context of AR amplification or at normal AR copy number and, furthermore, that some AR structural variations seem to be

associated with the presence of AR-V expression, whereas others are not. Further studies are needed to elucidate the functional importance of diverse structural AR alterations and the regulation of the RNA splicing program that seems to favour AR-V synthesis and/or stability as a survival strategy for PC cells in response to ADT.

Clinical relevance of truncated AR variants

As described earlier, AR-Vs can be detected in benign prostate tissue, hormone-naïve and CRPC, with the most frequent and highest expression detected in CRPC samples (20, 62, 63, 64, 65, 66, 67). AR-V7 is believed to be clinically relevant as it is frequently observed and abundantly expressed. In an early study of bone metastases from CRPC patients, we reported high AR-V7 mRNA expression to correlate with high AR-V protein levels and particular poor prognosis (65). In a recent study analysing AR-Vs in CTCs from CRPC patients, AR-V7 was reported as the most frequently occurring variant (12/15 patients), while the second most frequent variant, AR-V3 was more abundantly expressed (57). Expression of AR-V7 in primary prostate tumours has been shown to correlate with shorter time to disease relapse after radical prostatectomy (62, 63) and more rapid progression to CRPC (66). High levels of AR-V7 mRNA or nuclear AR-V7 protein or detectable expression of AR-V567es mRNA in CRPC tumours have been associated with poor patient survival (65, 66, 67). Thus, AR-V7 and possibly also other variants appear to be associated with the development of lethal PC.

In clinical CRPC specimens, individual AR-V transcripts are always co-expressed with the full-length AR transcript, but they are usually much less abundant (20). Nevertheless, metastasis levels of the AR-V proteins are comparable to that of the ARwt receptor in a substantial fraction of CRPC patients (65, 102). In samples where the AR-V7 mRNA levels constituted only 0.4–1% of the full-length AR mRNA levels, we observed a relative median AR-V protein expression of 32%, as determined by immunoblotting analysis using an antibody targeting the N-terminal of the AR. This could indicate post-transcriptionally stabilization of AR-Vs in selected CRPC tumours. Accordingly, *in vitro* studies have suggested that the AR-V7 protein may be more stable than the wild-type AR (101).

AR-Vs as therapy-predictive biomarkers

In a series of papers, detectable levels of AR-V7 and some other AR-Vs have been shown to predict poor

response to AR targeting therapies, as discussed below. In a prospective clinical study, Efstathiou and coworkers evaluated the prognostic impact of AR-V7 expression in metastasis biopsies. By using immunohistochemistry on FFPE specimens, AR-V7 protein levels were analysed in 60 patients with bone metastatic CRPC before and 8 weeks after enzalutamide treatment. The presence of AR-V7 staining was associated with primary resistance to enzalutamide (103). Sampling of metastasis biopsies from therapy responding and non-responding patients is extremely important to enable research studies where we can gain knowledge about the biology of CRPC. However, as CRPC metastases are primarily found in the bone marrow and in the skeleton and as sampling of such metastasis biopsies is logistically challenging, non-invasive liquid biopsies like CTCs and cfDNA may be preferred in studies aiming at monitoring the whole tumour burden of a patient.

In 2014, Antonarakis and coworkers measured mRNA expression of AR-V7 in CTCs from metastatic CRPC patients before starting treatment with enzalutamide or abiraterone. The presence of AR-V7-positive CTCs correlated with lower PSA response rate, shorter progression-free survival and reduced overall survival in both treatment groups (104). These findings were supported by another prospective study where the AR-V7 mRNA levels in CTCs were significantly higher in patients who had previously received abiraterone or enzalutamide compared to those who had not (48). Recently, Antonarakis and coworkers confirmed and extended their previous results by evaluating the predictive value of AR-V7-positive CTCs in a cohort of 202 CRPC patients starting enzalutamide or abiraterone treatment. Patients without detectable CTCs were found to have the longest progression-free and overall survival, while patients with AR-V7-positive CTCs showed the worst prognosis (105). The AR-V7 and AR-V567es transcripts have also been detected in CRPC patients by evaluation of whole blood (106, 107). In a retrospective study of 132 CRPC patients treated with either abiraterone or enzalutamide, KLK3 (PSA) and AR-V7 mRNA levels in whole blood were associated with short time to treatment failure and short overall survival (107). Novel data by Kohli and coworkers, however, provides evidence that previous measurements of the AR-V7 transcript using PCR-based assays may have misleadingly detected also the AR-V9 transcript due to a common 3' cryptic exon (69). By evaluating available RNA sequence data from metastases from two different CRPC cohorts, they found AR-V9 and AR-V7 mRNA levels in CRPC metastasis tissue to be highly correlated. They

furthermore found metastasis levels of AR-V9 together with AR-V3 to be significantly associated with short progression-free survival in 78 CRPC patients treated with abiraterone, while AR-V7 levels were borderline increased in poorly responding patients (69).

The role of AR-Vs in therapy resistance may not be limited to androgen-directed therapies. Preclinical studies have indicated that AR-Vs might also contribute to taxane resistance, although results are somewhat inconclusive (108, 109). In contrast, two prospective clinical trials showed no association between detection of AR-V7 in CTCs and primary resistance to taxane chemotherapy (110, 111). Instead, the clinical outcome for AR-V7-positive patients appeared to be better with taxanes than with AR-targeted therapies (110). This concept was also confirmed in a study where nuclear expression of the AR-V7 protein was measured in CTCs from 161 CRPC patients before treatment with antiandrogens or taxane chemotherapy. Patients with AR-V7-positive CTCs prior therapy exhibited superior clinical outcome with taxanes compared with antiandrogen therapy (112). These studies thus suggest that AR-V7 may serve as a predictive biomarker favouring docetaxel and cabazitaxel chemotherapy over treatments targeting the AR axis in CRPC patients. Still, CRPC patients would probably benefit from the development of more specific AR-V-targeting therapies.

Role of intra-tumoural steroidogenesis in CRPC

As described earlier, it is obvious that AR alterations in the epithelial tumour cells play a major role during development and growth of CRPC. In addition, intra-tumoural steroidogenesis may exist that potentiates AR activation in epithelial tumour cells but also has the possibility to affect AR-positive cells in the microenvironment, i.e. in stroma fibroblasts, smooth muscle cells, endothelial cells, osteoblasts and inflammatory cells, as reviewed in (14, 113). Numerous studies have reported persistent (or possibly returning) androgen levels in the prostate and in primary tumours after castration (114), and nuclear AR immunostaining can be seen not only in prostate epithelial but also in stroma cells after long-term castration (115). In both locally recurrent and metastatic CRPC, residual levels of testosterone and DHT have been reported at concentrations able to activate AR-mediated growth

in experimental PC models (116, 117). Also, adrenal androgens have been detected at biological relevant levels in PC after castration (118), and some studies suggest that circulating adrenal androgens contribute more than *de novo* synthesis to intra-tumoural steroid synthesis (102, 119, 120). In CRPC bone metastases, we found significantly increased expression of some enzymes; *AKR1C3* and *SRD5A1*, with the potential to convert the adrenal gland-derived steroids DHEA and androstenedione into testosterone and DHT, while levels of early steroidogenic enzymes converting cholesterol into DHEA and androstenedione (*CYP11A1*, *CYP17A1*, *HSD3B2*) were reduced compared to levels in prostate tissue (102). Notably, a large variation in androgen levels and in levels of steroid-converting enzymes has been reported between patients (102, 117). We found high *AKR1C3* protein levels to be almost mutually exclusive from detectable protein levels of constitutively active AR-Vs in CRPC bone metastases (102), suggesting that AR-Vs may not be selected for in metastases that have the potential to synthesize sufficient amounts of androgens to activate the wild-type AR.

Role of non-epithelial ARs in CRPC

When the AR is discussed in relation to castration resistance, it is generally assumed that only epithelial ARs are of importance, but this could be an oversimplification. Especially, cases with intra-tumoural steroidogenesis may have the possibility to affect AR-positive cells in the microenvironment, as discussed above. In the normal prostate, castration-induced tissue involution is dependent on effects in the androgen-regulated stroma and vasculature (14, 113, 121, 122, 123). Prostate tumours in patients are, for reasons largely unknown, characterized by a gradual loss of AR-positive cells in the tumour stroma, and this is in turn associated with a limited primary response to castration (124, 125, 126). Castration resistance can thus be linked not only to increased AR activity in tumour epithelial cells but also to decreased AR signalling in the tumour microenvironment. If these two events are functionally coupled is not known, but nevertheless, they may contribute to a limited castration response in metastatic cells adopted to a less androgen-dependent microenvironment than tumour cells within the prostate.

Accordingly, the primary response to castration in AR-positive tumour epithelial cells has proven to

be microenvironment dependent. In animals where AR-positive PC cells were injected both into the prostate and into the bone marrow, cells in the prostate responded considerably better to castration than those in the bone (127). This is apparently the case also in patients, as relapsed growth after castration and development of CRPC is far more common at metastatic sites (primarily in the bone marrow) than in the primary tumour. Cells in the bone marrow apparently influence tumour epithelial cells differently than those present in the prostate, and bone metastasis is thus the result of a complex reciprocal interaction between tumour cells and bone cells of different origin, as recently reviewed in (128). Metastatic cells in the bone environment are stimulated by survival factors released from activated osteoblasts and other cell types and by factors released from the bone matrix by resorbing osteoclasts. Cells of osteoblast and osteoclast origin have been shown to change AR co-regulator recruitment and activity (129). In turn, tumour cells feed this 'vicious cycle' through activation of osteoclastogenesis and subsequently the osteoblasts. Intra-tumoural steroidogenesis and local steroid secretion from metastatic cells probably affects AR-positive osteoblasts and may contribute to the sclerotic phenotype of PC bone metastases. Furthermore, the AR is present in various cell types of the immune system (130) and factors produced by macrophages (131, 132, 133, 134) and by lymphocytes (135) have been reported to influence AR signalling and the induction of castration resistance in PC cells.

Collectively, these studies suggest that even though the AR in tumour epithelial cells is of major importance in CRPC, the presence or loss of AR signalling in cells of the tumour/metastasis microenvironment also need to be considered. The importance of local steroidogenesis as well as of tumour cell interactions with androgen-responsive and non-androgen-responsive cells in the metastasis stroma for the establishment of bone metastasis and the development of CRPC need to be examined in more detail. With this, possibilities for novel therapies of CRPC targeting not only the tumour cells but also the metastasis stroma may arise.

Conclusion and future directions

Continuously accumulating evidence indicates that the AR is a main driver of CRPC. In addition to AR amplification and overexpression, also AR mutations, structural alterations and the enrichment of constitutively

active AR-Vs appear to be of high clinical importance during development of CRPC and acquired resistance to ADT and AR antagonists (Fig. 1). Currently, clinical trials are ongoing where the predictive value of AR mutations and AR-V expression is evaluated in relation to patient response/resistance to enzalutamide, abiraterone and other treatments for CRPC. Hopefully, those studies will demonstrate the value of measuring specific AR variants and mutations for therapeutic stratification. Furthermore, novel compound for targeting of constitutively active AR variants and/or receptors with activating mutations are under evaluation in clinical trials (Table 3 and www.clinicaltrials.gov). As recently reviewed (85, 136), preclinical studies have shown promising results for disrupting AR-V signalling by various strategies including targeting the AR NTD or DBD, reducing AR-V expression, promoting AR protein degradation, disrupting AR-V dimerization and chromatin binding, but further studies need to prove their clinical value. Alternatively, co-regulators to the AR and/or AR-Vs or their downstream gene products may be targeted. A novel AR antagonist, ODM-201, has been reported to overcome enzalutamide and apalutamide resistance and is currently in clinical trials (137, 138), so is also EPI-506, an AR antagonist that binds to

the AR NTD and thus has the potential to inhibit not only full-length AR but also LBD-truncated variants (139, 140, 141, 142). Response to EPI-506 will be evaluated in relation to AR-V7 CTC levels, in CRPC patients previously treated with enzalutamide and/or abiraterone (NCT02606123). Another highly interesting drug, galeterone, has been reported to promote degradation of the AR and AR-Vs and at the same time inhibit steroid synthesis and act as an AR antagonist (143, 144, 145, 146, 147). Galeterone is compared to enzalutamide for treatment of AR-V7 positive CRPC patients (NCT02438007). Also niclosamide is in clinical trial for treatment of CRPC patients with AR-V-positive tumors (NCT02532114), and has been shown to inhibit AR-V7 transcriptional activity and promote its proteasomal degradation to overcome enzalutamide and abiraterone resistance in preclinical models (148, 149, 150). Further compounds in clinical trials with suggested inhibitory effects on constitutively active AR variants include BET inhibitors, HSP90 inhibitors and antisense oligonucleotides (Table 3) (78, 101, 151, 152).

Novel treatments that will improve survival of men with metastatic PC may include not only more efficient drugs for treating CRPC, but also better strategies for primary treatment of M1 patients. Early chemotherapy

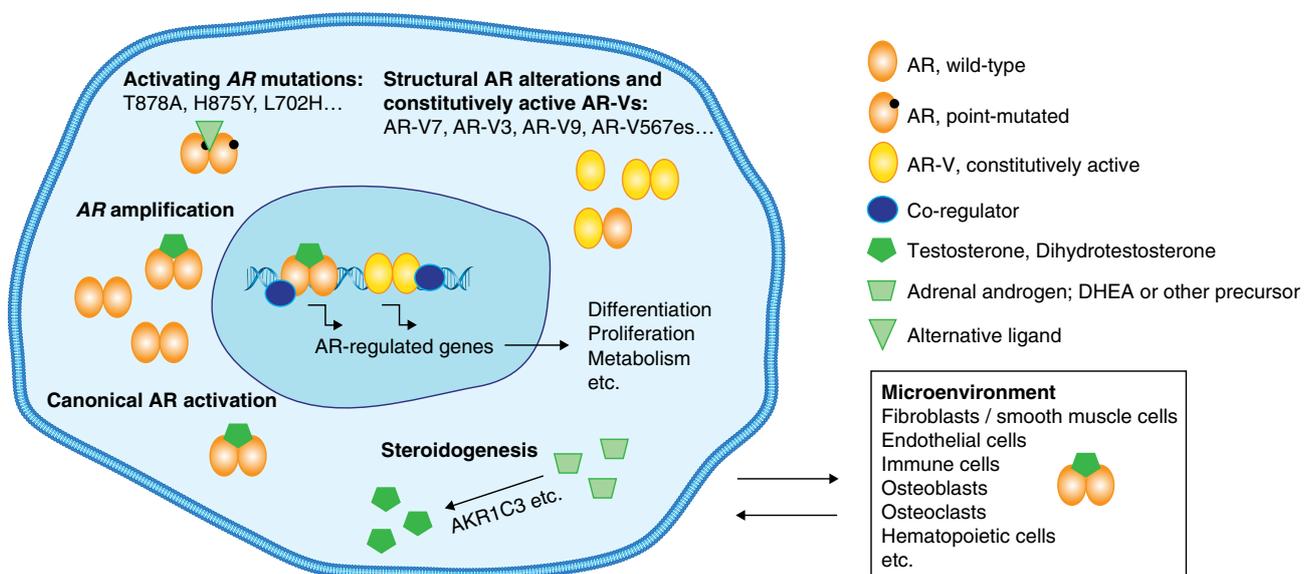


Figure 1

Different molecular explanations behind androgen receptor (AR) activation in castration-resistant prostate cancer, illustrated by an epithelial cancer cell interacting with AR-positive and non-AR-positive cells in the microenvironment of a bone metastasis. AR amplification results in AR overexpression and possibilities for canonical AR signalling at low androgen concentration. Activating AR point mutations result in promiscuous receptors activated by weak androgens, other steroids and glucocorticoids as well as in turning AR antagonists into agonists. Structural AR alterations give enrichment of constitutively active AR variants (AR-Vs) that are able to dimerize, translocate into the nucleus and activate transcription of AR-regulated genes without the need for ligand binding. Tumour cells have the machinery for metabolizing weak androgens (i.e. adrenal-derived DHEA and androstenedione) into the more potent androgens testosterone and dihydrotestosterone. Different molecular mechanisms behind AR activation are seen in individual bone metastases, giving possibilities for therapy stratification in individual patients.

Table 3 Novel compounds in clinical trials for treatment of CRPC with suggested mechanisms targeting the AR and/or its constitutively active variants.^a

Compound	Suggested mechanism	References ^a
ODM-201	<ul style="list-style-type: none"> AR antagonist, inhibiting nuclear translocation of AR including <i>T878A</i>, <i>W742L</i>, <i>F877L</i> mutants 	(137, 138)
EPI-506	<ul style="list-style-type: none"> AR antagonist binding the AR NTD 	(139, 140, 141, 142)
Galeterone	<ul style="list-style-type: none"> Inhibits CYP17 AR antagonist Induces proteasomal degradation of AR and AR variants 	(143, 144, 145, 146, 147)
Niclosamide	<ul style="list-style-type: none"> Inhibits AR-V7 transcriptional activity Promotes AR-V7 proteasomal degradation 	(148, 149, 150)
JQ1, OTX015	<ul style="list-style-type: none"> Bromodomain and extra-terminal (BET) inhibitors disrupting interactions between AR NTD, co-factors and chromatin, inhibiting transcriptional activity 	(152)
Onalespib	<ul style="list-style-type: none"> HSP90 inhibitor blocking AR-V7 mRNA splicing 	(101)
EZN-4176, AZD-5312	<ul style="list-style-type: none"> Antisense oligonucleotides (ASOs) targeting expression of AR and AR variants 	(78)

^aFor clinical trials see www.clinicaltrials.gov.

with docetaxel given directly following ADT was recently shown to improve survival (153) and is now in clinical praxis. Now, interim results from the LATITUDE and STAMPEDE studies combining ADT with abiraterone for upfront treatment of metastasized PC show promising results with improved progression-free survival compared to standard ADT treatment (154, 155). Early abiraterone treatment of M1 patients may have the benefit of lowering residual steroid levels in metastases by inhibiting not only testicular and adrenal gland, but also intra-tumoural steroidogenesis and, consequently, lead to a more pronounced castration effect in tumour cells and also in AR-positive cells in the tumour microenvironment (Fig. 1).

Possible benefits of combining ADT with other therapies in early treatment of M1 patients are highly underexplored. Recent studies have proven the existence of prostate tumours of diverse molecular subtypes, based on somatic genetic aberrations or differential gene expression pattern (22, 156). You and coworkers performed meta-analysis of RNA profiles in primary prostate tumours and identified three PC subtypes, PCS1–3, with different phenotypic characteristics and prognosis (156). Pathway analysis of subtype-enriched genes revealed diverse cellular processes in the different subtypes, indicating possibilities for treatment stratification. Importantly, metastases can also be differentiated with respect to PCS1–3 related RNA profiles. By analysing subtype-enriched genes in biopsies from 69 bone metastases (patients), we found PCS2 to be the most frequent subtype (Thysell E, Ylitalo EB, Jernberg E, Bergh A & Wikström P, unpublished observations). The PCS2 bone metastases (77%) were characterized by AR activity and metabolic processes associated with differentiated prostate epithelial cells (i.e. lipid and sterol biosynthesis, PSA expression), while PCS1 cases showed cellular dedifferentiation and high proliferation and PCS3 appeared

to be non-AR driven and more immunogenic (5; Thysell E, Ylitalo EB, Jernberg E, Bergh A & Wikström P, unpublished observations). AR amplification and high AR-V expression were seen in both PCS1 and PCS2 bone metastases (21; Thysell E, Ylitalo EB, Jernberg E, Bergh A & Wikström P, unpublished observations). Further studies are needed to explore if improved therapeutic results for PCS1 and PCS2 could be obtained by combining AR-directed therapies with treatment strategies selected based on tumour subtype. The PCS3 metastases will probably not respond to AR-directed therapies, but will need other therapeutic strategies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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