Review Article

# Decoding the androgen receptor splice variants

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**Abstract:** In the past five years, multiple structurally and functionally distinct androgen receptor (AR) splice variants have been decoded and characterized. The mature transcripts for the majority of the fully decoded AR splice variants contain a transcribed "intronic" sequence, capable of encoding a short variant-specific peptide to replace the AR ligand-binding domain (LBD). Functionally, AR splice variants represent a diverse group of molecules often demonstrating cell context-specific genomic functions that may or may not be coupled with the functions of the canonical full-length AR (AR-FL). However, the full spectrum of their functional diversity and the underlying mechanistic basis remains very poorly characterized. In clinical specimens derived from men treated with a variety of hormone therapy regimens, AR splice variants are almost always expressed at detectable, yet lower levels when compared to that of AR-FL. In spite of the collective *in vitro* data supporting the putative role of AR splice variants in therapeutic resistance to hormone therapies, the extent to which AR splice variants mediate resistance to each individual regimen is not known and awaits thorough investigations in a clinically relevant setting using specimens from men undergoing treatments. Among the AR splice variants, AR-V7 is more abundantly and frequently expressed in castration-resistant prostate cancer (CRPC) and remains the most important variant identified so far. The relative importance of different AR molecules, including AR-FL, should be functionally dissected in the setting of castration-resistant prostate cancer, particularly in tumors resistant to more potent inhibitors of AR-FL recently approved by the FDA. In this review, we will focus on the discovery and characterization of AR splice variants, their putative functions and roles in mediating constitutively active AR signaling, and key areas of investigation that are necessary to establish their clinical relevance.

**Keywords:** Androgen receptor (AR); AR Splice Variants; AR signaling; full-length AR (AR-FL); prostate cancer; castration-resistant prostate cancer (CRPC)

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## Introduction

Prostate cancer is a common cause of cancer-related death in aging males in the Western world. Because hormone therapy is the standard of care for men with metastatic prostate cancer, most men succumb to the disease following developing resistance to at least one of the hormone therapy regimens. Castration-resistant prostate cancer (CRPC) is a term used to describe prostate cancers that relapse following first-line hormone therapy (1). It is known that CRPC is not completely refractory to further hormonal manipulation, and AR signaling remains as a pivotal driver for disease progression despite castrate levels of androgens (2,3). Sustained AR signaling may be mediated by a number of mechanisms, including AR gene amplification and overexpression (4-6), intra-tumoral androgen synthesis (7), overexpression of AR coactivators (8), aberrant kinase pathway activation (9-11), AR mutation (12) , and constitutively active AR splice variants (13).

Novel therapies have been recently developed to treat CRPC patients by targeting overexpressed AR and intra-tumoral androgen synthesis. Abiraterone acetate, designed to inhibit CYP17A1, was approved on November 2011 for treating metastatic CRPC previously treated with

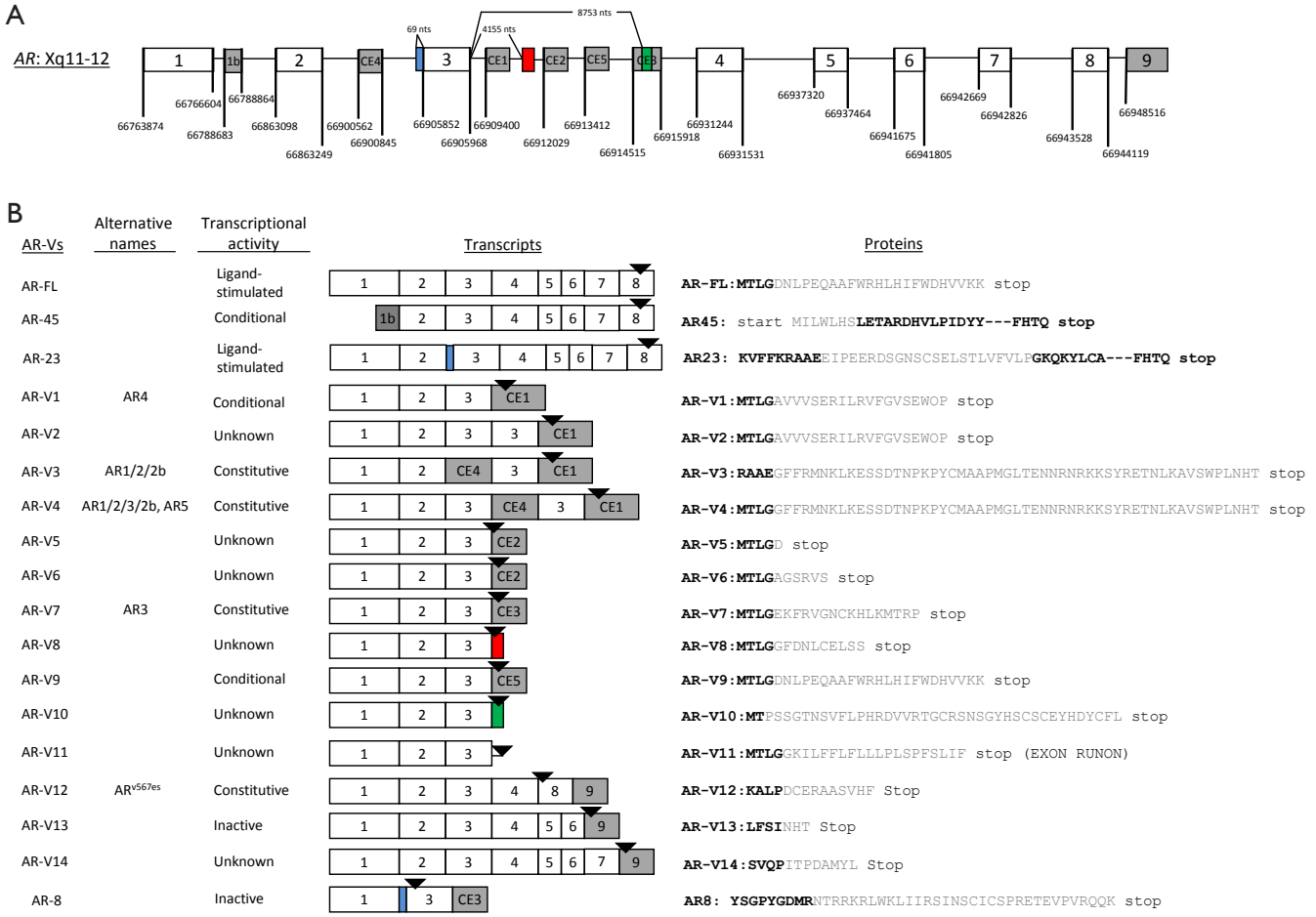


**Figure 1** Decoding the androgen receptor splice variant transcripts. (A) AR gene structure with canonical and cryptic exon splice junctions marked according to GRCh37/hg19 human genome seqeunces (not drawn to scale); (B) Nomenclature, functional annotation, exon compositions, and variant-specific mRNA (color matched to *Figure 1A*) and peptide sequences (in gray).

docetaxol (14), with expanded indication approved on December 2012 to include patients who did not receive prior docetaxol (15). Enzalutamide, a more potent anti-androgen, was approved on August 2012 for post-docetaxol metastatic CRPC (16). The successful clinical development of these two new agents (14-16) underscores the importance of understanding the mechanism of sustained AR signaling in CRPC. In this light, most AR splice variants identified so far do not contain the intended therapeutic target, the AR ligand-binding domain (LBD), for any of the existing hormone therapy regimens including the two new agents. In this review, we will discuss discovery and characterization of the structural and functional diversity of AR splice variants for which the key features have been documented in the literature (key features of the 18 AR splice variants are summarized in *Figure 1*), their potential roles in mediating constitutively active AR signaling, and key areas of investigation to establish them as a mechanism of CRPC, particularly in the setting of resistance to abiraterone and enzalutamide.

## The canonical AR-FL

In a normal male genome, there is only one copy of the AR gene located on Xq11-12. The AR gene is considered the most important gene in prostate cancer. The AR-FL cDNA was first cloned in 1988 (17). Structurally AR-FL resembles other nuclear receptors, containing a highly conserved DNA binding domain (DBD) encoded by Exon 2/3, a ligand binding domain (LBD) encoded by Exon 4-8 at C-terminus

 

with lower sequence homology, a poorly conserved N-terminal domain (NTD) encoded by Exon 1, as well as a hinge region encoded by Exon 3/4 (18). Unique to AR, it has a long NTD domain (~538 amino acids) harboring two transactivating regions, termed transcription activation unit 1 (TAU1) and 5 (TAU5), that are indispensible for AR activation (19).

## Earlier reports of AR splice variants: AR45 and AR23

In 2005, a NTD-truncated AR isoform with a deduced molecular weight around 45 kDa (so called AR45) was discovered by 5' rapid amplification of cDNA ends (5' RACE) from human placenta RNA (20). AR45 contains an intact DBD, hinge region, LBD, and a novel seven amino-acid long N-terminal peptide encoded by the novel exon 1b located ~22.1 kb downstream of AR exon 1 (*Figure 1*). The AR45 mRNA was found mainly in heart but also detected in skeletal muscle, uterus, prostate, breast, and lung (20). Exogeneously expressed AR45 did not stimulate the transactivation of androgen response element (ARE)-luciferase reporter in the presence of ligand. AR45 was proposed as a dominant negative AR that suppresses the function of AR-FL (20). In 2007, another AR splice variant, named AR23, was identified from a CRPC bone metastasis specimen (21). AR23 resulted from aberrant splicing of a 69 bp intron 2 sequence (corresponding to 23-amino acid residues), leading to in-frame insertion of a 23-amino acid sequence between two zinc fingers of the DBD (*Figure 1*). The genomic function of AR23 was not established because it does not translocate to the nucleus upon ligand binding, though cytoplasmic AR23 was partially active in androgen-responsive promoter reporter assays (21).

## AR Splice variants lacking LBD due to splicing of cryptic exons

AR splice variants drew more attention since 2008 primarily due to the discovery of a number of variants that lack LBD. Such variants have the potential to mediate constitutively active AR function, on the basis of earlier *in vitro* studies on AR deletion constructs generated in the laboratory (22). In 2008, Dehm *et al.* performed 3' RACE with primers anchored at exon 1 and identified a new exon (termed exon 2b) 37 kb downstream of exon 2, in the CWR22Rv1 cell line that demonstrated ligand-independent AR activity (23). Splicing of exon 2b yielded two novel C-terminally truncated AR variants, AR1/2/2b and AR1/2/3/2b (23). Due to the presence of stop codons in exon 2b, LBD was replaced by the variant-specific 11-aa peptide encoded by exon 2b. Both variants demonstrated constitutively active AR function by *in vitro* luciferase reporter assays. AR1/2/2b also lacks the second zinc finger of the DBD, while AR1/2/3/2b retains the entire DBD. Because the transcript structure of AR1/2/3/2b was explained by a duplicated DNA sequence unique to CWR22Rv1 cells (24), this variant was thought to be specific to this cell line. In contrast, AR1/2/2b was more commonly found in other PCa cell lines, including VCaP, LNCaP, and LAPC4, as well as PCa xenografts (23).

In 2009, Hu *et al.* reported the identification of more AR cryptic exons in both cell lines and clinical specimens (25). Using a strategy combining exhaustive analysis of expressed sequence tags mapped to the human AR locus and experimental cloning to determine the precise splice junctions, Hu *et al.* identified three cryptic exons named CE1, CE2, and CE3 in intron 3, and CE4, identical to exon 2b (23) discovered by Dehm *et al.* (25). Splicing of the cryptic exons generated seven AR splice variants (named AR-V1 to AR-V7) (*Figure 1*), all lacking LBD due to stop codons present in the transcribed "intronic" sequences (i.e., cryptic exons). Among these, AR-V1 and AR-V7 were readily detectable in clinical prostate cancer specimens, with ~20-fold higher levels detected in CRPC specimens compared to hormone naïve prostate tumors. Importantly, a variant-specific antibody was developed for AR-V7, and used to detect the translated product of AR-V7 in prostate cancer cell lines and xenografts. Both PSA reporter assays and expression microarray analysis confirmed that AR-V7 was constitutively active in driving expression of canonical androgen-responsive genes (e.g., KLK3, KLK2, and NKX3.1) in an androgen-independent manner (25).

Guo *et al.* reported the discovery of LBD-truncated variants AR3, AR4, and AR5 using 3' RACE in 2009 (26). AR3, AR4, and AR5 contained coding sequences identical to those in AR-V7, AR-V1, and AR-V4, respectively. A variant-specific polyclonal antibody was also developed for AR3 (AR-V7), and used to detect protein expression in both hormone naïve and CRPC specimens. In addition, knockdown of AR3 in CWR22Rv1/CWR-R1 cells revealed a set of 117 genes that were preferentially regulated by AR3 (26). This study also reported the cloning of multiple additional variants that were not further characterized.

 

## AR splice variants discovered by other approaches

Combining 3' RACE with next generation sequencing, Watson *et al.* not only confirmed the known AR-V1 and AR-V7, but also found 4 more AR splice variants named AR-V8 to AR-V11 (27) (*Figure 1*). This experiment was carried out in the VCaP cells, a prostate cancer cell line derived from vertebral metastatic lesion of a CRPC patient (27,28) that was shown to express the AR-V7 protein (25). These four new AR variants show splicing junctions between exon 3 and different regions of intron 3, with the predicted AR variant proteins truncated after AR DBD with 10-39 amino acid extension before the stop codon (*Figure 1*). Using VCaP xenograft in SCID mice, Watson *et al.* found that AR-Vs (AR-V7 and AR-V1) and AR-FL were upregulated by castration in both mRNA and protein levels, while re-administration of testosterone suppressed the expression of both AR-FL and AR-V7 in VCaP cells. Similar regulation of AR-FL and AR-Vs by androgens was also demonstrated in LuCaP35 xenografts with modest variation. However, only AR-V7, but not AR-V1, conferred gain-of-function on accelerating the LNCaP xenograft growth in castrated mice and colony formation in soft-agar assay (27).

More recently, Hu *et al.* employed a modified RNA amplification method, termed selective linear amplification of sense RNA (SLASR), for unbiased detection of transcribed AR sequences using arrayed 60-mer probes tiled across the human *AR* gene locus, directly in clinical CRPC specimens (29). This study provided a snapshot of the expression peaks along genomic sequences downstream of AR exon 3 and identified 3 new variants named AR-V12 to AR-V14 (*Figure 1*). Importantly, this study revealed expression peaks within intron 3 as well as sequences further downstream of exon 8 (named exon 9). These previously unappreciated expressed sequences have the potential to participate in AR splicing. One example is AR-V12 (*Figure 1*), which has the same open reading frame with $AR^{v567es}$ (see below) but contained untranslated sequences mapped to exon 9.

## $AR^{v567es}$ and AR8

Sun *et al.* investigated the AR isoforms in a panel of 25 LuCaP prostate cancer xenografts (30). With a primer set anchored on exons 2 and 8 in RT-PCR, a short AR transcript spanning exon 2 to 8 was discovered. Sequencing revealed a novel AR variant arising from skipping of exons 5 to 7 while retaining the full sequence of exons 1 to 4 and exon 8. This new variant is named $AR^{v567es}$ (30). This exon combination (1/2/3/4/8) shifts the open reading frame (ORF) of $AR^{v567es}$ to an early stop codon just after the first 29 nucleotides of exon 8 (*Figure 1*). $AR^{v567es}$ is unique in that it retains the full hinge domain encoded by part of exons 3 and 4. The AR hinge domain contained important sequences for AR localization and activity (31). Similar to AR-V7, $AR^{v567es}$ activates androgen-responsive genes (such as *KLK3*, *TMPRSS2*, and *NKX3.1*) in a hormone-independent manner when ectopically expressed in LNCaP cells (30). The coding sequence for $AR^{v567es}$ is identical to AR-V12, which is encoded by a transcript containing exons 1/2/3/4/8/9 as later reported in Hu *et al.* (29) (*Figure 1*). However, more in-depth studies of $AR^{v567es}$ have been hampered by lack of a variant-specific antibody, as well as lack of suitable sequences to target for variant-specific knockdown that is an important tool to determine protein translation and function.

In CWR-R1 cells, Yang *et al.* identified a membrane-associated AR variant, named AR8 by RACE (32). Using an alternative splicing acceptor site 186 bp upstream of exon 3, the deduced protein of AR8 (Exon1-3'-3-3b/CE3) contained 33 unique amino acids after the NTD domain. Higher expression level of AR8 was detected in castration-resistant cell lines (C4-2, C4-2B, CWR22Rv1). This C-terminal truncated AR-V has no DBD or LBD and no transactivating function in ARE-luciferace reporter assay. Possibly due to palmitoylation of two cysteine residues within its unique C-terminal sequence, this protein was found mainly in plasma membrane when overexpressed in COS-1 cells or PCa cells (LNCaP and CWR-R1). Membrane-bound AR8 complexes with AR-FL and EGFR and may serve as a mediator in Src-induced AR activation (32).

## Nuclear localization of AR splice variants

A prerequisite for AR to exert its genomic function is to enter the nucleus. Upon androgen binding to LBD, AR-FL exposes a nuclear localization signal (NLS) within C-terminal end (CTE) of DBD and hinge region to interact with importin proteins for translocation through the nuclear pore complex (33). A canonical bipartite nuclear localization signal (NLS) was mapped at the junction of DBD and the hinge region (amino acid 617-RKCYEAGMTLG--ARKLKK-633) (34). With the exception of AR-12/$AR^{v567es}$, other AR isoforms may have variable capability in nuclear import due to loss of NLS. Evidence provided by

 

immunofluorescent staining supports constitutive nuclear localization of AR-V7/AR3 and AR-12/AR$^{v567es}$ in the absence of androgens, while AR-V1, AR-V9, and AR-V13 are mainly cytoplasmic (25,27,29,30), possibly due to lack of basic amino acids characteristic of the bipartite nuclear localization sequence (NLS) (35). Interestingly, genomic functions of AR-Vs do not always parallel to their localization. For example, AR-V1 and AR-V9 showed ligand-independent activity in LNCaP cells but not in PC-3 cells (29). Such variants were termed "conditionally active" variants (29), to differentiate them from constitutively active variants (see below), because their functions are conditional on the cellular context. To further understand the nuclear transport of AR-V7 and its relation with AR-V transcriptional function, Chan *et al.* showed that part of its unique sequence at C-terminus (aa 628-EKFRVGNCKHLKMTRP-643) resembles the truncated bipartite AR NLS. Mutation of amino acid residues K629 and R631 to alanine in AR-V7 shifted its expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern; while alanine mutation at K636 or K639 had no effect on nuclear localization of AR-V7 (36).

## Diverse and cell-specific functions of AR splice variants

Among the AR-Vs listed in *Figure 1*, AR-V7 (also named AR3) and AR$^{v567es}$ have received more attention due to their unequivocal constitutively active nuclear functions (25,26,30). Both AR-Vs activate transcription of canonical AR-FL target genes when overexpressed in cell lines with or without activated AR-FL. Other AR-Vs may be conditionally active, i.e., their transcriptional activities are cell type-specific (37). For example, AR-V1 and AR-V9 demonstrated transcriptional activity when introduced in AR-FL positive LNCaP cells but not in the AR-FL negative PC-3 cells (37). It is possible that the conditional activity of AR-V1 and AR-V9 may require nuclear localization that was not readily detected by immunofluorescence. Previous studies showed androgen receptor (AR) deletion mutants that retain a partially truncated LBD did not have constitutive activity (22,35,38). Hu *et al.* demonstrated examples of inactive AR splice variants that retain a partially truncated LBD, including AR-V13 and AR-V14 (37) (*Figure 1*).

Expression levels of AR-V7 are dramatically increased after suppression of AR-FL signaling by androgen depletion, AR-FL knockdown, or treatment with enzalutamide in VCaP cells and LNCaP95 cells but not in LNCaP and CWR22Rv1 cells, suggesting that in addition to cell-context specific functions of AR splice variants, the regulation of AR variant levels may also depend on a specific cellular context (39).

## Molecular origin of AR splice variants

In clinical specimens, AR splice variants coexist with AR-FL, and the expression levels of individual AR variants almost always constitute a small fraction of the expression level of AR-FL (25,27). In addition, AR splice variants are also expressed in benign prostate epithelium (25,30), again at a much lower level relative to AR-FL. AR-FL is often overexpressed in CRPC due to AR gene amplification (40,41) or other genomic changes (42). In addition, elevated AR expression in CRPC may involve AR self regulation. Cai *et al.* showed that lysine-specific demethylation-1 (LSD-1) was recruited to AREs in intron 2 of the AR gene and acts as a repressor when AR-FL was activated. This recruitment was abolished when androgen was depleted (43). It is therefore possible that expression of AR splice variants are generally coupled with the transcriptional output from the AR gene locus (44). Supporting this possibility, AR variant levels were downregulated by testosterone replacement in castrated mice in parallel with a decrease of AR-FL in VCaP and LuCaP35 xenografts (27). In cell line models (VCaP and LNCaP95) with higher levels of AR-FL and low levels of AR variants, suppression of AR-FL signaling by enzalutamide resulted in an unequivocal increase of AR-V7, and a relatively moderate increase of AR-FL (39). In addition, increased expression of AR-FL, AR-V7 and AR$^{v567es}$ was also observed in castration-resistant LuCaP xenograft (LuCaP23CR and LuCaP35CR) when AR-FL signaling was inhibited by abiraterone (45). Thus, although AR-V expression may not strictly parallel that of AR-FL, and the magnitude of AR-V mRNA increase is generally greater than that of AR-FL in CRPC and in experimental models (39), AR-V expression is strongly coupled with AR-FL expression.

In some cell line and xenograft models (e.g., CWR22 and LuCaP86.2), AR intragenic rearrangement or deletions may be responsible for high AR variant expression. In CWR22Rv1 cells, an intragenic copy number increase occurred in an approximately 35-kb AR genomic segment between introns 2 and 3, with the rearranged segment flanked by long interspersed nuclear element (5'-LINE-1 and 3'-LINE-1) (46). To further investigate the association of focal imbalance of the AR gene and AR variant expression,

 

Li *et al.* demonstrated a complex pattern of focal copy number imbalance with or without AR gene amplification. In LuCaP 86.2 xenograft cells, an 8579-bp deletion of AR exons 5, 6, and 7 may be responsible for the high level of $AR^{v567es}$ variant expression (47). The extent to which similar genomic alterations contribute to the generation of AR splice variants in clinical specimens remains unclear.

## Detection of AR splice variants in clinical specimens

The majority of AR-Vs listed in *Figure 1* can be detected in prostate cancer tissue specimens by RT-PCR (25-27, 32,37). Alternatively spliced transcripts containing premature stop codons may be degraded through the nonsense-mediated decay (NMD) mechanism (48). Therefore it is also critical to demonstrate protein expression by detecting the corresponding variant protein product in order to draw functional relevance. Variant-specific antibodies have been reported for AR-V7 (AR3) (25,26), AR8 (49), and AR1/2/2b (50). In all these efforts, the variant-specific c-terminal peptides were used (*Figure 1*) as antigens. Among these, AR-V7/AR3 remains the only AR splice variant with a proven protein product that can be detected in clinical specimens using variant-specific antibodies (25,26,39), including a monoclonal antibody to AR-V7 (39). An alternative approach to detect the potential existence of LBD-truncated AR variants is to combine data obtained using antibodies recognizing AR-NTD and AR-LBD, respectively. For example, Zhang *et al.* showed a wide distribution of the AR-NTD/LBD ratio in clinical CRPC specimens (51). Higher ratios of NTD/LBD were detected in more aggressive tumors. However, this approach is based on the assumption that excess AR-NTD detected in CRPC specimens originated from the expression of AR-Vs.

## Genomic functions of AR splice variants

A key question in relation to the genomic functions of AR splice variants is whether they active the same transcriptional programs directed by AR-FL. Hu *et al.* showed data suggesting that endogenously induced AR variants are not sufficient to "rescue" the suppressed AR-FL, when a set of canonical AR-FL target genes are evaluated (39). Instead, increased expression of AR variant paralleled the increased expression of cell cycle genes, and forced expression of both AR-V7 and $AR^{v567ES}$ induced the same set of cell cycle genes in both the presence of absence of canonical AR-FL signaling (39). Li *et al.* performed gene expression profiling in rearrangement-driven AR-V positive cells following specific knockdown of the AR-FL and AR-Vs to differentiate genes activated by the two different receptor molecules (52). AR-V-dependent cell cycle genes were found to demonstrate a biphasic response. They were induced at low AR-V levels but repressed when higher AR-Vs were expressed in the cells. This observation mirrors the canonical biphasic androgen-stimulated (i.e., AR-FL-mediated) growth response observed in cell line models. The findings suggest that AR-V expression reactivates and restores the AR-FL transcriptional programs, rather than by targeting a unique set of genes. These seemingly opposing findings may be explained by cell-context differences as well as the different methodologies used in the studies. More in-depth analysis will address cell-context specific genomic functions mediated by the AR splice variants.

## Future directions and priorities

In spite of intense interest in the putative role of AR splice variants in CRPC and the years that have elapsed since their discovery and characterization, the field is still in infancy and investigations encompassing the full spectrum of mechanistic characterization and clinical translation are still at a nascent stage. Successful clinical development of abiraterone and enzalutamide (14-16), both intended to target the AR LBD (which is missing in AR-Vs), is directly driven by laboratory mechanistic studies establishing intra-tumoral androgens and AR protein overexpression as the key molecular determinants of CRPC (2). Thus we envision that mechanistic studies dissecting the genomic functions of different AR molecules will facilitate efforts in developing new therapies to overcome resistance to abiraterone and enzalutamide. Given the expanded clinical use of abiraterone and enzalutamide, there is an urgent need to dissect the various putative mechanisms of resistance to these new, more potent inhibitors of AR-FL signaling. Although AR splice variants provide a biologically plausible explanation for therapeutic resistance, the concept has not been validated in clinical specimens due to the recent approval of the two new agents, and consequently lack of sufficient number of relevant specimens collected from treated patients. Nevertheless, the discovery of AR splice variants has already stimulated efforts to develop novel agents that target all AR molecules to overcome resistance (53-64). Further conceptual advances in the field will provide a sustained impetus for such efforts.

 

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## Footnote

*Conflicts of Interest*: The authors have no conflicts of interest to declare.

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