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REVIEW

The Link Between Androgen Receptor Splice Variants and Castration-Resistant Prostate Cancer

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Abstract Resistance to the latest advanced prostate cancer therapies, including abiraterone and enzalutamide, is associated with increased expression of constitutively active androgen receptor splice variants (AR-Vs). The exact mechanism by which these therapies result in AR-Vs is unknown, but may include genomic rearrangement of the androgen receptor gene as well as alternative splicing of the AR pre-messenger RNA (mRNA). An additional complication that hinders further development of effective AR strategies is that the mechanisms by which the directed therapies are bypassed may vary. Finally, the question must be addressed as to whether the androgen receptor remains to be the driver of most castration resistant disease or whether truly AR-independent tumors arise after successful androgen ablation therapy. In this review, we will examine androgen receptor splice variants as an alternative mechanism by which prostate cancer becomes resistant to androgen receptor-directed therapy.

Introduction

In 1941, Huggins first demonstrated that castration was an effective treatment for men with prostate cancer [1]; since then, efforts have continued to focus on ablating androgen receptor (AR) signaling in recurrent prostate cancer. Unfortunately, although androgen ablation is initially effective in suppressing prostate cancer growth in over 80 % of patients, cancer recurs in almost all of these patients. Furthermore, the recurrences most commonly are associated with the reactivation of AR signaling [2]. This seems to be the case regardless of the mechanism used to inhibit AR transactivation, e.g., inhibition of ligand binding with agents such as bicalutamide, enzalutamide (MDV-3100), or AR509; or inhibition of hormone synthesis with CYP17 agonists such as ketoconazole or abiraterone (Fig. 1) [11–14]. Development of effective inhibitory AR treatments is further hindered by that fact that mechanisms of resistance to current therapies vary from amplification of the AR to production of intratumoral hormones to the appearance of constitutively active AR splice variants or a combination thereof (Fig. 1) [15, 16]. Finally, it is not known if AR remains the driver of most castration resistant disease or whether truly AR-independent tumors arise after successful androgen ablation therapy; the proportion of recurrences that develop a more neuroendocrine (non-AR driven) phenotype are unknown but are estimated to be much rarer, 10–15 % of cases [10, 17]. Recent data on circulating tumor cells identified a classic full-length AR (AR-FL) “on” signature (characterized by positive prostate-specific antigen (PSA) staining) versus an AR-FL “off” signature (characterized by positive prostate-specific membrane antigen (PSMA) staining) and suggested that the classic AR signature was lost in patients who recurred following effective androgen ablation therapy [18]. In this review, we will examine the role of AR splice variants as a mechanism by which prostate cancer becomes resistant to AR directed therapy.

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Androgen Receptor Structure and Function

The AR belongs to the class I nuclear steroid receptors that include the glucocorticoid receptor, estrogen receptors α and β, progesterone receptor, and mineralocorticoid receptors.

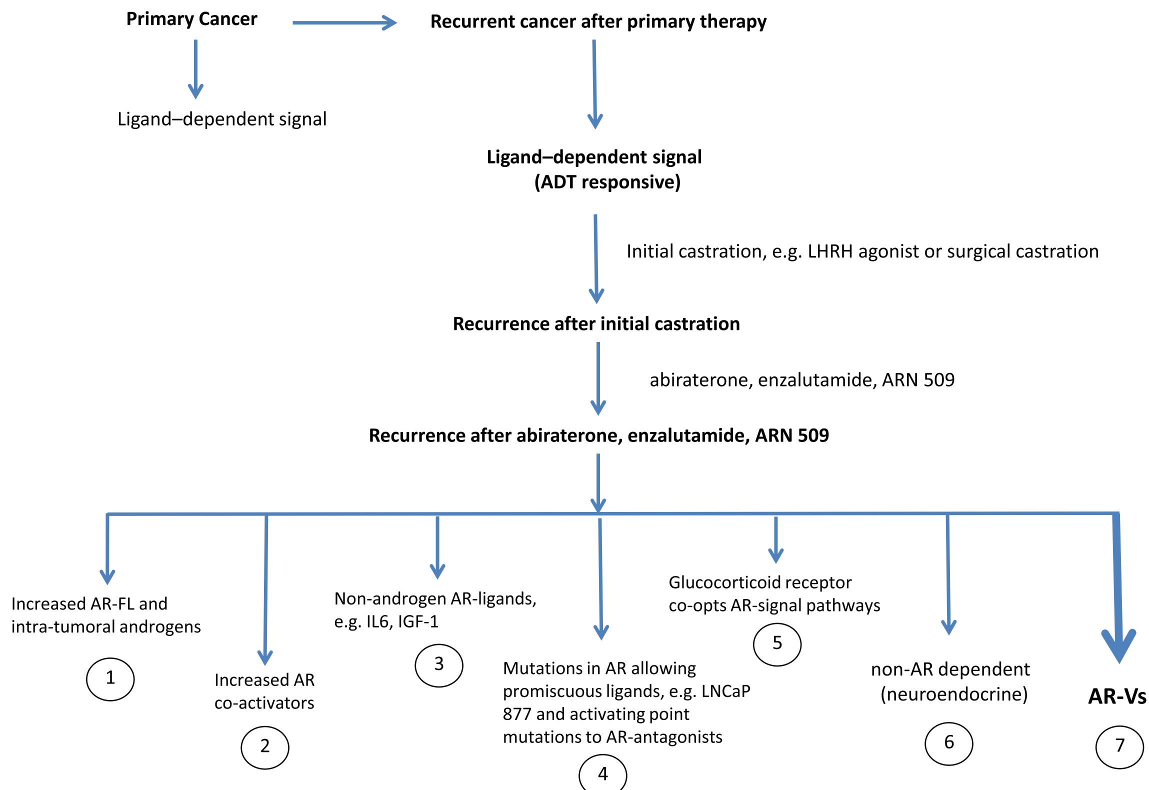

Fig. 1 Proposed pathways by which prostate cancer cells activate AR signaling, including variants (AR-Vs), and thus acquire resistance to AR-directed therapy. *Pathway 1* denotes resistance that has occurred by an increase in AR-FL expression along with association to persistence of intratumoral androgens. The intratumoral androgens may be derived from incomplete suppression of androgen production from the testes, conversion of adrenal androgens to dihydrotestosterone in the prostate, or intratumoral production of androgens [3–5]. *Pathway 2* indicates the AR-FL receptor can also exhibit enhanced transactivation due to an increase in AR co-activators [6]. *Pathway 3* indicates that non-androgen ligands may also enhance activation of the AR-FL receptor (IL-6, IGF-I) [7]. *Pathway 4* indicates mutations that develop in the LBD of the AR. In the case of LNCaP cells, mutations can result in a promiscuous receptor or resistance to AR targeting agents including enzalutamide, ARN509, bicalutamide, and flutamide [8]. *Pathway 5* shows that resistance to enzalutamide and ARN509 may develop by upregulation of the glucocorticoid receptor, which in turn activates a similar but distinct pattern of AR genes that stimulate tumor growth in response to glucocorticoid ligands [9]. *Pathway 6* demonstrates the observation that neuroendocrine-like cancers arise more frequently following treatment with the newer anti-AR therapies [10]. Finally, in *pathway 7*, we note the development of constitutively active AR-Vs that are the basis of this review

Binding to their cognate ligands activates the receptors; activated receptors are then transported to the nucleus where they bind to DNA as homodimers to two hexameric half sites. The dimerization of the AR can occur through N-terminal/C-terminal (N-C) interactions, dimer to dimer (D-D) interactions, and ligand binding domain (LBD) interactions [19]. The classic AR–ligand-driven activation of the AR requires ligand-induced N-C interaction. Once steroid ligand binds to the receptor's LBD, a highly conserved AF-2 interface is formed in the C-terminus. The AF-2 interface is the mediator of transcriptional activity in most class 1 steroid receptors; however, the AR is different in that upon binding of ligand the AF-2 domain interacts with the L*XX*LF and W*XX*LF domains in the N-terminus to initiate transcription. These interactions confer AR co-regulator binding to the transactivation unit 1 (TAU-1) and transactivation unit-5 (TAU-5). Thus, the AR is unique in that mediation of its transcriptional activity occurs through the N-terminal domain rather than the C-terminal domain, as occurs in the other class 1 nuclear steroid receptors. Importantly, the N-C interaction of the AR is ligand-dependent and the dominant interaction of the AR-driving transcription is the AF-2 L*XX*LF interaction. In contrast, the W*XX*LF-AF2 interaction is a minor contributor to ligand-induced AR transcriptional activity and the interactions between AF-2 and the W*XX*LF motif have been thought to be more important in ligand stabilization of the AR. However, recent mutation and deletion studies of the AR have shown that in the absence of the LBD, AR continues to have ligand-independent transcriptional activity that is primarily driven through the W*XX*LF motif in the TAU-5 domain (for more information, see these reviews on AR domain interactions [19, 20]). The significance of this brief introduction to the AR-transcriptional structure is to point out that among the class 1 nuclear steroid receptors, the AR is unique in that it can generate ligand and C-terminal-independent transcription though its N-terminal and DNA binding domains.

AR Splice Variants

Splice Variant Structure and Function Multiple AR splice variants (AR-Vs) have been described from prostate cancer cell lines, xenografts, and tissue (Fig. 2 and Tables 1 and 2) [21–26]. The majority of these variants were discovered in the 22Rv1 prostate cancer cell line. One of the first AR variants to be described, however, was originally found in placental tissue with the highest expression in cardiac tissues [27, 28]. AR45 is a highly conserved variant that is inhibitory to full-length AR (AR-FL) in the heart, but alternatively it has been reported to increase AR-FL activity in the prostate, demonstrating that the function of AR variants may differ depending on tissue type [27, 28]. Of the AR-Vs found in prostate cancer, some have no function while others appear to enhance the effect of AR-FL [21–25, 29]. Most significant among these splice variants are those in which the AR ligand binding domain (LBD) is lost, resulting in ligand-independent constitutive AR activation. Of the constitutively active splice variants found in prostate cancer, AR-V7 (also known as AR3) and AR^{v567es} are the most commonly found and thus the most studied. RNA and protein for both variants has been found in prostate cancer cell lines, xenografts, and human tumor specimens. Similar AR variants have also been described in mouse

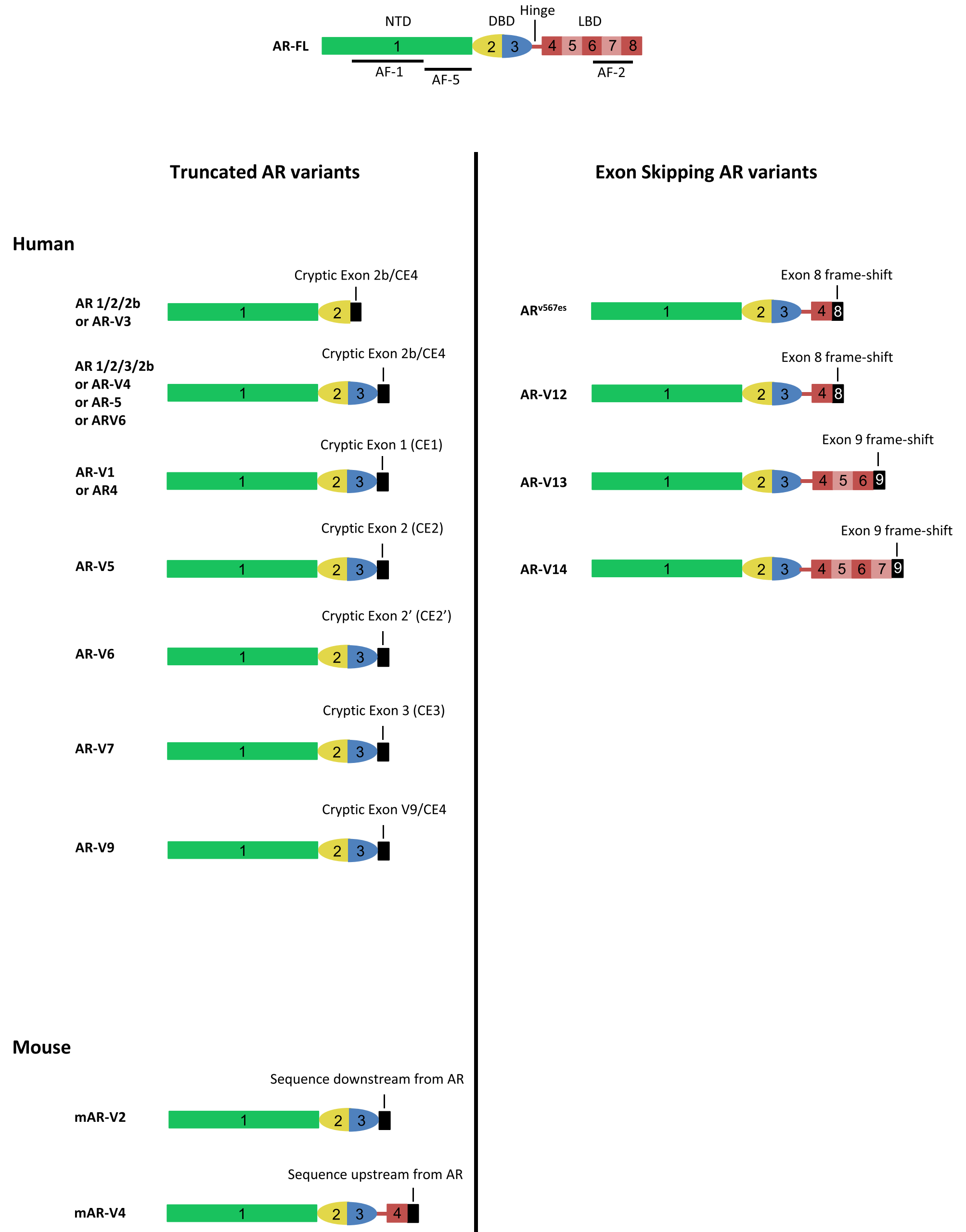

Fig. 2 Diagram depicting full-length androgen receptor (AR-FL) and AR variants. Variants fall into two categories based on structure: truncated variants (e.g., AR-V7) and exon-skipping variants (e.g., AR^{v567es}). Note that AR^{v567es} and AR-V12 transcripts differ by the addition of exon 9 at the end of AR-V12, although the proteins are predicted to be the same

Table 1 Truncated AR variants

Truncated variants	Alternate names	Source	Original references
AR-V1 (exons 1/2/3/CE1)	ARV4	Cell lines, 22Rv1; tissues, PCa (higher in CRPC)	[24, 25]
AR-V2 (exons 1/2/3/3/CE1)		Cell lines, 22Rv1	[24]
AR-V3 (exons 1/2/CE4[a]/3/CE1)	AR 1/2/2b (exons 1/2/CE4)	Cell lines, 22Rv1 and VCaP; xenografts, LuCaP 23.1 and 35	[24, 29]
AR-V4 (exons 1/2/3/CE4[a]/3/CE1)	AR 1/2/3/2b, AR5 ARV6	Cell lines, 22Rv1; tissues, benign prostate and PCa	[24, 25, 23, 29]
AR-V5 (exons 1/2/3/CE5[b])		Cell lines, 22Rv1	[24]
AR-V6 (exons 1/2/3/CE5[c])		Cell lines, 22Rv1	[24]
AR-V7 (exons 1/2/3/CE3)	AR3	Cell lines, 22Rv1, VCaP, and LNCaP androgen-independent lines; xenografts, LuCaP series and VCaP; tissues, benign and PCa (up in CRPC)	[24, 25]
AR-V8 (exons 1/2/3/CE6)		Xenografts, VCaP	[21]
AR-V9 (exons 1/2/3/CE5)		Xenografts, VCaP	[21, 45]
AR-V10 (exons 1/2/3/CE7)		Xenografts, VCaP	[21]
AR-V11 (exons 1/2/3)		Xenografts, VCaP	[21]

[a] Translation stops at CE4

[b] Translation stops after MTLGD

[c] Translation stops after MTLGAGSRVS

CE cryptic exon

models of prostate cancer: mAR-V2 has sequence homology to human AR-V7 while mAR-V4 is most homologous to human AR^{v567es}. However, unlike the human AR variants, the unique C-terminal sequences of the murine variants are not derived from AR introns/exons but rather from DNA sequences on the X chromosome distal to the murine AR gene [21]. Of the two mouse variants, only mAR-V4 is constitutively active and confers a growth advantage to prostate cancer cells [21].

While AR variants lacking the LBD are transcriptionally active when placed in AR-negative cell lines, the expression of AR variants has generally been reported to occur in conjunction with ongoing expression of AR-FL. Interestingly, Sun et al. [22] have demonstrated that in the absence of ligand, the AR^{v567es} variant forms a heterodimer with AR-FL causing it to be translocated to the nucleus and that, in the presence of ligand, AR^{v567es} enhances the activity of AR-FL [22]. Similar data have been seen with AR-V7 and AR-FL [30]. These data suggest that while AR variants can activate an AR transcriptional program by themselves, they also can potentiate the activity of AR-FL under low-ligand conditions; such conditions may be present in tumors of castrate men. In support of this view, Minges et al. [31] suggested that an AR co-factor, MAGE-A11, allows AR-Vs to dimerize with AR-FL by binding to the AF1 and F*XX*LF motifs in the N-terminus. When cells containing the dimerized AR-Vs/AR-FL were placed in low levels of androgens, this dimerization enhanced transcriptional activity of the AR-FL [31]. In a similar regard, Watson et al. [21] demonstrated that in the presence of both truncated and full-length AR, targeting AR-FL with the anti-androgen enzalutamide suppressed AR activity and cell growth as efficiently as when only AR-FL was present, suggesting activity of the AR splice variants is mediated through the full-length AR [21].

While AR-Vs can enhance AR-FL transcription, others and we have also demonstrated that there are unique gene sets expressed by the AR variants compared to AR-FL (Supplemental Table 1) [16, 32]. These unique gene sets are

Table 2 Exon skipping AR variants

Exon splicing variants	Source	Original references
AR^{v567es} (exons 1/2/3/4/8)	Cell lines, VCaP and LNCaP androgen-independent lines; xenografts, LuCaP 86.2 and castration resistant LuCaP series; tissues, PCa (highest in CRPC and metastases)	[22]
AR-V12 (exons 1/2/3/4/8/9)	Tissues, CRPC	[44]
AR-V13 (exons 1/2/3/4/5/6/9)	Tissues, CRPC	[44]
AR-V14 (exons 1/2/3/4/5/6/7/9)	Tissues, CRPC	[44]

associated with mitotic and anti-apoptotic pathways and, therefore, suggest that variant expression could enhance progression through the cell cycle in the face of therapies that normally cause apoptosis of cancer cells. Clinically such biological effects are suggested by the rapid progression and shorter time to death of men whose tumors express variants (see "Clinical Associations of AR Variants"). Whether there are specific variant-regulated differences in promoter and enhancer effects upon target genes compared to the AR-FL or differences due to resident time on chromatin and other epigenetic effects of the variants is not known. We have clearly shown that the time course of nuclear entry of variants versus AR-FL receptor is markedly different [22]. However, a complication of most of the expression studies reported with the variants is that they have either been done in the LNCaP human prostate cancer cell line, which contains mutated AR-FL and PTEN genes, or in cell lines that do not otherwise express AR. In this latter circumstance, we have found that cells that have lost or never had AR expression often have low levels of the co-regulators needed for AR transactivation; thus, common AR-regulated genes such as PSA or TMPRSS2 are poorly expressed in these cells after introduction of AR.

It is clear that multiple forms of AR variants exist in prostate cancer and that the function of most variants is unknown. However, it is of interest that the structure of the variants is relatively similar between human and mouse models of prostate cancer, suggesting that these variants are structurally related and selected to potentially bypass the androgen deprived environment.

Formation of AR Splice Variants Currently, it is hypothesized that the formation of the AR splice variants can occur through two different mechanisms: genomic rearrangements and alternative splicing events. Dehm's group has used paired-end sequencing to examine the AR gene in 22Rv1 and several LuCaP human prostate cancer xenografts to demonstrate that in cell lines/xenografts which consistently express AR-Vs, there is an intragenic re-arrangement of the AR gene that may account for the presence of AR-V7 or AR^{v567es} [33, 34]. Further, using a combination of FISH and deep sequencing, these studies propose that individual prostate cancer cells can express either AR-FL or AR-Vs but not both. These data suggest that initially AR-Vs may only exist in subsets of cancer cells, but following castration and inhibition of ligand-driven AR-FL, the AR-V clones emerge to drive the tumor.

Evidence also exists to support a second mechanism of AR-V generation, alternative splicing. Alternative splicing is a common mechanism for generating the variety of proteins expressed in cells [35–37]. In malignancies, differential splicing of pre-messenger RNA (mRNA) is a frequent mechanism used by the cell to generate protein variants that have oncogenic activity [35–37]. We have demonstrated that transcriptional-associated alternative splicing generates AR-V7 in the VCaP human prostate cancer cell line but not in the LNCaP human prostate cancer cell line [38]. Neither of these lines has been shown to have an intragenic rearrangement or inversion of the AR gene [34]. However, LNCaP does have a PTEN mutation and a mutation in the LBD of the AR that increases the promiscuity of ligands that can activate the AR [39, 40]. VCaP cells have a TMPRSS2-ERG fusion and a marked increase in the copy number of AR-FL [41, 42]. When the LBD of VCaP cells was blocked by a small interfering RNA (siRNA), enzalutamide, or growth in charcoal-stripped serum, AR-V7 and AR^{v567es} were generated in as little as 4–12 h. In contrast, LNCaP cells failed to generate AR-Vs under these conditions [34, 41, 42]. ChIP assays performed on VCaP cells treated with enzalutamide demonstrated that proteins comprising the splicesome (U1A, U2AF, ASF/SF2, and p54nrb), along with *Pol*-II, were recruited to the 3′ and 5′ splice sites of the introns/exons needed to generate AR-V7. These data indicate that the splicing occurs with transcription elongation [30, 43]. Further, under androgen deprivation conditions, it was not an increase in message of these splicing factors that led to creation of AR-V7 but rather an association of the splice factors with different splice sites within the AR pre-mRNA. This study identified one intronic splicing enhancer (ISE) and one exonic splicing enhancer (ESE) near the 3′ splice site of AR exon 3B (also known as cryptic exon 3 (CE3)). Following treatment with enzalutamide, U2AF65/hnRNP I bound to the ISE, while ASF/SF2 bound to the ESE. Liu et al. [38] observed that siRNA knockdown of splice factors or mutation of the ISE and ESE sites decreased the generation of AR-V7. While LNCaP does not express AR-Vs under androgen deprivation conditions, LNCaP 95, a castration-resistant LNCaP sub-line , does express high levels of AR-Vs [38]. These cells are maintained long term in an androgen-depleted medium, suggesting that LNCaP cells can become castrate resistant once they express AR-Vs. The LNCaP 95 cell line has a single copy of the AR gene with no intragenic rearrangements, but this line does express high levels of the androgen-glucuronidating enzymes UGT2B-15 and UGT2B-17, which maintain low levels of intracellular androgen and thus favor formation of AR-Vs by a transcriptional splicing mechanism. Of further note, Hu et al. [16] have described an additional non-transcribed 3′ sequence, termed exon 9, in AR exon-skipping variants (AR-V12, V13, and V14) [44]. The presence of exon 9 provides the potential for additional splice sites in the AR that may be involved in formation of the different exon-skipping AR-Vs. Together, the splicing data provide further evidence that androgen deprivation generates AR splice variants.

The possibility of both alternative splicing and intragenic AR gene rearrangements as mechanisms for the formation of AR-Vs following androgen deprivation indicate that there are rapid transcriptional mechanisms for AR-V formation as well

as longer-term somatic mechanisms for the selection of AR-V-positive tumor cells. Regardless of the mechanism responsible for the generation of the constitutively active AR-Vs, their increase in response to androgen deprivation therapy (ADT) is clear and whether through clonal selection or transcriptional associated splicing, ADT is the prime enhancing factor. However, whether constitutively active AR-Vs are responsible for progression to castrate-resistant prostate cancer (CRPC) through AR signaling or are epiphenomena of AR response when it loses its stabilizing ligand is not yet known. In the next portions of this review, we will review clinical and laboratory evidence for or against the AR-Vs as potential mediators of CRPC.

Clinical Associations of AR Variants

Others and we have recently described the appearance of posttranscriptional AR splice variants following castration [15, 21, 22, 24, 45, 46]. The consistency with which AR-V7 and AR^{v567es} are found in tumors and metastases from castrate men suggests that production of these variants may occur in response to castration, and it is notable that the variants most prevalent in human tissues are those most consistently found following androgen deprivation in vitro [26, 29]. We have found that the generation of AR^{v567es} is especially sensitive to suppression of intratumoral androgens, suggesting growth of these tumors is associated with generation of AR variants in the presence of castrate levels of androgen [15, 16, 22, 47].

Expression of the two constitutively active AR-Vs, AR-V7 and AR^{v567es}, has been most commonly associated with castrate-resistant prostate cancer and its metastases. In our experience, their expression is unusual in benign prostate tissue or primary prostate cancer. Interestingly, Sun et al. [22] demonstrated the presence of both variants in prostate tissue from healthy men treated with anti-androgens as part of a male contraceptive study. Such a finding further indicates that androgen deprivation drives the formation of the AR-Vs [22]. Other studies have seen expression of AR-V7 mRNA in 80 % of benign prostate tissues from men with no evidence of cancer [32]. Likewise, AR-V7 protein has been detected in benign basal epithelial cells using a specific antibody to AR-V7; however, these data have not been reproducible using other available AR-V7 antibodies. If AR-V7 expression does arise from intragenic re-arrangements, then it would not be surprising to see low levels of AR-V7 mRNA and protein in normal prostate tissue; upon castration, the cells expressing AR-V7 would then have a growth advantage and tissue-wide levels of AR-V7 mRNA and protein would increase.

In a recent study, Zhang et al. [26] performed AR staining on tissue microarrays that contained prostate cancer tissue from 55 non-castrate men at the time of radical prostatectomy (RRP) and 144 metastases from 43 castrate men who died from prostate cancer. C- or N-terminal-specific AR antibodies were used. Staining intensity as well as localization, nuclear (N) or cytoplasmic (C), was determined for each antibody [26]. Because the AR-Vs lack the C-terminal portion of the AR, the AR C-terminal specific antibody will not detect variants. In addition, since the two most common AR-Vs are constitutively active, samples with AR-Vs would be expected to have more nuclear staining with the N-terminal AR antibody. In the primary tumors, the ratio of nuclear to cytoplasmic AR staining was not significantly different, whereas in the metastases significantly more nuclear staining occurred. Although variant-specific antibodies were not used, these data show that there was a significant alteration in the levels of C-terminal containing ARs in the metastases of men who are castrate and die from their disease. These data are consistent with a high prevalence of C-terminal AR loss and AR nuclear localization, as is seen with the AR variants, and is consistent with the presence of constitutively active AR activity in castrate resistant prostate cancer.

Although a number of studies in the literature have shown an association between AR-Vs and prostate cancer metastases, only two clinical studies have demonstrated an association between AR-V7 expression and subsequent progression to castrate-resistant prostate cancer (CRPC) [24, 32]. In the first study, tissue microarrays of primary tumors were stained for AR-FL or AR-V7. There was no association between AR-FL expression and subsequent progression to castrate resistant disease; however, AR-V7 expression in the primary tumor correlated with subsequent chemical recurrence [48]. In the second study, tumor tissue was collected from men who presented with pathologic fractures due to prostate cancer. Two thirds of these men had been castrated, and one-third presented with fracture as the initial manifestation of their disease. AR-V7 and AR^{v567es} were assessed in the tumor tissue. Men who expressed any AR^{v567es} or who were in the upper quartile for AR-V7 expression died on average 2 months after diagnosis (with a maximum survival of only 3.3 months), whereas those patients who did not express the variants lived an average of 8 months following diagnosis (with a maximum survival of 36 months). Furthermore, tumors from patients who expressed the variants had a more mitotic gene expression pattern [26]. These data do not indicate that the AR-Vs cause resistance to therapy but do suggest that variant expression is associated with a significantly rapid progression of the tumor. However, because each of these biomarker studies had relatively small patient numbers, at this point, no definite conclusions can be drawn as to whether AR-V7 expression will serve as a biomarker to define prostate cancers that will progress. Therefore, as better antibodies are developed to detect more of the AR-Vs, it will be important to examine the potential of AR-Vs to serve as biomarkers of low-grade tumors with progressive abilities. This is especially important as more patients enter active surveillance protocols.

Castration-Resistant Prostate Cancer and AR Variants

Several studies indicate that the AR-Vs are drivers of castration-resistant prostate cancer and function independently of the full-length AR [22]. If correct, then current and future therapy targeting the ligand-binding domain would be ineffective since these AR-Vs lack the LBD. Unfortunately, this latter suggestion is not universal as the function of constitutively active AR-Vs is a more complex relationship that includes not only dimerization with AR-FL but also the differential effect of ubiquination and proteosomal degradation on AR-Vs expression. Others and we have shown that even potent inhibitors of the LBD, such as enzalutamide, do not inhibit the AR activity of prostate cancer cells driven by AR-Vs [29, 34]. Each of these factors appears to be altered in the progression to CRPC such that the role of AR-Vs is of greater significance as the LBD is compromised. Of further importance, the splicing mechanisms, which include specific components of the spliceosome that generate the constitutively active AR-Vs, can be *activated* by inhibition of AR signaling and conversely suppressed by transactivation of the AR. These data indicate that the generation of the constitutively active AR-Vs are not simply random splicing events but are probably specific tumor survival events. Finally, there is the issue of whether the variants actually lead to expression of a unique set of AR-regulated genes, distinct from the AR-FL transcriptome [22, 32, 34]. Again, this is a controversial but important issue.

Nuclear Localization of AR Variants The nuclear localization sequence (NLS) of the AR-FL is thought to be bipartite: sequences are located in the transcriptional domain of exon 3 and in the hinge region in exon 4 (Fig. 3). In the case of AR-FL, the non-transcriptionally active receptor resides in the cytoplasm where it is bound by heat-shock protein 90 (Hsp90); when activated by ligand, the receptor sheds Hsp90, associates with Hsp27, and binds to the dynein motor of microtubules. AR-FL is transported to the nucleus and through interactions with importin enters the nucleus where it binds as a homodimer to androgen response elements (AREs) on DNA and becomes transcriptionally active. This process has been described in depth by a number of reviews [49, 50]. Recently, it has been shown that interruption of the dynein motor on the microtubules with taxanes, nocodazole, or dynamitin will inhibit transport of AR-FL to the nucleus [49, 50]. AR-FL binds to the dynein motor through the portion of the NLS located in exon 4 [51]. As shown in Fig. 3, the truncated AR variants, e.g., AR-V7 and AR-V1, lack exon 4 and thus contain only the portion of the NLS located in exon 3. AR exon-skipping variants, however, contain the hinge region of exon 4 and therefore have the canonical NLS. Individually, these variants should be transported into the nucleus in a mechanism similar to AR-FL [52]. Because the Hsp90

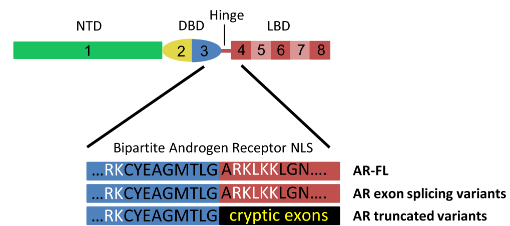

Fig. 3 Diagram depicting the bipartite nuclear localization sequence in the androgen receptor. Only the AR exon-skipping variants have exons 3 and 4 and thus the complete NLS. Truncated AR variants lack exon 4 and therefore are missing the second NLS site

binding site is located in exon 7, the AR-Vs are not tethered by this Hsp in the cytoplasm and may be transported directly to the nucleus where they are constitutively active [53]. However, since both AR-V7 and AR^{v567es} are constitutively active but AR-V7 lacks the NLS in the hinge region, they must have different modes of nuclear transport. Recently, we have shown that this is the case. Treating prostate cells in vitro with taxanes or nocadazole inhibited nuclear transport of AR-Vs that contain the complete NLS, e.g., AR^{v567es}. Further, taxane treatment of patient-derived prostate cancer xenografts that are driven by AR^{v567es} significantly reduced in vivo tumor volume and growth [51]. In contrast, AR-V7, which does not contain the consensus NLS and does not engage the microtubule dynein motor system as part of its nuclear transport mechanism, instead relies on non-canonical sequences in the N-terminus as well as the cryptic exon 3 for nuclear transport [52]. Truncated AR-Vs containing cryptic exons other than CE3, such as AR-V1, cannot be transported to the nucleus, and therefore they lack constitutive activity [52]. The currently available data then indicate that although the various constitutively active AR-Vs demonstrate activity in the absence of ligand, they do not behave in an identical manner; these differences should be taken into account when planning patient treatment. As we have shown, taxane treatment may not affect AR-V7 activity but may significantly impact the activity of AR-FL and AR^{v567es}.

In addition to the ability of the AR-Vs to translocate to the nucleus independent of ligand, we have also shown in the absence of ligand that AR-FL can dimerize with both AR-V7 and AR^{v567es}. This dimerization can result in the nuclear localization of the heterodimer with subsequent binding to AREs [22, 30]. Sequential chromatin immunoprecipitation (Re-ChIP) analysis with an AR-V7 antibody followed by an AR-FL antibody was performed in 22Rv1 cells, which express endogenous AR-V7 [31]. Co-occupancy of AR-V7 and AR-FL was shown on the promoter of the PSA gene. This co-occupancy was unaffected by androgen or enzalutamide treatment. However, the promoter of ubiquitin-conjugating enzyme E2C (UBE2C) was only bound by AR-V7. ChIP assays demonstrated that AR-FL knockdown did not significantly affect this binding, consistent with UBE2C being an AR-Vs

specific target [14, 15]. These studies demonstrate that the mechanism by which the constitutively active AR-Vs translocate to the nucleus in the absence of ligand is AR-V specific and depends, in part, on whether the particular variant sequence contains both components of the bipartite NLS. Additionally, regardless of mechanism, the ability of the variants to heterodimerize with AR-FL results in nuclear translocation and ARE binding in the absence of ligand and, importantly, in the presence of enzalutamide.

In addition to the mechanisms responsible for nuclear localization of the AR-Vs, most AR variants lack the putative nuclear export signal (NES) located in the LBD (aa 743–817) [54]. This suggests that once in the nucleus, chromatin binding by the variants will be significantly prolonged and signaling altered. Additionally, components of the NES are associated with AR polyubiquination and AR degradation [55]. Thus, there is a potential for decreased rates of degradation of AR-V protein compared to AR-FL. The stability/degradation of the AR-Vs, however, has not been well defined at this point.

AR Variant Transcriptome Initial studies demonstrated that LNCaP cells in which either AR-V7 or AR^{v567es} were constitutively expressed were castration resistant when grown subcutaneously in immunocompromised mice [21, 22, 24, 25]. Mostaghel et al. [15] reported that the effects of AR-Vs in the presence of AR-FL appear to be regulated by the levels of intratumoral androgens [15]. Using a series of LuCaP xenografts in which mice were treated with the CYP17 inhibitor abiraterone, the authors demonstrated that if intratumoral androgens could be detected by mass spectrometry (MS) in the recurrent tumors, then AR-Vs, while detectable, were not significantly upregulated compared to pre-treatment levels. Furthermore, tumor recurrence in this case was associated with an increase in AR-FL ligand-dependent genes, e.g., PSA and TMPRSS2. However, when abiraterone recurrence was associated with the absence of MS-measureable intratumoral DHT, then the levels of AR-Vs increased significantly and were associated with mitotic genes, e.g., UBE2C. Hu, et al. [16] further demonstrated in clinical samples that expression of AR-V7 protein was also correlated with expression of UBE2C [16]. In the same study, prostate cancer cells treated with either a siRNA to the LBD or enzalutamide had decreased AR-canonical gene expression but increased mitotic gene expression [16]. These studies suggest that in the absence of ligand, AR-Vs function independently of AR-FL and their actions cannot be blocked by anti-androgen inhibitors. These studies also suggest that AR-Vs may express their own transcriptome.

Therefore, we still have the quandary as to what the contribution of the AR constitutively active splice variants are when they exist in association with AR-FL. Another criticism that has been voiced to show that the AR-Vs are not relevant in the presence of AR-FL is that the level of expression of the variants in tissue and most cell lines is approximately 0.1–2.0 % of the AR-FL [21]. The low levels of variant may, in part, be due to a lower affinity of N-terminal AR antibodies to the variants. Development of variant-specific antibodies is underway and is necessary to more accurately determine AR variant levels. For example, when an AR-V7-specific antibody was used for IHC, AR-V7 protein was readily detected in human prostate cancer tissues [16]. Even if specific antibodies show lower levels of AR variant proteins compared to AR-FL, low levels do not necessarily indicate a lack of functional importance for the variants. Unlike AR-FL, the constitutively active variants translocate directly to the nucleus where they function as transcription factors; therefore, comparing their levels to the AR-FL level, which must exist in the cytosol in order to bind ligand, is not an equivalent comparison. Recently, Cao et al. [30] confirmed the data of Sun et al. [22] by demonstrating that in androgen deprivation conditions when AR-FL associates with either AR-V7 or AR^{v567es}, the heterodimers are transported into the nucleus and bind to canonical ARE promoter elements, e.g., PSA and TMPRSS2 [22, 30]. Importantly, transportation and binding were not blocked by enzalutamide. Further, in the absence of AR-FL, the AR-Vs bound to enhancer and promoter elements on genes shown to be upregulated when AR-Vs are highly expressed (e.g., UBE2C) [16]. Since UBE2C does not have a consensus ARE binding site in its promoter or enhancer elements, this suggests that long-range chromatin looping may play a role in its transcription [56]. These data indicate that the AR-Vs may require the presence of AR-FL for their activity on AR-canonical gene expression but that they can also act independently of AR-FL to generate a distinct AR-V transcriptome.

Whether the AR-Vs have a unique transcriptome remains unclear. Hu, et al. [16] recently published that the AR-Vs have a unique mitotic transcriptome; some of the upregulated genes in this transcriptome (e.g. UBE2C) are upregulated in a CRPC transcriptome published by Cuzick et al. [16, 48, 57]. In contrast, Li et al. [34] found prostate cancer cells driven by the AR-Vs have a biphasic classic androgen transcriptome, albeit in the absence of ligand. These studies, however, used different prostate cancer cell lines and different methods of expressing the variants. The difference in transcriptomes then is not surprising and may point to cell-specific transcriptomes. Further studies using a variety of prostate cancer cell lines that endogenously express AR-Vs as well as human prostate cancer tissues are necessary. Studies using the new AR-Vs transgenic mice could also highlight whether or not AR-Vs have a unique transcriptome.

AR Variant Transgenics Current studies using prostate cancer xenografts in mice have resulted in conflicting data regarding the importance of AR-Vs. Watson et al. [21] did not see a

significant effect of AR-Vs on tumor progression or in treatment resistance [21]. On the other hand, others and we have seen an association between increased AR-Vs expression and treatment resistance and prostate cancer progression [22, 24]. All of these studies, however, have examined what happens in cells or xenografts after exposure to AR-Vs for a few days to a few weeks; in this short amount of time, it may be difficult to discern the effect of the variants. Recently, two studies have addressed what happens in vivo in transgenic mice engineered to express either AR^{v567es} or AR-V7 (AR3). In both studies, AR variant complementary DNA (cDNA) was inserted into a vector containing a rat probasin promoter (ARR2), which resulted in AR variant expression specifically in the prostate epithelium. The background of the mice, however, differed in the two studies: AR^{v567es} was placed in the C57/Bl6 background while AR-V7 (AR3) was placed in the FVB mouse. While the FVB mouse tends to result in more aggressive cancer progression in transgenics, in this case, AR variant expression in the different backgrounds resulted in similar phenotypic alterations [58, 59].

Liu et al. [59] created a transgenic AR^{v657es} mouse model. Using PCR and a human specific N-terminal antibody (AR441), AR^{v567es} was detected in all four lobes of the transgenic prostate, with the highest expression in the lateral and ventral lobes. No AR^{v567es} was detected in wild-type littermates. At 16 weeks of age, hyperplasia was evident in the transgenic prostate, with adenocarcinoma developing by 1 year. In castrate transgenics, AR^{v567es} mRNA was still detectable by PCR. Immunohistochemistry, using a human-specific AR antibody, demonstrated positive nuclear AR staining pointing to the constitutive activity of AR^{v567es}. Importantly, castration increased the aggressiveness of the carcinomas in year-old mice, with invasive carcinomas detected in two thirds of mice and liver metastases detectable in one out of six mice. Immunohistochemistry on transgenic prostates revealed increased levels of proteins associated with epithelial to mesenchymal transition, including vimentin and Twist. cDNA arrays revealed upregulation of genes commonly found in AR-V-positive tissues, including UBE2C. Gene set enrichment analysis (GSEA) further revealed upregulation of five oncogenic signatures associated with different stages of prostate cancer progression (Kras, NF-κB, β-catenin/Wnt, Gli1, and Stk33). Together, these data suggest that AR^{v567es} plays a role in development of CRPC.

Sun et al. [58] reported that by 4 weeks of age, prostate-specific expression of AR-V7 (AR3) led to alteration in prostate gland structure in addition to upregulation of factors associated with both prostate stem cells (e.g. Sca-1, TGF-β) and an epithelial to mesenchymal transition (e.g., Snail, Slug, Twist, N-cadherin, and vimentin). By 1 year, the mice had developed prostate intraepithelial neoplasia (PIN). In addition, the authors noted a significant decrease in miR-29, a micro RNA associated with tumor suppressive activity in the prostate. In vitro studies demonstrated that miR-29 binds to the 3′UTR of IGF-1 thereby decreasing its expression and likewise decreasing the levels of pAKT, a downstream target of the IGF-1 signaling pathway. Examination of miR-29, IGF-1, and pAKT levels in the transgenic prostates in turn demonstrated a correlation between decreased levels of miR-29 and increased levels of IGF-1 and pAKT. In contrast to the AR^{v567es} study, Sun et al. [58] performed early castration studies on 4-week-old mice; 2 weeks later, testosterone was reintroduced to half of the mice. AR-V7 transgenic mice showed more rapid regeneration following testosterone supplementation than wild-type littermates. It would be of interest to see if castration at 1 year would lead to development of carcinoma in the AR-V7 mouse as it did in the AR^{v567es} transgenic mouse.

Future studies crossing these models with other prostate cancer transgenic models will further elucidate the role of AR variants in prostate cancer progression, especially their role in lethal CRPC.

New Agents Targeting AR Variants

Since the C-terminus of the AR can be crystalized, we have seen the recent introduction of very effective AR-FL inhibitors such as enzalutamide and ARN-509. However, because the N-terminus is intrinsically disordered and has not been crystalized, agents with a similar degree of activity are not yet available [48]. Further, it is unlikely that agents will be able to selectively inhibit each of the AR-Vs. In place of AR-V-specific agents, naturally derived compounds such as EPI-001 or mahanine that inhibit both AR-FL and AR-Vs are under study as new prostate cancer therapies [55, 60, 61]. While these agents are not yet in clinical trials, in preclinical studies, they demonstrate the best hope of inhibiting the AR-Vs. As indicated, a number of agents are under development that potentially can inhibit AR-V activity. Since the variants may be an important mechanism for initial tumor survival in response to ADT [3, 38], treatments targeting AR-Vs should be considered at the time androgen ablation therapy is initiated, when AR-Vs are likely to arise, rather than delayed until after recurrence and additional mechanisms of resistance such as point mutations in the LBD of the AR or a neuroendocrine phenotype develop.

Conclusion

Constitutively active AR splice variants are commonly increased in recurrent prostate cancer following castration. In cell lines and xenografts, these variants can continue to drive tumor progression in the face of potent C-terminal AR

inhibition agents, such as enzalutamide and abiraterone. However, when AR-Vs are co-expressed with AR-FL, significant inhibition of xenograft growth can still occur in response to enzalutamide. This effect is likely due to heterodimerization between AR-Vs and AR-FL as well as the continued presence of intratumoral androgens. The answer to the question as to whether or not AR-Vs are causal mediators of CRPC or biomarkers of aggressive disease clinically is still in question with the majority of the preclinical and transgenic mouse data favoring causality. However, until N-terminal inhibitors of the AR are available for clinical use, it is unlikely the question will be resolved experimentally. To further complicate the issue is the idea that while different AR-Vs may be constitutively active, there appears to be some variation in function. For example, mechanisms of nuclear transport differ between AR-V7 and AR^{v567es}; these differences can affect how the cells respond to cancer therapies such as taxanes. Regardless of the outcome of these functional questions, though, the overwhelming evidence points to AR-Vs playing a role in the development of castration resistant prostate cancer.

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