




Review

Androgen Receptor Activation in Castration-Recurrent Prostate Cancer: The Role of Src-Family and AckI Tyrosine Kinases

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Abstract

There is growing appreciation that castration-recurrent prostate cancer (CR-CaP) is driven by the continued expression of androgen receptor (AR). AR activation in CR-CaP through various mechanisms, including AR overexpression, expression of AR splice variants or mutants, increased expression of co-regulator proteins, and by post-translational modification, allows for the induction of AR-regulated genes in response to very low levels of tissue-expressed, so-called intracrine androgens, resulting in pathways that mediate CaP proliferation, anti-apoptosis and oncogenic aggressiveness. The current review focuses on the role played by Src-family (SFK) and AckI non-receptor tyrosine kinases in activating AR through direct phosphorylation, respectively, on tyrosines 534 or 267, and how these modifications facilitate progression to CR-CaP. The fact that SFK and AckI are central mediators for multiple growth factor receptor signaling pathways that become activated in CR-CaP, especially in the context of metastatic growth in the bone, has contributed to recent therapeutic trials using SFK/AckI inhibitors in monotherapy or in combination with antagonists of the AR activation axis.

Key words: Src-family tyrosine kinases, Ack1, androgen receptor, prostate cancer, castration-recurrence.

Introduction

In 2013, 238,590 new cases of prostate cancer (CaP) are estimated to be identified, typically through increased levels of serum prostate specific antigen (PSA), digital rectal exams and pathologic analyses of biopsy samples. Roughly 12-13% of these cases will result in death (29,720 estimated in 2013), making it the second leading cause of cancer deaths in U.S. men (<http://seer.cancer.gov/statfacts/html/prost.html>). Since the initial description by Huggins and Hodges in 1941 [1] that CaP can be clinically reduced by castration, which causes deprivation of testicular androgen, most treatment-naïve CaP cases have been treated with some combination of androgen-deprivation therapy (ADT) plus surgical prosta-

tectomy or radio-ablation. This has resulted in high initial cure rates of early, localized disease and palliation of metastatic disease. However, the so-called lethal clinical phenotype of CaP relates to disease recurrence following ADT, producing castration-recurrent (CR) metastatic disease that is found primarily in the bones and lymph nodes, and that responds poorly to standard chemotherapy and radiation [2].

CR-CaP: Dependence on AR signaling

Most CR-CaP cases continue to express the androgen receptor (AR) as well as significant androgen- and AR-driven genes such as PSA [3]. Indeed, CR-CaP

cases typically express increased levels of AR protein [4-6], sometimes attributable to AR gene amplification [7], and as well, increased levels of androgen-responsive genes such as PSA and TMPRSS2 [8]. The notion that CR-CaP is AR-dependent is supported by the recent clinical success of AR antagonists such as enzalutamide (MDV3100), which increases survival rates in CR-CaP patients but only for several months [9]. The ultimate failure of AR antagonists to produce durable cures has been associated with the generation of AR splice variants lacking the ligand-binding domain (LBD) [10] or a F876L mutation in the LBD [11, 12]. Less understandable, however, are enzalutamide-resistant cases that continue to express WT-AR; these may reflect that AR overexpression may convert AR antagonists to agonists [13]. In addition to AR activation by direct tyrosine phosphorylation by SFK and Ack1 kinases- which will be the focus of the current review, other studies have identified additional AR-related changes that may contribute to CR-CaP: i) AR mutations (primarily in the LBD) that increase binding for non-androgen agonists [14], ii) AR stabilization [15], iii) induction of AR co-regulators and iv) post-translational modification [14]. These CR-associated changes are thought to facilitate AR-driven tumor progression in response to the post-castration expression of low levels tissue androgens [16].

The notion that the sustained expression of AR-driven genes is required for CR-CaP generation is supported by several studies using mouse models of human CaP. For example, Yuan et al. [17] showed that growth of a cell line (R3) derived from CR-growths of the human CWR22 xenograft in castrates was suppressed by the shRNA-mediated knockdown of AR, even though growth was not inhibited by the anti-androgen, bicalutimide. Campagno et al. [18] showed that intratumoral injections of AR-siRNA inhibited growth of LNCaP-C4-2 and CWR22Rv1 cells, two CR-CaP lines. Similar findings were demonstrated by Snoek et al. [19] using a tetracycline-regulated AR-shRNA in LNCaP-C4-2 cells and by Guo et al. [20] using AR-shRNA in CWR22Rv1 cells in castrates. An interesting twist on this concept comes from the study of Gan et al., [21], who showed that the ability of paclitaxel to inhibit the CR growth of CWR22Rv1 cells correlated with the downregulation of AR caused by the expression of the PTEN tumor suppressor and the nuclear accumulation of the AR suppressive transcription factor, FOXO1. Thus, siRNA-mediated FOXO1 knockdown attenuated the inhibitory effect of paclitaxel on AR-driven CR-promoting genes.

Several studies have demonstrated that androgen-independent or CR-CaP cell lines, or CR-CaP

tumor samples, have AR-driven gene expression signatures that correlate with CR-CaP. Work from the Chinnaiyan and Brown labs [22, 23] used AR-ChIP-chip or -ChIP-seq combined with gene expression analyses to identify AR cistrome genes in an androgen-independent LNCaP variant cell line whose expression pattern correlated with CR disease in humans. For example, the study of Wang et al. [23] identified UBE2C, an AR-driven cell cycle regulated gene that overrides an M-phase checkpoint, as a marker of androgen-independent growth. Interestingly, by comparing the AR cistrome in CaP cells expressing full-length (FL) AR vs. a splice variant (V) of AR typically upregulated in abiraterone and enzalutamide-resistant CR-CaP cells and tumors, Hu et al. [24] also identified UBE2C expression as a marker of specific for CR-CaP. A more recent study by Sharma et al. [25] analyzed the AR cistrome in human clinical CR-CaP samples, identifying a 16-gene signature with stronger correlation to CR-CaP than the aforementioned Chinnaiyan and Brown lab studies. Importantly, these and other studies identified transcription factors such as GATA, OCT, PU1, TEF1 [26], FOXA1 [25], and the methyltransferase, EZH2 [27] as potential AR co-factors based on the proximity of their ChIP sites with those of AR. Some transcriptional co-factors, such as FOXA1, may only play a role in ligand-dependent AR function, since androgen-independent AR binding sites in CR-CaP, whose sequences increasingly diverge from the classic androgen responsive element (ARE: 5'-GGA/TACANNNTGTTCT-3'; [28]), form under conditions of FOXA1-independence [29].

CR-CaP: Increased Protein Tyrosine Phosphorylation, SFK and Ack1 activity

The regulation of AR transcriptional activity by post-translational modifications such as phosphorylation, acetylation, sumoylation, ubiquitination and methylation, have been well described [30]. CR-CaP clinical samples exhibit increased relative protein tyrosine phosphorylation levels compared to levels in AD-CaP [31]. This correlates with increased activation levels on non-receptor tyrosine kinases [32] as well as receptor tyrosine kinases such as Met [33], c-Kit [34] and the EGFR family [35, 36]. However, the role of AR tyrosine phosphorylation may have been underappreciated because it is not induced by androgens such as dihydrotestosterone (DHT), whereas it is induced by EGF, heregulin, IL-6 or serum [20, 37, 38]. In contrast, androgens induce IGF-1R activity in prostate cancer progression [39].

Although Src-family kinases are rarely mutated in human cancers [40, 41], there is growing appreciation that they play critical roles in cancer progression,

especially associated with recurrent and metastatic disease [42-45], due to gene amplification [46], over-expression or activation by post-translational modification (reviewed in [47]). Indeed, SFK play central roles in mediating oncogenic signaling downstream of many receptor tyrosine kinases, which are themselves activated in cancer by multiple mechanisms [48]. *FGR* is the only SFK member gene that has been shown to be amplified in prostate cancer, specifically, in 37% of hormone-refractory disease [49]. In contrast, SFK members *Src* and *Lyn* are activated in CaP cell lines [50, 51] and tumor tissues [51], and *Fyn* is upregulated in primary prostate cancer vs. benign lesions [52], and even higher in metastases as evidenced by *in silico* analysis of Oncomine studies (Figure 1). Most SFK members share an autophosphorylation site (termed Y416 for chicken *Src*, Y419 for human *Src*) that is recognized by Src-poY416-specific antibodies and that is an appropriate surrogate marker for kinase activity in cells [53]. A limited set of studies has demonstrated a ~2.5-fold increase in relative Src-poY416 staining in CR- vs. AD-CaP samples [20, 54], and in metastases (bone and lymph node) vs. primary tumor sites (Figure 2, unpublished data, Gary Gallick, MD Anderson Cancer Center). Taken together, these data strongly support a role for increased tyrosine kinase and SFK activity for progression to CR- and metastatic CaP.

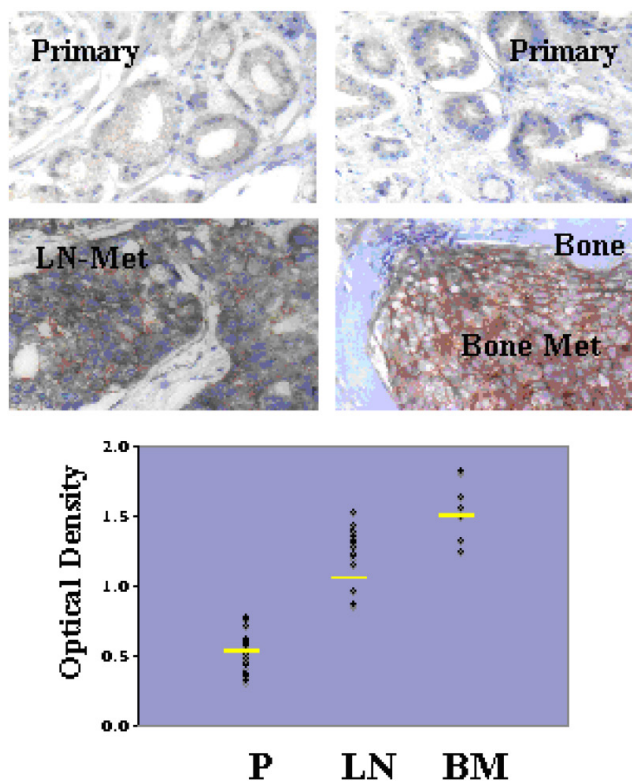


Figure 2. Increased levels of activated Src in lymph node (LN) and bone metastases (BM) compared to primary-site (P) CaP. De-identified patient formalin-fixed paraffin sections from pathology archives (MD Anderson Cancer Center, under IACUC approval) were stained using Src-poY416-specific antibody (Cell Signaling Technologies, Inc., cat. # 201, 1:100, as described in [58]). *Top images*-Immunohistochemistry staining analysis for Src-poY416 in typical P, LN and BM clinical samples. *Bottom panel*-Scoring for relative Src-poY416 staining using the 0 to 3+ system, with the mean values shown as yellow lines. Published with permission from Gary E. Gallick, M.D. Anderson Cancer Center.

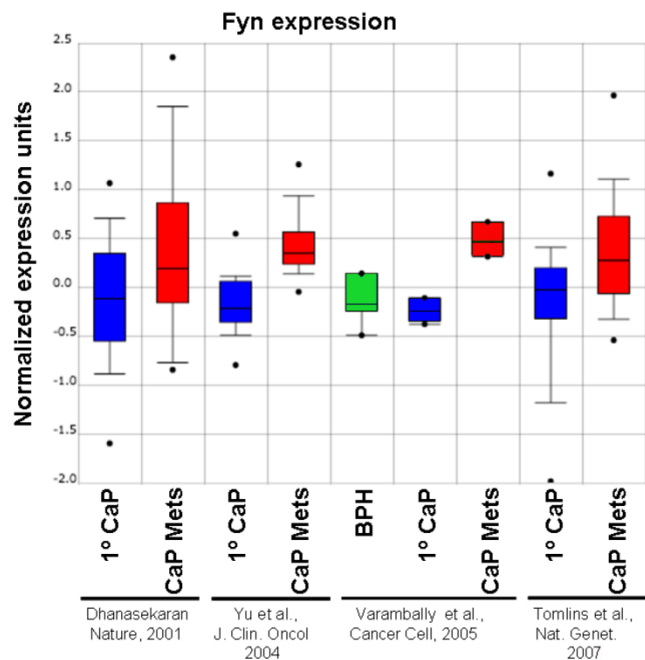


Figure 1. Relative expression level of Fyn in BPH, primary CaP (1°) and metastases ("Mets") from Oncomine (<http://www.oncomine.org>) from the studies of Dhanasekaran et al. [116], Yu et al. [117], Varambally et al. [118] and Tomlins et al. [119].

In addition to the role of SFK in prostate cancer progression, several groups have reported that the non-receptor tyrosine kinase, Ack1 (Activated Cdc42-associated Kinase 1) may facilitate CaP progression through the direct activation of AR. Several mechanisms for Ack1 activation in prostate cancer have been identified, including gene amplification [55] or kinase hyperactivation [38] that occurs downstream of multiple receptor tyrosine kinases [56]. As shown in the study of Taylor et al. [57] (Figure 3), increasing levels of Ack1 (TNK2) message are found in primary site CaP compared to normal or benign prostate hyperplasias (BPH), and even higher levels are found in lymph node metastases.

Several lines of evidence indicate that the expression of specific SFK or Ack1 can drive the formation of CaP or progression to CR-CaP. Although not the main focus of this review, there is a large body of evidence showing that SFK play key roles in facilitating proliferation of CaP induced by various growth factors and in promoting oncogenic migration parameters such as invasiveness [58] (reviewed in [32, 59]). Indeed, *Src* is required for the lymph node metastasis of a metastatic variant of PC-3 CaP cells alt-

though its knockdown has no effect on primary tumor growth [58]. Gelman et al. [60] recently demonstrated that TRAMP mice, whose prostate cancer progression is induced by the prostate-specific transgenic expression of the SV40 Tag [61], had greatly diminished prostatic adenocarcinoma and metastasis formation rates when crossed into Src-null, and to a lesser extent, Lyn-null backgrounds, but no change in the rate or extent of conversion to neuroendocrine cancer in the prostate. The loss of Fyn had no effect on primary tumor or metastasis formation in TRAMP mice exhibiting CaP. However, an interesting finding, and one that merits further investigation in the context of human disease, is that in rare cases where primary CaP failed to form within the typical onset period (<20 weeks of age), the loss of Src, Lyn or Fyn resulted in highly aggressive metastatic disease exhibiting markers of adenocarcinoma. This might suggest that SFK suppress the growth of metastases in the absence of paracrine factors secreted by primary tumors, a phenomenon described in the TRAMP model [62] and in human cancers [63].

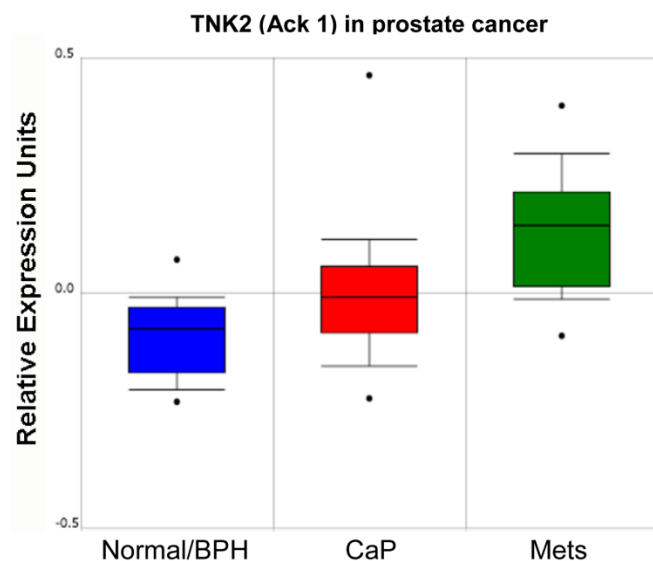


Figure 3. Relative expression level of Ack1 (TNK2) in normal/BPH, primary CaP and lymph node metastases (mets) from Oncomine (<http://www.oncomine.org>) from the study of Taylor et al. [57].

The notion that activated Src is sufficient to drive CaP initiation comes from the study of Cai et al. [64] who used a tissue recombination model to show that Src, and to lesser extents, Fyn and Lyn, can induce prostatic basal epithelial cells to form CaP tumors when mixed with urogenital sinus mesenchymal cells. Subsequent phosphoproteome analyses of mouse tumors induced by activated AKT plus AR, ERG, or activated K-Ras, as well as of metastatic CR-CaP patient tumors, showed evidence of Src-driven pathways [31, 65]. Interestingly, even the overexpression

of non-mutated c-Src was able to induce CaP initiation in the context of AR overexpression [66], an important finding given that Src kinase-activating mutations are not readily found in primary or CR-CaP [67-69]. A recent paper by Su et al. [70] demonstrates that the frequency and time-to-onset of spontaneously generated CR-CaP in the CWR22 xenograft model are decreased by the siRNA-mediated knockdown of Src.

Activation of AR by Direct Phosphorylation: Role of SFK and Ack1

The landmark study by Guo et al. [20] demonstrated that AR activation could be induced by direct phosphorylation by Src on Y534, as identified by mass spectrometry. Kraus et al. [71] confirmed that the Src-mediated tyrosine phosphorylation of AR was positively regulated by the PKC scaffolding protein, RACK1. The concept that AR might serve as a Src substrate is strengthened by evidence that a Src-AR complex is required for androgen-induced CaP cell proliferation *in vitro* [72] and that Src kinase inhibition blocks AR-dependent transactivation of known androgen-inducible genes [20, 73]. Moreover, expression of activated Src and the subsequent phosphorylation of AR on Y534 are sufficient to induce androgen-independent growth *in vitro* in LNCaP and LAPC-4 cells [38] and CR growth *in vivo* [20]. Activated Ack1 could also facilitate androgen-independent CaP cell proliferation *in vitro* through the direct phosphorylation of AR on Y267 [38, 74]. EGF treatment of LNCaP or LAPC-4 cells could induce AR phosphorylation on both Y267 and Y534, whereas other pro-proliferative stimulants could only activate AR through either Ack1 or Src pathways: AR-poY267 could be induced by heregulin or the Mer receptor ligand, Gas6, whereas AR-poY534 could be induced by IL-6 or bombesin [37]. Importantly, increased AR-poY267 and -poY534 staining levels correlate with CR-CaP disease progression and worse survival prognosis [20, 75], strongly suggesting that the increased activation of SFK and Ack1, and subsequent AR activation through direct phosphorylation by these kinases, facilitates CaP malignancy, especially CR progression.

Several non-genotropic AR functions have been described and are thought to occur at the plasma membrane, i.e.- not through AR's transactivation function [76]. Interestingly, Src may control these functions, which are poorly understood but which may regulate cell survival pathways, by direct binding to AR [77, 78] although data are lacking as to whether this control axis is dependent on Src phosphorylation of AR.

Tyrosine Kinase Antagonist Treatment in CR-CaP

Studies corroborating the involvement of activated Src in progression to androgen-independence or castration-recurrence [70, 73, 79, 80], increased invasiveness [66], or metastatic growth in bones [81], as well as preclinical studies demonstrating critical roles for SFK in prostate cancer metastasis [51, 58, 82-85] have spawned clinical trials using SFK-specific or pan-tyrosine kinase inhibitors (reviewed in [2, 36]).

Indeed, a large set of review papers have addressed the rationale of targeting of SFK and Ack1 in CR-CaP, especially in its main manifestation as bone metastatic growths [36, 42, 43, 58, 86-90].

The effects of kinase inhibitors on CaP biology *in vitro* and *in vivo* depends, in most cases, on the specificity of the drugs studied. For example, Dasatinib (BMS-354825), originally described as a Src/Abl-specific inhibitor [91], likely inhibits a wide range of receptor- and non-receptor tyrosine kinases [92]. Thus, whereas initial reports demonstrated that Dasatinib could inhibit Src/FAK-mediated signaling pathways that control prostate cancer cell adhesion, motility and invasiveness [50, 93], subsequent reports showed that it could also inhibit CaP growth as boney metastases [94], androgen-independent growth [54] associated with the site-specific tyrosine phosphorylation of AR by Src or Ack1 [37], or spontaneous formation of CR (CWR22) tumors [70]. Although early clinical trials showed some efficacy of using Dasatinib as a monotherapy or in combination with docetaxel [95-99], the recent READY Phase III trial showed that adding Dasatinib had no greater effect on survival in cases of chemotherapy-naïve metastatic CR-CaP [100]. Importantly, serum markers, such as insulin-like growth factor-1 (IGF-1), have been identified that correlate with efficacy by Dasatinib in metastatic CR-CaP cases [101], prompting a study by Dayyani et al. [102] showing superior inhibition of CR-CaP growths in mouse models when combining the IGF-1receptor/insulin-receptor inhibitor, BMS-754807, with Dasatinib.

The argument that Dasatinib's broad tyrosine kinase specificity undercuts its clinical efficacy [103] has resulted in the increased focus on more specific SFK inhibitors in CR-CaP. For example, Saracatinib (AZD0530), a Src/Abl inhibitor which showed anti-CaP efficacy in preclinical CaP models [104, 105] and in PC-3 bone metastases [106], showed efficacy as a monotherapy in CR-CaP patients [107]. Bosutinib (SKI-606) is another Src/Abl inhibitor [108] that has shown efficacy against prostate cancer in preclinical models [109], has passed Phase I testing [110] and was FDA-approved for use in Gleevec-resistant chronic

myelogenous leukemia, yet is not part of any current Phase II trials for prostate cancer. Cabozantinib (XL184), an inhibitor of the Met receptor tyrosine kinase, which manifests its oncogenic signaling in prostate cancer through Src activation [83], has shown efficacy in Phase II trials against CR-CaP [111]. KXO1 (KX2-391) is a novel non-ATP-competitive Src/tubulin polymerization inhibitor which, in recent Phase II studies [112], failed to reduce tumor burden in bone-metastasis CR-CaP cases, although a caveat noted was that the dosing was insufficient to achieve the drug's tubulin polymerization inhibition activity. Lastly, several new Ack1-specific inhibitors have been identified [113], including AIM-100, which has shown efficacy against CR-CaP cell lines *in vitro* and *in vivo* [75, 114].

Future Directions

The growing corpus of evidence showing that SFK and Ack1 are potent drivers of CR-CaP and metastasis via AR-dependent mechanisms has led to a renewed focus on developing specific tyrosine kinase inhibitors as mainline or adjunct therapies. In addition to solving issues of increased toxicities when combining such antagonists with newer generation inhibitors of AR or androgen synthesis, such as enzalutamide or abiraterone, respectively, there is no consensus *a priori* as to whether SFK-specific drugs might work better than those with broad specificity for tyrosine kinases. As the use of SFK/Ack1 inhibitors increases in CR-CaP populations, one area needing improvement is the identification and development of bio- or genetic markers of drug efficacy. One such example might be the development of immunohistochemistry-grade antibodies specific for AR-poY267 and -poY534. Indeed, newer pharmacogenetic paradigms are being developed to identify individualized response and toxicity signatures that will help stratify patient-specific treatments [115] with the aim of improving patient survival outcomes.

Competing Interests

The authors have declared that no competing interest exists.

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