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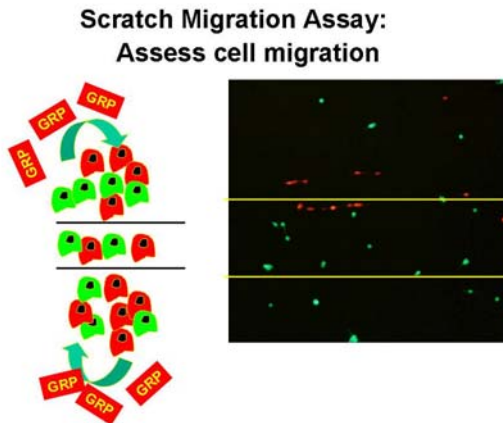
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14. ABSTRACT We have made headway into understanding the paracrine relationship between neuropeptide expressing, androgen-insensitive CaP cells and their ability to support the proliferation and migration of androgen sensitive CaP cells. Critically, we have identified src kinase as a molecule central to the process. We have been awarded a NIH CTEP phase II trial to study a novel, oral src kinase inhibitor AZD0530 in androgen-insensitive prostate cancer patients based upon our work.					
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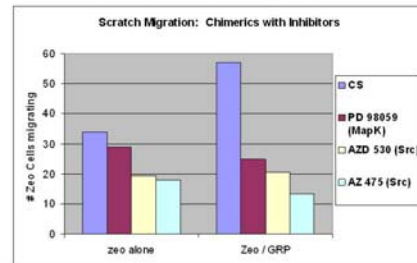
Table of Contents

Introduction.....	4
Body.....	4-9
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	12
References.....	12
Appendices.....	12

migration assay. This assay was conducted with the help of fluorescence tags and microscopes. LNCaP-Zeo cells do not migrate in an unstimulated environment to any significant degree. LNCaP-Zeo-GFP migrated 1.7 fold more to the scratch region when plated together with LNCaP-GRP-Red cells than alone (bottom left). MEK1 inhibitor, PD98059 and Src kinase inhibitors, AZM475271 and AZD0530 all partially inhibited this stimulated migration of LNCaP-Zeo-GFP cells (bottom right).



Scratch Migration:
Chimeric stimulation of AS cells to migrate

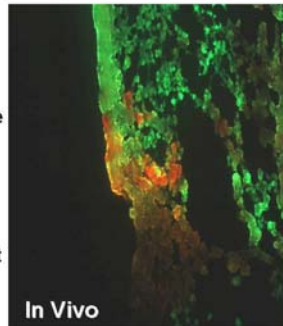


• GRP AI cells promoted migration of AS cells into lanes
• Src and MAPK inhibitors significantly reduced migration

d. Study the paracrine effect using the *in vivo* xenograft model with regard to growth and metastasis. Co-injection of LNCaP-Zeo cells with LNCaP-GRP cells in castrated SCID mice produced tumors in the prostate regions. LNCaP-Zeo cells are not normally

Growth of LNCaP supported in AI conditions by LNCaP-GRP

- Zeo-GFP and GRP 4-9-red cells, 2 million each, were implanted into prostates of castrated SCID mice.
- Tumor growth was palpable in 2 months.
- Frozen sections of the tumor were visualized by fluorescence microscopy.
- Cells of different origins with green or red fluorescent tags are clearly visible in tumor sections.

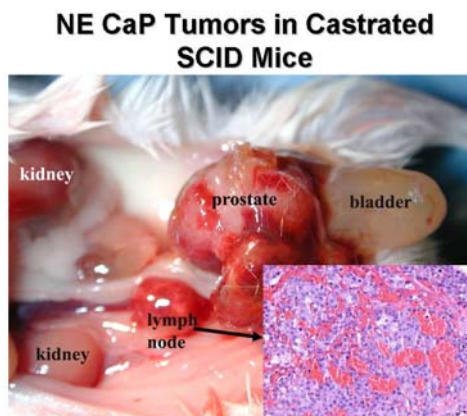


tumorigenic in the *in vivo* castrate environment. The Zeo cells were tagged with green fluorescence protein (GFP) and the GRP with red (Red). Frozen sections of tumor vividly showed patches of green and red colors under the fluorescent microscope. Taken together, both overexpression of GRP may stimulate growth of androgen sensitive Zeo cells both *in vitro* and *in vivo* through paracrine effect.

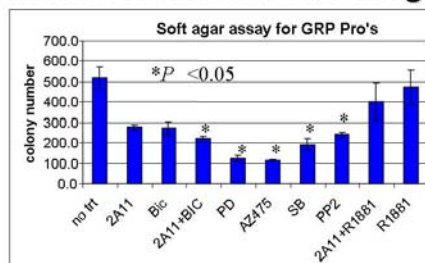
Aim 2. To evaluate the mechanisms of AR involvement in our NE model.

a. *Testing of inhibition of neuropeptides, signaling molecules and AR inhibitors individually and in combination on soft agar growth of GRP clones and xenograft cells.*

Tumors harvested from GRP implanted



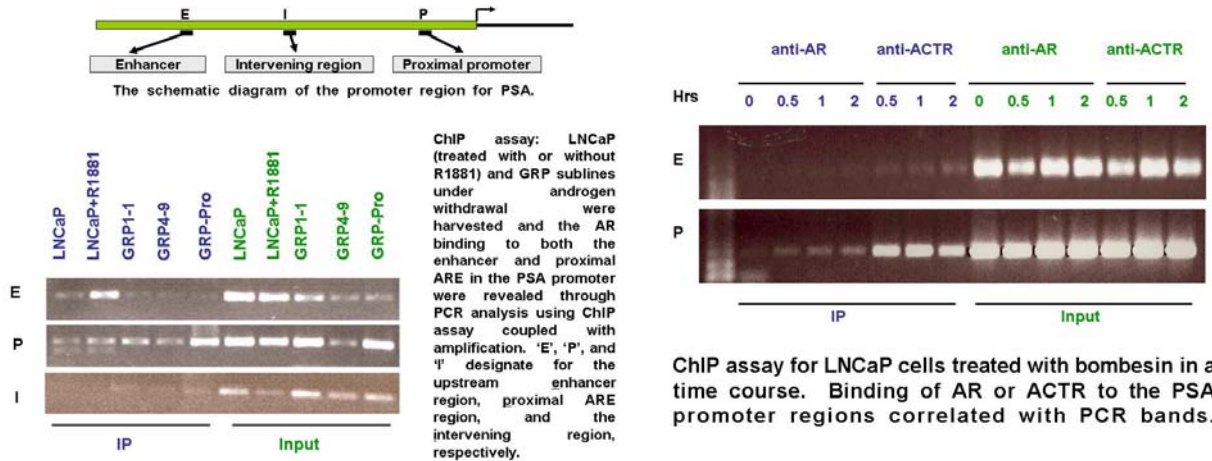
Inhibition of Recultured Xenografts



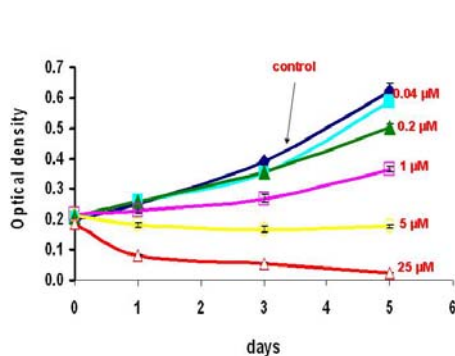
Soft agar assay of the recultured GRP Pro's xenograft. Treatments include: monoclonal antibody to bombesin/GRP, 2A11 (1 mg/ml), anti-androgen bicalutamide (BIC, 5 μ M), MEK1 inhibitor PD98059 (PD, 10 μ M), Src inhibitors AZM457271 (5 μ M) and PP2 (10 μ M), P38MAPK inhibitor SB 203580 (SB, 10 μ M), synthetic androgen R1881 (1 μ M).

mice were re-cultured in vitro to establish xenografts termed as GRP-Pro (derived from Prostate). The expression of human AR, PSA and GRP in tumor xenograft GRP-Pro was analyzed by RT-PCR analysis and supports the authenticity of the clones. Soft agar assay using GRP-Pro showed their aggressive nature as manifested by their androgen- and anchorage- independent growth in 2 weeks. This growth was partially inhibited by the mAb specific to bombesin, 2A11, the androgen inhibitor, bicalutamide, and in combinations (with significant difference $p \leq 0.05$) supporting that the growth is dependent on both the neuropeptide GRP and AR. When synthetic androgen was added with 2A11, the colony formation ability of GRP-Pro resumed to a level similar to control. This further supports the overlapping effect of GRP and AR to the growth of GRP-Pro. Based on the tyrosine kinase display, Src kinase is present in LNCaP cells and involved in signaling via phosphorylation upon bombesin stimulation. Src kinase was constitutively active in LNCaP GRP and its xenograft GRP-Pro when cultured in androgen-free CS serum media. We thus subjected growth of LNCaP GRP-Pro to the inhibitors for Src kinases, AZM475271 from AstraZeneca and PP2. Since MEK1/2 is downstream to Src activation, we also tested the effect of PD98059. Finally, we included the MAPK P38K inhibitor SB203580 because P38 displayed activation in LNCaP cells upon androgen withdrawal. All kinase inhibitors tested decreased the growth 60-80% of control, with significant differences ($p \leq 0.05$). This suggests that the androgen-independent growth of GRP-Pro involves both Src and MEK in a GRP stimulated AR-dependent manner.

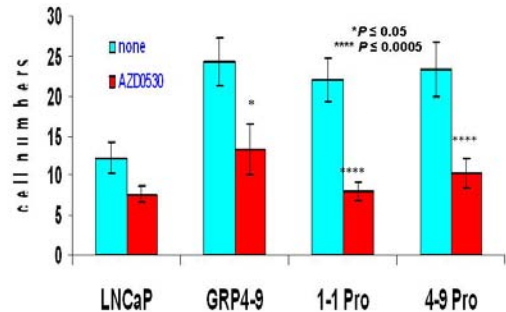
The mechanisms of neuropeptide-mediated AR activation were then investigated in more detail. We performed chromatin immunoprecipitation (ChIP) assay and discovered that bombesin-stimulated AR binds preferentially to the proximal ARE site in the promoter region rather than the enhancer region bound by the androgen-stimulated AR. GRP-Pro cells constitutively expressing GRP have the AR occupied on the proximal ARE constantly. This bombesin/GRP-stimulated preferential binding of AR to the proximal site of the PSA promoter is assisted by the AR co-activator ACTR 30 min from addition of bombesin.



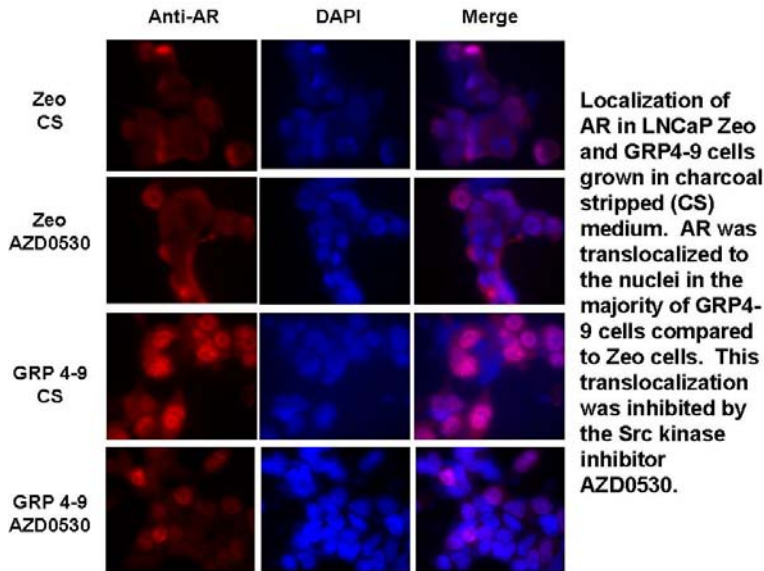
As reported last year, growth of GRP cells in soft agar may be inhibited by the specific Src inhibitor AZD0530. We performed a dose-response growth inhibition curve using GRP-Pro cells grown in CS media and treated with various doses of AZD0530. The IC₅₀ for this inhibition is slightly higher than 1 μ M. The LNCaP GRP cell lines have demonstrated promoted migratory activities than their parental cells. Src kinase inhibitor AZD0530 inhibits the migration assayed by the Boyden chamber assay to the levels similar to the basal activity in the LNCaP cells.



GRP-Pro cells were plated in CS medium with and without the Src inhibitor AZD0530 and their growth was monitored by MTT assay over 7 days. Various concentrations of AZD0530 from 0.04 to 25 μ M were added from day 0. Error bars represented standard error of means.



Inhibition of migration of LNCaP-GRP and GRP-Pro cells by AZD0530. Migration assays were carried out in the modified Boyden chamber. Migration assays were performed in a Boyden chamber with 8 mm Nucleopore membrane coated with human plasma fibronectin (50 mg/ml). 2×10^4 LNCaP cells were placed in the upper wells, CS conditioned media with or without 500 nM AZD0530 in the lower wells, and the chamber was incubated at 37°C for 4 hours to allow cell migration. The entire field was counted under a microscope and each experiment was performed in triplicate.

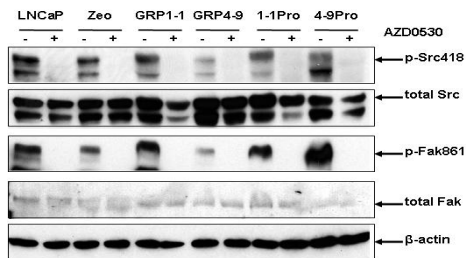


LNCaP GRP cells showed translocation of AR into the nuclei in the absence of androgen stimulation (in CS growth media) compared to the mock-transfected LNCaP Zeo cells. Addition of Src kinase inhibitor AZD0530 abolished the AR nuclear translocation as shown in the left. This result suggests that AR is activated through autocrine stimulation of GRP that is dependent of Src activation.

We surveyed the status of Src and FAK in the LNCaP and GRP subclones and found similar levels of phosphorylated Src and FAK kinases. However, when these two kinases were co-immunoprecipitated by anti-FAK antibodies, stronger phospho-Src levels were detected in GRP subclones than their mock control Zeo cells. These findings confirm our hypothesis that in the absence of AR, bombesin/GRP bind to their receptors, activate Src and FAK kinases in the complex and activate AR through phosphorylation.

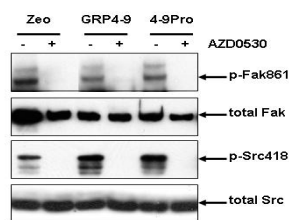
b. *Small hairpin RNA (shRNA)-based silencing of NE cells in vitro and in vivo.* We are in the process of designing the shRNA. Once we get the shRNA construct, we will start experiments in this section. We have requested the no-cost one-year extension to

Src and FAK status in cell lysates



Phosphorylation of Src and FAK kinases was inhibited in parental LNCaP, LNCaP-GRP and GRP-Pro cells by AZD0530 as probed by antibodies specific to Tyr(418)-Src and Tyr(861)-FAK, respectively. The ability of AZD0530 to inhibit Src-mediated phosphorylation of FAK kinase was explained by co-immunoprecipitation (co-IP) of FAK and Src kinases with anti-FAK antibody.

CO-IP of Src and FAK



complete this and the in vivo study.

c. *Testing of inhibitory treatments on chimeric tumors in soft agar and in vivo.* We have demonstrated inhibition of paracrine migration. We are presently testing inhibition of chimeric tumor growth and metastasis in vivo.

d. *In vivo testing of inhibitory treatments at different time points.* Since we have identified Src kinase as the key player in neuropeptide-mediated AR activation, we tested the effect of Src kinase inhibitor AZD0530 in vivo with LNCaP GRP-Pro cells. After almost two months of AZD0530 administration to castrated mice injected with LNCaP GRP-Pro cells, we observed a complete inhibition of metastasis by AZD0530. Although inhibition of primary tumor growth was not significant as reported by other researchers working on various cancers, AZD0530 demonstrated potent inhibition on tumor metastasis. None of the treated animals had metastases to regional lymph nodes but both surviving control animals did.

In vivo study: Ten male SCID mice were castrated and orthotopically implanted with 4×10^6 GRP-Pro cells into the prostate. AZD0530 (50 mg/kg) treatment was administered to seven mice (treatment group) while buffer was administered to three (control group) 16 days after surgery. The study was terminated 70 days after injection, mice from both groups were examined for primary tumor growth and metastasis. At the end of study, two remaining control mice both bore tumors and metastasis to lymph nodes, while five out of seven treated mice produced tumors but with NO metastasis.

	Tumor	Tumor weight (g)	Metastasis
Control	3/3 (one died before tumor collection)	1.04 ± 0.34	2/2
Treatment	5/7	0.73 ± 0.29	0/5

Other Research Accomplishments

We have characterized the expression of the NE induced expression of src, FAK and STAT3 in all major prostate cancer cell lines. We have also validated the action of Src kinase inhibitor AZD0530 through the Src signaling pathway in two androgen-independent prostate cancer cell lines PC-3 and DU-145 by examining the status of phosphorylation of the downstream kinases and substrates. Through this study, we have identified the molecular mechanism of AZD0530. In vivo inhibitions of tumor progression by AZD0530 are also underway. These data are presently being combined for publication submission.

We have determined the downstream signaling cascades from NE activation and delineated the effect of a novel oral src kinase inhibitor AZD0530 at these signaling points. This is presently in preparation for publication.

Key Research Accomplishments

We have demonstrated that Src kinase is the key player in neuropeptide-mediated AR activation. Together with our studies in the chimeric growth of androgen-sensitive and androgen-insensitive cells, we are more confident with our proposed hypothesis. A paracrine effect exists for androgen insensitive CaP cells to support the survival and proliferation and migration of androgen sensitive CaP cells in a castrated environment. We have further delineated the impact of NE differentiation in prostate cancer.

Reportable Outcomes

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Conclusions

We have made headway into understanding the paracrine relationship between neuropeptide expressing, androgen-insensitive CaP cells and their ability to support the proliferation and migration of androgen sensitive CaP cells. Critically, we have identified src kinase as a molecule central to the process. We have been awarded a NIH CTEP phase II trial to study a novel, oral src kinase inhibitor AZD0530 in androgen-insensitive prostate cancer patients based upon our work.

References

None

Appendices – See Attached

1. Nelson EC, Cambio AJ, Yang JC, Ok JH, Lara PN Jr, Evans CP.
Clinical implications of neuroendocrine differentiation in prostate cancer.
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REVIEW

Clinical implications of neuroendocrine differentiation in prostate cancer

EC Nelson, AJ Cambio, JC Yang, J-H Ok, PN Lara Jr and CP Evans

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The cellular signaling pathways of the prostate play a central role in the induction, maintenance, and progression of prostate cancer (CaP). Neuroendocrine (NE) cells demonstrate attributes that suggest they are an integral part of these signaling cascades. We summarize what is known regarding NE cells in CaP focusing on NE cellular transdifferentiation. This significant event in CaP progression appears to be accelerated by androgen deprivation (AD) treatment. We examine biochemical pathways that may impact NE differentiation in a chronological manner focusing on AD therapy (ADT) as a central event in inducing androgen-independent CaP. Our analysis is limited to the common adenocarcinoma pattern of CaP and excludes small-cell and carcinoïd prostatic variants. In conclusion, we speculate on the future of treatment and research in this area. *Prostate Cancer and Prostatic Diseases* (2007) 10, 6–14. doi:10.1038/sj.pcan.4500922; published online 31 October 2006

Keywords: androgen-independent prostate cancer; hormone refractory; neuroendocrine cells; neuroendocrine differentiation

Introduction

Prostate cancer (CaP) is the most common non-cutaneous malignancy in American men and is predicted to be the third leading cause of cancer deaths for 2006.¹ Although local therapy for CaP is relatively effective, androgen deprivation therapy (ADT) remains the mainstay of treatment for disseminated disease and is principally palliative in nature. Introduced in the 1940s,² ADT removes androgen stimulation, initially inducing apoptosis in CaP. However, the disease eventually progresses to an androgen-independent (AI) state with an associated life expectancy of only 15–20 months. Despite continuous research efforts, limited progress has been made in the treatment of advanced CaP in the last 50 years and life expectancy associated with metastatic disease has not changed significantly.³ ADT, while extending length and quality of life for many patients, also induces biological changes in CaP that may promote progression to an AI state.

The role of prostatic neuroendocrine (NE) cells in this biologic process has recently become the focus of much attention. Known changes in the number, histology, and functions of NE cells during CaP progression indicate that they may play a regulatory role. The fact that the majority of NE cells may not exhibit androgen receptors (ARs) is of special interest in the androgen-deprived

patient.^{4,5} In these patients, NE cells may allow continued CaP growth through paracrine stimulation of neoplastic epithelial cells. Indeed, mitogenic and oncogenic activity has been demonstrated for many of the factors NE cells are known to produce.

The purpose of this review is to summarize the latest developments in understanding the role of NE cells in the normal prostate, in CaP, and the effects on potential treatment modalities related to this. Data suggest that ADT may facilitate NE differentiation (NED) and thereby accelerate cellular mechanisms that contribute to the AI state. This review will be structured chronologically around this central event.

NE histology and differentiation

The normal prostate contains a glandular epithelium within intervening fibromuscular stroma. The epithelium can be further subdivided into tall columnar cells that secrete into the lumen of the gland, and cuboidal cells forming a basal layer against the basement membrane. A third type of epithelial cell was first described by Pretl in 1944.⁶ These cells are identified by their neurosecretory granules and expression of neuron peptide hormones such as bombesin/gastrin-releasing peptide (GRP), neurotensin (NT), serotonin, calcitonin and parathyroid hormone-related peptide (PTHrP).^{7,8} Based on these findings, they were labeled NE cells, part of the larger amine precursor uptake and decarboxylation (APUD) lineage.

In other organs, the origin of NE cells has been shown to be endodermal stem cells,^{9,10} and a similar model was

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thought to apply to prostatic NE cells. Bonkhoff *et al.*¹¹ demonstrated a possible development progression for NE cells from stem cells using immunohistochemical staining to demonstrate intermediate phenotypes. Recently, neural crest cells have re-emerged as the putative source for NE cells.¹² Although disagreement exists regarding the embryonic source of NE cells in the prostate, it is clear that prostatic epithelial cells are remarkably plastic and have the capability to differentiate into NE cells. As described below, the NE cells associated with CaP are phenotypically dissimilar to normal NE cells and function in different ways leading to the conclusion that they probably emerge from transdifferentiation of epithelial cells rather than malignant NE precursors.

Normal function of NE cells

Many secreted types of NE granules are identified by immunohistochemical staining and indicate that different subsets of NE cells exist. This introduces much complexity to the question of their normal function. A general understanding may be obtained by comparisons to NE cells in other organ systems and examination of the individual products secreted by prostatic NE cells.

Prostatic NE cells are part of a larger histological genre known as the APUD system, present in many organs of the body. For example, stomach D cells and G cells produce somatostatin and gastrin, respectively, and many intestinal NE cells secrete various hormones that regulate gut function. In a similar way, it may be inferred that the factors produced by prostatic NE cells regulate prostatic growth, function, and cellular differentiation. Indeed, many of the factors shown to be produced by NE cells are known to support growth and differentiation in the prostate (Table 1). For example, bombesin/GRP receptors are members of the superfamily of heterotrimeric G-protein-coupled transmembrane-spanning receptors.¹³ Binding of these receptors elicits calcium mobilization, thereby promoting growth and cell invasiveness through proteolytic activities in cell lines.^{14–17}

If NE cells exert regulatory control over prostatic tissue, the question arises as to what regulates the NE cells. Although some NE cells may express AR,¹⁸ many are AI as they do not contain ARs.^{4,5} However, they do have receptors for epidermal growth factor (EGF) and ErbB2, which suggests they are controlled more by local growth factors from the prostatic stroma than systemic hormones.¹⁹ The expression of the EGF receptor itself is under the control of PTHrP produced by both epithelial cells and NE cells. It has been reported that interleukin

(IL)-1 β and IL-6 upregulate CgA expression in CaP cell lines,²⁰ and IL-6 has been shown to induce morphologic change toward an NE phenotype in epithelial cells.²¹

To summarize, NE cells express potent neuropeptides that mediate diverse biological processes such as cell growth, differentiation and transformation. In addition, their morphology and distribution within the prostate epithelium suggest a regulatory role similar to APUD cells in other organs of the body. In contrast to other epithelial cells, they are generally AR negative and probably rely on paracrine growth factor control.

Role of NE cells in early CaP

The NE cells in CaP appear morphologically different than those seen in benign tissue and co-express epithelial markers such as prostate-specific antigen (PSA) and NE markers (CgA).^{22,23} It is believed that these cells are the result of transdifferentiation of epithelial cells.²⁴ Such NED has been experimentally demonstrated in several CaP cell lines using cyclic AMP (cAMP), epinephrine, forskolin, the cytokines IL-1 and IL-6, and as will be seen later, AD conditions.^{20,21,25,26} These changes were shown to be reversible when the substances were removed,²⁷ emphasizing the incredible degree of plasticity exhibited by prostatic epithelial cells. Based on this likely mechanism for generating malignant NE cells, some NE cells may express neuropeptide growth factors before changing morphologically and/or expressing CgA, NSE, etc. In support of this, Iwamura *et al.*²⁸ showed increased PTHrP in high-grade prostatic intraepithelial neoplasia before much NED had taken place as measured by common NE markers.

NED is very common in CaP specimens. For example, Bostwick *et al.*²⁹ reported a prevalence of 92%. Although wide ranges have been reported (30–100%), this is probably due to different sampling techniques and testing methods.³⁰ Several varieties of prostatic NED have been described. Two very rare types are small-cell carcinoma and carcinoid tumor, both of which express large numbers of malignant NE cells. The most common type is the typical adenocarcinoma with individual NE cells surrounded by small foci of epithelial CaP cells. This arrangement (Figure 1) suggests that the NE cells are producing growth factors supporting surrounding (proliferating) cells.^{31,32}

The prognostic significance of NED is controversial. Many studies before 1990 showed a worse prognosis with increasing NED, but subsequent examinations failed to find any correlation independent of tumor

Table 1 Selected NE cellular products

Products	Action in CaP	References
Calcitonin gene family	Growth modulation, <i>in vitro</i> resistance to apoptosis, stimulates PTHrP release	45,66
GRP	AI growth factor, mediates migration, <i>in vitro</i> resistance to apoptosis, activates NF- κ B	66,94,95
Neuropeptide Y	Possible angiogenic effect through MAP kinase	96
Parathyroid hormone-related protein	Mitogenic, regulates EGF receptor, overexpressed early in CaP	28,77
Proadrenomedullin N-terminal peptide	Angiogenesis and GRP actions through GRP receptor binding	97,98
Serotonin	Mitogen, facilitates AI growth	24,66,99
VEGF	Angiogenesis, promotes growth and motility in AI manner	48,100

Abbreviations: AI, androgen-independent; CaP, prostate cancer; GRP, gastrin-releasing peptide; MAP, mitogen-activated protein; NF- κ B, nuclear factor-kappa B; PTHrP, parathyroid hormone-related peptide; VEGF, vascular endothelial growth factor.

8 grade or androgen responsiveness.⁷ The controversy continues with some recent studies showing an independent negative correlation between serum CgA and survival in AI CaP,^{33,34} but others showing no prognostic correlation,³⁵ or improved outcomes with higher CgA.³⁶ These authors all agree that the serum CgA continues to be a valid marker of progression, but the complex biology of NED makes direct correlation with prognosis difficult. Cussenot *et al.*³⁷ studied CgA and NSE serum levels in CaP patients before ADT. Although most elevations of serum markers were found in AI tumors, some were not, leading them to theorize a role for NED in the progression of CaP before AD. Their

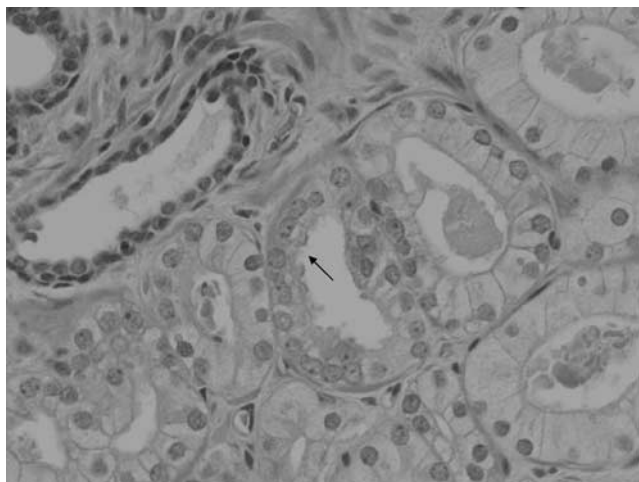


Figure 1 NED in Gleason 7 CaP specimen (courtesy of Dr Gandour-Edwards).

study corroborated findings by Hoosein *et al.*³⁸ that NED markers correlated more with metastasis than locally advanced disease.

The role of NE cells in the development and progression of CaP is suggested by their central role in cell signaling pathways and several of these will be briefly outlined including bombesin/GRP, serotonin, PTHrP and possible pathways involved in angiogenesis (Figure 2). A negative regulator of proliferation, somatostatin, will also be discussed.

Bombesin/GRP is a potent mitogenic neuropeptide shown to stimulate CaP growth in cell culture,³⁹ probably through its ability to induce c-fos and c-myc, thereby deregulating the cell cycle.⁴⁰ GRP receptors are known to be distributed throughout the human prostate and Markwalder and Reubi⁴¹ showed they are overexpressed in CaP. Bombesin/GRP also causes CaP cell lines PC-3 and LNCaP to acquire greater invasive potential.⁴²

Serotonin is produced by most NE cells and is known to be associated with malignant transformation.^{7,43} Dizeyi *et al.*⁴⁴ showed several types of serotonin receptors exist in CaP tissue and cell lines. Higher grade cancer was shown to express a greater number of receptors and tissue growth was regulated by serotonin agonists and antagonists. The pathways by which serotonin acts are complex due to multiple receptor binding capabilities. It has been suggested that serotonin may be related to the potent oncoprotein ras¹⁰ downstream from EGF receptors.

PTHrP is predominantly expressed in fetal tissues but is also produced by NE cells. It is overproduced by CaP tissue lines and can stimulate growth in a paracrine manner.⁴⁵ PSA cleaves PTHrP destroying its ability to

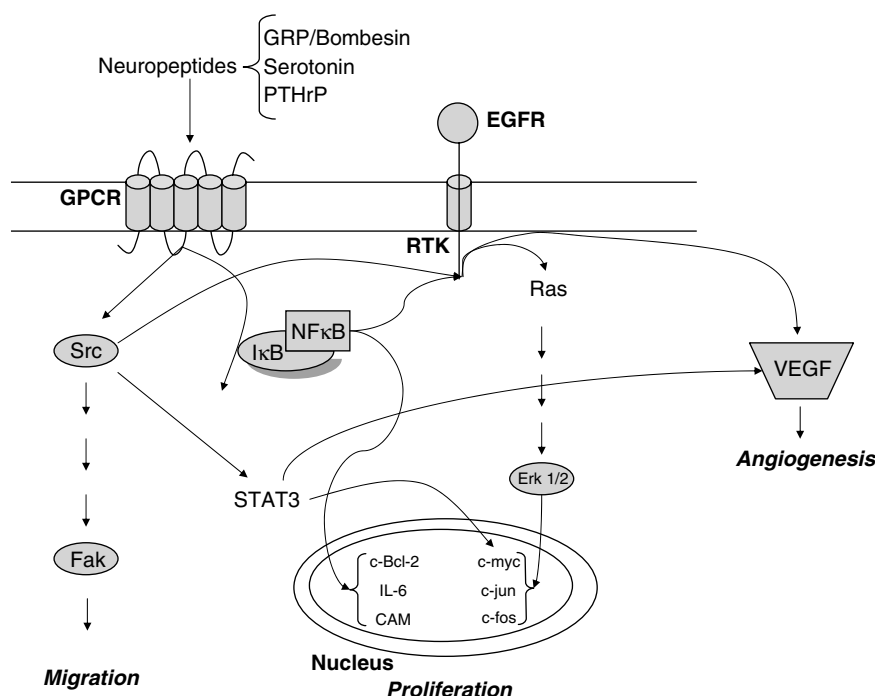


Figure 2 Summary of possible pathways involving NE cellular products in the development and progression of CaP. Neuropeptides activate G-protein-coupled receptors activating Src and NF- κ B. In addition to their direct downstream effects, they may transactivate growth factor receptors. Abbreviations: CAM, cell adhesion molecule; EGFR, epithelial growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; GPCR, G-protein-coupled receptor; NF- κ B, nuclear factor-kappa B; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor.

bind receptors. This has led to the suggestion that as PSA expression decreases with ADT and CaP progression, PTHrP's growth-promoting activity increases.¹⁹

Angiogenesis is a necessary component of neoplastic growth because of increased energy requirements. NED is correlated with overall microvessel density in CaP. In addition, increased microvessel density is seen surrounding areas of NED and this effect is independent of tumor grade.⁴⁶ Several products of NE cells are possible mediators of this effect. Vascular endothelial growth factor (VEGF) is produced by some NE cells,⁴⁷ and VEGF staining NE cell density correlates with microvessel density.⁴⁸ Although bombesin/GRP probably does not directly stimulate angiogenesis through tyrosine kinases,⁴⁹ it may stimulate the nuclear factor-kappa B (NF- κ B) angiogenesis pathway or enhance the angiogenic effects of growth factors by transactivating the EGF receptor.^{50–52} The fact that the EGF receptor is overexpressed in CaP may enhance this effect.⁵³

Somatostatin is the one neuropeptide that may have a restraining influence upon prostatic growth and possibly neoplastic transformation. NE cells not only produce somatostatin, they also have receptors indicating autocrine as well as paracrine function.⁵⁴ In CaP cells, somatostatin induces cell-cycle arrest and apoptosis,⁵⁵ perhaps through receptor type 3, which induces Bax.⁵⁶ Somatostatin may inhibit neovascularization and prostatic growth both directly and through indirect effects mediated by insulin-like growth factor (IGF)-1. Somatostatin decreases growth hormone (GH) release by the liver, which in turn decreases IGF-1 release.⁵⁶ Acromegalic patients have increased GH and IGF-1, and the somatostatin agonist octreotide has been shown to decrease prostate size in a cohort of these patients.⁵⁷

To summarize, NE cells express potent neuropeptides that mediate diverse biological processes such as cell growth, differentiation, transformation and invasion. Although NE cells do not stain for proliferative antibodies, they may be a source of paracrine factors that support CaP growth and progression. All of these complex interactions between signal-transduction pathways are undoubtedly involved in prostatic homeostasis. This fine balance may be disturbed not only by pre-existing genetic faults but also via environmental toxins and carcinogens, diet, and the stress response, all of these acting through the microcellular hormonal milieu. Many of these microcellular environmental pathways converge through G-protein-coupled receptors via cAMP, protein kinase A and tyrosine kinases to activate mitogen-activated protein (MAP) kinases. Increasing aberrant activation of these pathways is independent of androgens, utilizing instead a complex interplay between classical growth factors and neuropeptides.

NE cells during ADT

The current treatment of metastatic CaP consists of medical or surgical AD. The prostatic microenvironmental conditions brought on by ADT apparently play a central role in the progression of CaP to the AI state. NE cells are thought to play an important part in effecting

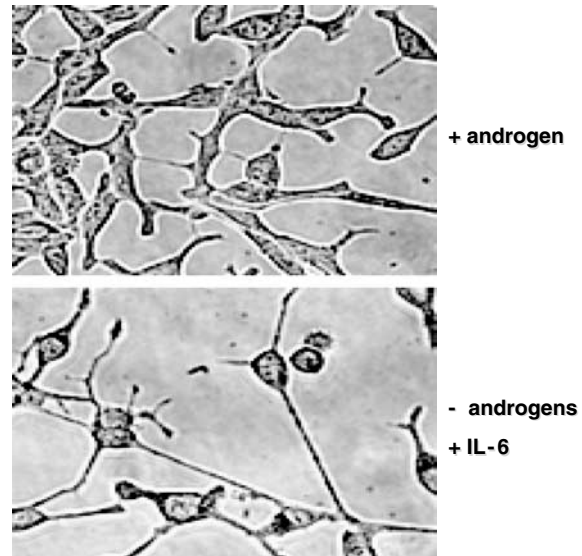


Figure 3 Morphological changes in LNCaP undergoing NED. In the presence of androgen, cells show normal fusiform morphology with unbranched cellular processes. Under AD conditions, cell bodies become compact and cellular processes lengthen and demonstrate a branching morphology.

this change based on several lines of evidence from both *in vitro* and *in vivo* studies.

Jongsma *et al.*⁵⁸ demonstrated that PC310 cells differentiate along NE lines when they are androgen deprived. AD of LNCaP cells results in NED (Figure 3), with the addition of IL-6,²¹ IL-8⁵⁹ and neuropeptides.⁶⁰ Human prostatic epithelial cells are similarly plastic and undergo NED in an AD mouse xenograft.⁶¹ CaP patients treated with ADT demonstrate higher levels of CgA compared to androgen normal controls.⁶² When patients undergo surgical resection, prostatic specimens showed significantly increased NED in patients treated with neoadjuvant ADT compared to surgery only.⁶³ Examination of gene expression shows higher levels of CgA mRNA in androgen-deprived CaP vs benign tissue.⁶²

Feldman and Feldman⁶⁴ have described a system for classifying mechanisms of AI growth during ADT into five categories. These are by no means mutually exclusive and may all be operative. Two of their categories form a useful framework for discussing the various possible actions of NE cells in the larger tissue microenvironment, specifically their possible role in supporting AI growth of CaP epithelial cells.

The 'outlaw receptor pathway' causes androgenic effects via crosstalk between the AR and other signaling pathways. The final common pathway for these effects seems to be phosphorylation of residues on the AR causing activation of downstream effects, probably through stimulation of MAP kinase.^{59,65} Theoretically, these signaling pathways can be activated by various biogenic amines produced by NE cells.⁷ Jongsma *et al.*⁶⁶ showed that some androgen-depleted CaP cell lines can proliferate when stimulated by GRP, a neuropeptide produced by NE cells.

The gradual shift of normal to uncontrolled stimulation of proliferation may be accelerated by ADT through stimulation of greater growth factor production as shown by Culig *et al.*⁶⁷ for EGF. Alternatively, decreased

binding protein production may increase available growth factors. Androgens may regulate bioavailability of neuropeptides through regulation of binding proteins as has been shown for IGF/insulin-like growth factor-binding protein (IGFBP).^{68,69}

The 'bypass pathway' includes mechanisms that do not require androgens or the AR. The inhibition of apoptosis is an important mechanism for the progression of neoplasia and several mechanisms in CaP have been studied. Bcl-2 is a gene product that blocks apoptosis and is not normally expressed by prostate epithelium.⁷⁰ The expression of this gene directly correlates with androgen responsiveness and has been shown to be induced upon ADT in a mouse xenograft model.^{71,72} Epithelial cells surrounding NE cells have higher levels of Bcl-2, suggesting that the microcellular environment produced by them induces greater cell survival.⁷³ In addition, the NE cells express survivin, another antiapoptotic substance.⁷⁴

Growth factors may not only promote growth through outflow receptor pathways, they may also inhibit apoptosis. Neuropeptides endothelin-1, bombesin, and growth factor IGF-1 activate the IGF-1 receptor, which phosphorylates AKT, a serine/threonine kinase. Activated AKT induces a strong antiapoptotic cellular signal.⁷⁵ The activity of AKT is opposed by PTEN (phosphatase and tensin homolog) and loss of PTEN is correlated with high-grade CaP.⁷⁶ If IGF-1 activity is increased because of the AD-mediated decrease in IGFBP, the AKT pathway would overcome the inhibition of PTEN even if it has not been lost to mutation. Activation of the AKT pathway is probably important in AI progression.⁷⁷

To summarize the role of NE cells in the AI state, it has been demonstrated that the products of NE cells stimulate AI growth and increasing anaplasia. All CaP cells from cell lines and patient samples have receptors for bombesin or NT.⁴¹ PC-3 cells display a growth response to NT¹⁶ and invasive/motility responses to bombesin.^{17,42,78} Elevated expression of GRP receptors are found in CaP specimens.^{41,79} Likewise, androgen-sensitive LNCaP cells were shown to become invasive after bombesin treatment.⁴² These and other findings suggest that NED of CaP cells may be a central link in supporting AI CaP growth under AD conditions.

NE cells after ADT

Following ADT, NE features are an independent prognostic factor for progression of CaP.⁸⁰ Following neoadjuvant ADT, surgical specimens showed greater NED compared to non-treated controls.⁶³ In a retrospective study of CaP patients treated with chemotherapy, Cabrespine *et al.*³⁵ showed that CgA serum levels following ADT were independently related to treatment duration and were helpful in assessing patient response to chemotherapy.

It is unlikely that ADT always initiates NED or that this is an important feature in all CaP patients. However, if it is true that ADT of CaP tends to promote NED and supports continued progression of the tumor towards AI, then the natural question arises, can CaP associated with NED be treated?

Treatment

Treatment of AI CaP is the focus of intense research. Only those strategies that directly impact NE cellular signaling will be discussed here. These treatments can be separated into adjunctive and salvage categories. The former combines with ADT to prevent NED from taking place, while the latter attempts to block the biochemical pathways that result from existing NED tumors.

Adjunctive treatment strategies are very limited. We are not aware of any currently used adjunctive medications intended to prevent NED. So far, the only treatments intended to do this involve variations in the method or temporal aspects of AD. Sciarra and Di Silverio⁸¹ have randomized patients with biochemical progression following prostatectomy into two monotherapy groups: medical castration or antiandrogen. They showed a significantly lower CgA level in the group treated with antiandrogen, although both groups showed significant increases.

Intermittent androgen deprivation (IAD) was developed as an attempt to delay the biochemical events that lead to AI during continuous ADT. The known side effects of ADT and concern for quality of life in advanced CaP have also fueled interest. At least one study has shown that IAD may also prevent or delay NED in locally advanced disease when compared to CAD.⁸² Metastatic disease also showed a trend toward lower serum CgA levels.

There are many treatments attempting to inhibit NED or at least block pathways NE cells use to drive CaP progression. Three known pathways that have excited interest are bombesin/GRP, serotonin and somatostatin. Antibodies against bombesin/GRP were shown to inhibit prostate cell line growth through MAP kinase pathways.⁸³ Several studies have shown *in vitro* inhibition of CaP growth using serotonin inhibitors.^{44,84,85} Somatostatin has been used for various endocrine tumors for some time with varied success.⁷ The actions of somatostatin in CaP are more complex and the treatment effect may be through secondary mediators such as decreasing certain growth factors from NE cells.⁸⁶ In addition, multiple somatostatin receptor types exist and different medications show different affinities. A recent review of the literature including seven studies using somatostatin analogues as monotherapy showed 'negative results'.⁸⁷ Direct growth factor antagonists of many varieties have been tested with mixed results. For example, suramin binds several growth factors and has shown moderate activity in CaP.⁸⁸

Targeting downstream effectors of the pathways listed above may allow inhibition of multiple growth factors with one treatment. Src, a non-receptor tyrosine kinase activated by G-protein-coupled receptors, activates signal transducers and activator of transcription 3, which in turn activates transcription of VEGF, cyclinD1 and c-myc. Research at our institution has demonstrated the importance of Src as a central signal-transduction molecule in NED.⁶⁰ An NCI-sponsored phase II trial of the Src inhibitor AZD0530 as treatment for AI CaP is planned to start by early 2007.

Another indirect method of targeting growth factors is growth hormone-releasing hormone (GHRH) antagonists. These medications have recently undergone

improvements in efficacy and duration of action and have shown activity *in vitro* and in xenografts.⁸⁹

Several combination therapy protocols have been used. GHRH antagonists in conjunction with bombesin/GRP antagonists showed additive interference with IGF and EGF pathways in PC-3 cell lines and xenografts.⁸⁹ The authors suggest this may allow future adjuvant use of these types of medication. Sciarra *et al.*⁹⁰ suggest that somatostatin may influence the microenvironment in which CaP cells reside, allowing other treatments to more effectively destroy the malignancy. Recognizing the direct cytotoxic effects of estrogen on CaP,⁹¹ they used the somatostatin agonist lanreotide in combination with ethinyl estradiol, theorizing a synergistic effect. Fourteen of 20 stage D3 patients demonstrated extended response time and symptomatic improvement. In addition, serum CgA decreased significantly, suggesting that a decrease in NE cell number or activity may be partially responsible for their results.

Chemotherapy targets dividing cells to induce genomic damage and apoptosis. Although NE cells are typically thought to be post-mitotic,⁵ at least one paper claims otherwise.⁹² Modern chemotherapy regimens may be useful according to a recent report,³⁵ which demonstrated significant decreases in CgA in treated AI CaP patients.

One tremendous difficulty in developing new treatment strategies is assessing effectiveness in reaching the intended target. The focal nature of NED makes direct tissue analysis less accurate than serum markers such as CgA.⁹³ However, all currently used serum markers are expressed by non-NE cells and therefore are affected by overall prostatic tissue volume rather than only NE cell number. Measuring patient outcomes, although helpful in identifying useful treatments, gives no information on specific pathways. Development of new markers for NED is a needed area of research. Other potential methods include radiolabeled monoclonal antibody imaging studies such as somatostatin receptor scintigraphy. Non-invasive visualization of various receptors in NE tissue shows great promise in treatment assessment.

Conclusions

In summary, AR-negative NE cells are present in normal prostatic tissue and may play a role in supporting initial neoplastic changes. ADT may induce microenvironmental changes that increase the activity of these cells. It is at least certain that they are selected for due to the lack of androgen. NE cells are capable of inducing transdifferentiation toward an NE phenotype in surrounding epithelial cells. The substances excreted by the increasing number of NE cells support the proliferation of existing CaP in an AI manner progressively increasing independence from androgen control. Greater understanding of these early post-castration molecular events will allow targeted adjunctive treatment of NED, thus decreasing the number of CaP cells that escape from hormonal control. New markers and associated imaging techniques for NED will allow molecular expression profiling, thus individualizing treatment based on the patient's unique microcellular environment.

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Nonreceptor Tyrosine Kinases in Prostate Cancer

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Abstract

BACKGROUND: Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer in men in the United States. Signal transduction molecules such as tyrosine kinases play important roles in CaP. Src, a nonreceptor tyrosine kinase (NRTK) and the first proto-oncogene discovered is shown to participate in processes such as cell proliferation and migration in CaP. Underscoring NRTK's and, specifically, Src's importance in cancer is the recent approval by the US Food and Drug Administration of dasatinib, the first commercial Src inhibitor for clinical use in chronic myelogenous leukemia (CML). In this review we will focus on NRTKs and their roles in the biology of CaP. **MATERIALS AND METHODS:** Publicly available literature from PubMed regarding the topic of members of NRTKs in CaP was searched and reviewed. **RESULTS:** Src, FAK, Jak1/2, and ETK are involved in processes indispensable to the biology of CaP: cell growth, migration, invasion, angiogenesis, and apoptosis. **CONCLUSIONS:** Src emerges as a common signaling and regulatory molecule in multiple biological processes in CaP. Src's relative importance in particular stages of CaP, however, required further definition. Continued investigation of NRTKs will increase our understanding of their biological function and potential role as new therapeutic targets.

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Keywords: Nonreceptor tyrosine kinase, prostate cancer, Src, FAK, ETK.

Introduction

Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer in American men, consisting of more than 33% of all new cancer cases. Though many patients are diagnosed with CaP, it has a relatively low mortality rate when compared to other cancers. Nevertheless, it remains the third leading cause of cancer-related deaths in men in the United States, with about 27,350 estimated CaP-related deaths in 2006 in the United States [1]. Because CaP growth is facilitated by androgen exposure and because androgen withdrawal leads to apoptosis of CaP cells, the current treatment of choice for recurrent or metastatic CaP includes castration through chemical or surgical means. Nearly all patients, however, relapse with androgen-

independent (AI) disease after androgen ablation therapy. Ultimately, the uncontrolled growth of AI metastatic tumors leads to patient mortality.

Tyrosine kinases (TKs) are signaling molecules well known for their roles in human diseases such as diabetes and cancer. Indeed, v-Src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src), a nonreceptor tyrosine kinase (NRTK), was the first proto-oncogene discovered. More than a quarter of a century has passed since the discovery of Src, and the studies on TKs are coming to fruition with the development and use of tyrosine kinase-based target-specific therapy such as Gleevec, Iressa, and Herceptin for therapy against chronic myelogenous leukemia (CML), lung cancer, and breast cancer, respectively. Dasatinib, a dual Src/v-Abl Abelson murine leukemia viral oncogene homolog (Abl) inhibitor with anti-migratory activity in prostate cancer cells in culture was recently approved by the US Food and Drug Administration for use in patients with CML [2]. Further underscoring the importance of NRTKs, AZD0530 is another dual Src/Abl inhibitor that is currently in multicenter phase II clinical trials for multiple types

Abbreviations: Abl, v-Abl Abelson murine leukemia viral oncogene homolog; AI, androgen-independent; Akt, v-akt murine thymoma viral oncogene homolog 1; AR, androgen receptor; ARG, Abelson-related gene; Bcr, breakpoint cluster region; Brk/PTK6, breast tumor kinase/protein tyrosine kinase 6; BPH, benign prostatic hypertrophy; BRCA1, breast cancer susceptibility gene 1; CaP, carcinoma of the prostate; CML, chronic myelogenous leukemia; CRKII, v-crk avian sarcoma virus CT10 oncogene homolog; CSK, C-terminal Src kinase; DOC-2/DAB2, differentially expressed in ovarian cancer-2/disabled-2; EGF, epidermal growth factor; ER, estrogen receptor; ERK1/2, extracellular signal-regulated kinase 1/2; ET1, endothelin; ETK/BMX, endothelial/epithelial tyrosine kinase/bone marrow X kinase; FAK, focal adhesion kinase; FeR, FpS/FeS-related tyrosine kinase; FeS/FpS, feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog; FGR, Gardner-Rasheed feline sarcoma viral (v-FGR) oncogene homolog; Fyn, Fyn oncogene related to Src, FGR, Yes; HIF-1 α , hypoxia-inducible factor 1 α ; IGF-1, insulin-like growth factor 1; IL, interleukin; Jak, Janus kinase; KAI1/CD82, Kangai 1/cluster designation 82; Lck, lymphocyte-specific protein tyrosine kinase; Lyn, v-Yes-1 Yamaguchi sarcoma viral-related oncogene homolog; LPA, lysophosphatidic acid; Met, met proto-oncogene (hepatocyte growth factor receptor); MMP, matrix metalloproteinase; NEP, neutral endopeptidase; NRTK, nonreceptor tyrosine kinase; p130CAS, p130 CRK-associated substrate; PAK1, p21-associated kinase 1; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; PYK2/CAK β , proline-rich tyrosine kinase 2/cell adhesion kinase β ; Raf, v-raf-1 murine leukemia viral oncogene homolog 1; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; SH, Src homology; Src, v-Src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog; STAT, signal and transducer of transcription; SYK, spleen tyrosine kinase; Tec, Tec protein kinase; TGF, tumor growth factor; TIMP, tissue inhibitor of metalloproteinase; TKIP, tyrosine kinase inhibitor peptide; TnK, tyrosine kinase nonreceptor; Tyk2, tyrosine kinase 2; VEGF, vascular endothelial growth factor; Yes, v-Yes-1 Yamaguchi sarcoma viral oncogene homolog 1

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of malignancies, including prostate cancer. In this review we will focus on each of the NRTKs and what is known about their respective roles in the biological processes of cell proliferation, migration, invasion, apoptosis, and angiogenesis in CaP.

There are several NRTK families. These are classified based on their structural similarities (Figure 1): Abl, tyrosine

kinase nonreceptor (TnK), C-terminal Src kinase (CSK), focal adhesion kinase (FAK), feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog (FeS), Janus kinase (JaK), Src, Tec protein kinase (Tec), and spleen tyrosine kinase (SYK). Though these NRTK families are extensively and individually reviewed elsewhere, this

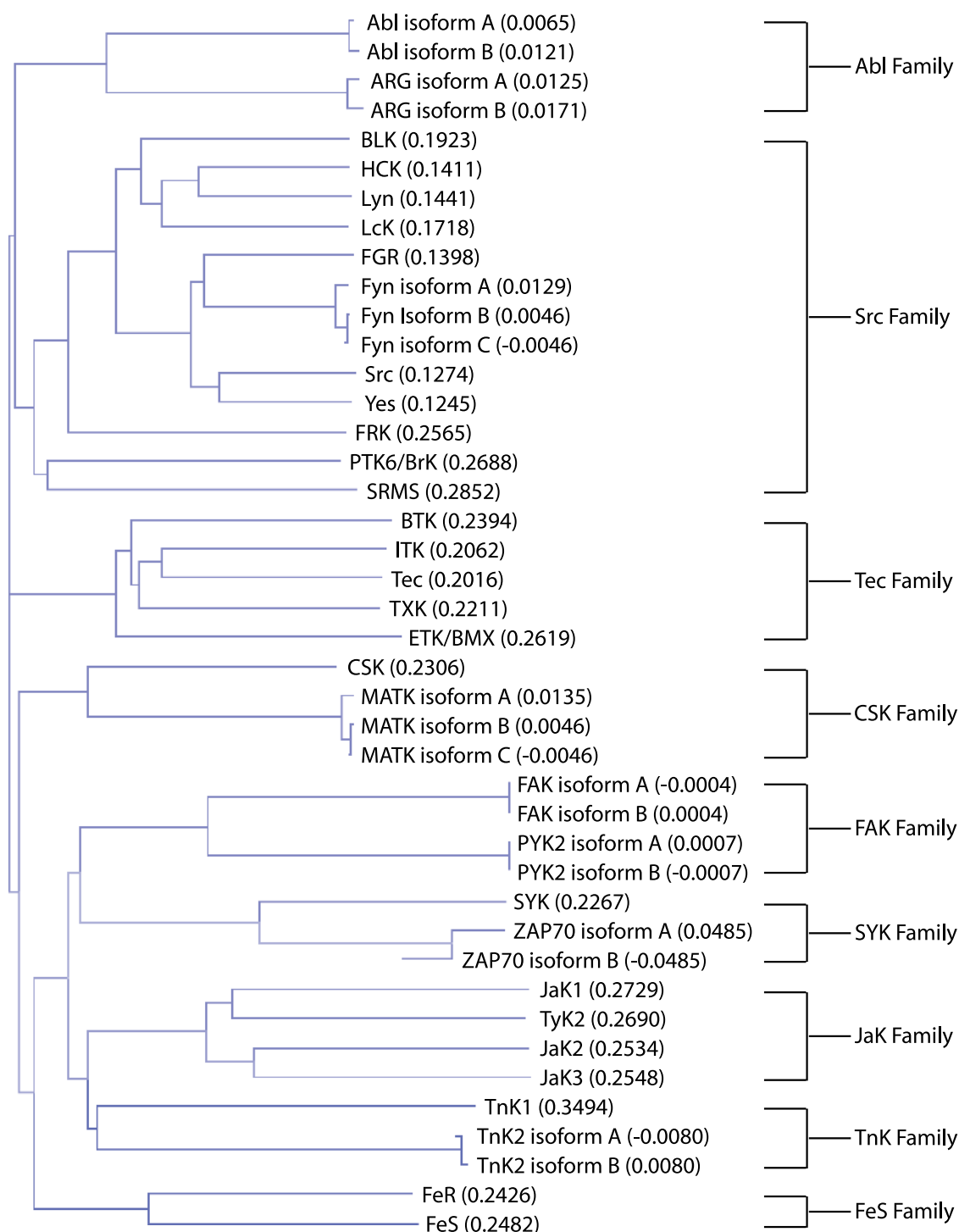


Figure 1. NRTK families and their members in a guide tree. Protein sequences are obtained from Entrez Gene and aligned using Vector NTI Advance software (Invitrogen, Carlsbad, CA). Vector NTI Advance uses the neighbor-joining method of phylogenetic tree construction by Saitou and Nei [127]. The numbers in parentheses after each kinase reflect the calculated distance values between pairs of analyzed sequences.

is the first time they are summarily discussed in relation to CaP.

Profiles of NRTKs in CaP

In 1996, Robinson et al. [3] led the first attempt at profiling the expression of TKs in CaP. Using a modified and improved reverse transcriptase–polymerase chain reaction approach, they identified nine NRTKs expressed in CWR22, a CaP xenograft. NRTKs include lymphocyte-specific protein tyrosine kinase (LcK), v-Yes-1 Yamaguchi sarcoma viral oncogene homolog 1(Yes), Abl, Abelson-related gene (*ARG*), Jak1, tyrosine kinase 2 (TyK2), and endothelial/epithelial tyrosine kinase/bone marrow X kinase (ETK/BMX). Fur-

thermore, *ARG* was found in several other CaP cell lines, which include PC-3, DU145, and LNCaP. In a similar study, Moore et al. [4] used degenerate polymerase chain reaction against conserved kinase catalytic subdomains and found that Abl, Jak1, Jak2, and TyK2 are expressed in surgically removed CaP tissues. In CWR22Rv1, DU145, LNCaP and PC3 cell lines, 18 NRTKs are expressed. This was confirmed by our internal data and also cross-referenced with several published reports (Figure 2).

Src Family

As the first human proto-oncogene discovered, Src's history spans nearly a century and has been extensively reviewed [5–22]. Members of the Src family include B lymphoid

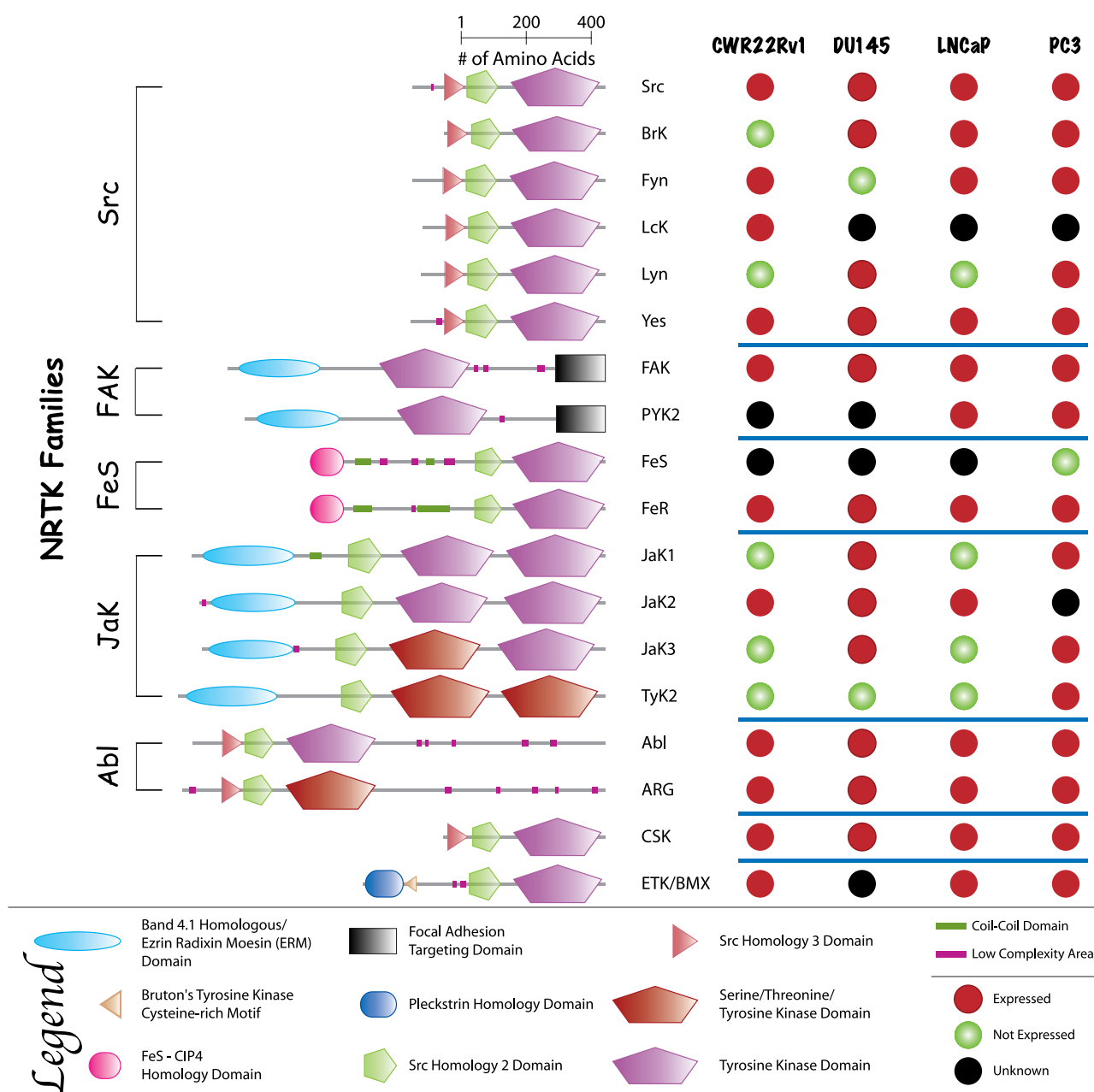


Figure 2. Summary of NRTK mRNA or protein expression in CWR22Rv1, DU145, LNCaP, and PC3 cell lines based on internal data and published reports. NRTK domain drawings and domain information were derived from Simple Modular Architecture Research Tool (SMART, Heidelberg, Germany).

tyrosine kinase (BLK), breast tumor kinase/protein tyrosine kinase 6 (BrK/PTK6), Gardner-Rasheed feline sarcoma viral oncogene homolog (FGR), Fyn oncogene related to Src, FGR, Yes (Fyn), hemopoietic cell kinase (HCK), LcK, v-Yes-1 Yamaguchi sarcoma viral-related oncogene homolog (Lyn), Src, Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites (SRMS), Yes, and Yes-related kinase (YRK). Of these, FGR, Fyn, LcK, Lyn, Src, and Yes are expressed in either CaP tumor samples or cell lines. Src, FGR, Fyn, LcK, and Lyn in particular have been the most widely studied in CaP.

Src The premier member of its namesake family, Src is extensively studied in cancer biology. Less is known, however, about Src biology in CaP. Though there are no published reports of Src expression or activation levels in clinical CaP specimens, Src is implicated in CaP through its association with factors that correlate positively with the presence or the progression of CaP disease, such as protein kinase C (PKC) ϵ , endothelial-derived gene 1 (*EG-1*), and a truncated form of c-kit [23–25]. As further evidence of Src's possible involvement in CaP, DRS, a negative Src regulator, is down-regulated in CaP tissues and in prostate intraepithelial neoplasia relative to normal and benign prostate hyperplasia (BPH) tissues [26]. Thus, there is circumstantial clinical evidence that Src plays a role in CaP through its interactions with other factors of significance in CaP.

More is known about Src in CaP *in vitro*. Src is expressed in commonly used CaP cell lines CWR22Rv1, DU145, LAPC-4, LNCaP, and PC-3 (Figure 3). At first glance, Src protein expression levels in CaP cell lines do not positively correlate with the aggressiveness, AI state, or the proliferation rates of these cell lines. It is important to note, however, that wild-type cellular Src is not normally constitutively active. Its main role is to transduce signals of upstream activators. In cancer, the upstream signals may be aberrant, thus leading to improper activation of Src and its downstream pathways. One such pathway in CaP is Src activation by neuroendocrine ligands [27].

Neuroendocrine differentiation in CaP is theorized to be in part responsible for the development of AI CaP through the secretion of neuroendocrine ligands. There is evidence that

Src takes part in AI cell proliferation. Cyclic adenosine monophosphate (cAMP) analogs are able to activate Src following neuroendocrine differentiation, perhaps secondary to secreted neuroendocrine factors such as gastrin-releasing peptide and lysophosphatidic acid (LPA) [28–31]. LPA is thought to promote cell proliferation through the v-Ha-ras Harvey rat sarcoma viral oncogene homolog (Ras)–v-raf-1 murine leukemia viral oncogene homolog 1 (Raf)–ERK1/2 pathway in Src-dependent fashion. Bombesin, a *Xenopus* gastrin-releasing peptide homolog, can also activate ERK1/2 through Src, possibly through epidermal growth factor (EGF) receptor transactivation [32]. Once ERK1/2 has been activated, it can then activate the androgen receptor (AR) in an AI manner, which promotes cell growth [27,33]. In addition to LPA and bombesin, non-neurotrophic factors such as interleukin-8 (IL-8) and insulin-like growth factor-1 (IGF-1) also promote AI cell growth through Src [34,35].

In addition to cell proliferation, Src also takes part in antiapoptotic pathways in CaP. Bombesin, endothelin (ET1), met proto-oncogene (*Met*), and dihydrotestosterone-activated AR all inhibit apoptosis through Src activation [26,36–38]. There is, however, no consensus mechanism by which Src promotes cell survival. Nuclear factor κ B (NF- κ B)–v-akt murine thymoma viral oncogene homolog 1 (Akt)–p21-associated kinase 1 (PAK1) pathway, MEK1/2–ERK1/2–CREB pathway, and signal and transducer of transcription 3 (STAT3)–dependent down-regulation of B-cell lymphoma leukemia (BCL-xL) and myeloid cell leukemia sequence 1 (MCL-1) are all pathways by which Src inhibits apoptosis [39].

Src is involved in other aspects of CaP biology: cell migration and adhesion. Src interacts with the extracellular signals through the IL-8 receptor, Met, β_1 integrins, Kangai 1/cluster designation 82 (KAI1/CD82), and CD44 [23,34,40,41]. CD44 is a cell surface glycoprotein involved in cell–cell and cell–matrix adhesions. KAI1/CD82 functions as a metastasis suppressor, disrupting integrin-induced Src activation [42]. Intracellularly, Src modulates cell migration and adhesion through its interaction with FAK and p130 CRK-associated substrate (p130CAS) [2].

In addition to cell migration, Src also assists in tumor invasion through its regulation of matrix metalloproteinases (MMPs). MMPs aid in invasion through the degradation of the extracellular matrix. Bombesin promotes Src-dependent tumor progression and metastasis through the activation of MMP9 in conjunction with β_1 integrins [43]. Src inhibition, on the other hand, decreases MMP9 activity levels [2,44].

Induction of angiogenesis by malignant cells is required for continued cell proliferation and metastasis, and vascular endothelial growth factor (VEGF) is a critical angiogenic factor. Src participates in angiogenesis in CaP through the Jak1–STAT3–VEGF pathway [45]. Src activation is also required for VEGF expression in simulated hypoxia environment through increased levels of hypoxia-inducible factor 1 α (HIF-1 α) and activation of STAT3; as additional evidence of Src's involvement in angiogenesis, overexpression of active Src leads to increased VEGF expression [46]. Expression of the melanoma-differentiation–associated gene-7, a Src

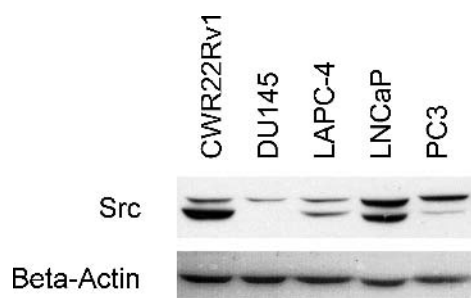


Figure 3. Western blot analysis of total Src protein expression levels in prostate cancer cell lines. Src is shown as a doublet upon probing in most cell lines. Internal overexpression data (not shown) indicate that both bands are Src and that the doublet is not a result of nonspecific probing of other Src family kinase members.

inhibitor, on the other hand, inhibits the subsequent downstream STAT3–VEGF pathway [46,47].

Src is also of particular interest in CaP in part because of its interaction with steroid receptors. There is evidence that low amounts of AR and androgen lead to Src activation in the cytoplasm, thereby triggering downstream signaling events independent of AR's transcriptional and DNA-binding activity [38,48]. In fact, dominant negative Src can inhibit DNA synthesis following stimulation with low amounts of synthetic androgen. AR overexpression and higher concentrations of androgen, however, seem to bypass the Src pathway, leading to AR translocation to the nucleus and AR-transcriptional activity–based DNA synthesis.

In addition to the aforementioned activation of Src by androgen-activated AR, Src also associates with AR and estrogen receptor (ER) upon stimulation with estradiol, ultimately resulting in increased cell proliferation [38,49,50]. It is thought that Src serves as a scaffolding protein for the AR–ER complex. Steroidal ligand, however, is not necessary for AR–Src complex formation. Upon EGF stimulation, preformed heterodimers of ER α and AR form a complex with EGF receptor and Src, resulting in the activation and phosphorylation of EGF receptor, DNA synthesis, and cytoskeletal changes [51]. On the other hand, DOC-2/DAB2, a tumor suppressor and a negative Src regulator protein, is reported to inhibit AR's mitotic effects through the disruption of the AR–Src complex [52,53]. Thus, taken together with reports of AI AR activation by Src, AR and Src seem to be able to reciprocally transactivate, depending on the concentration and type of stimulatory ligand.

There are few published reports on cellular elements that negatively regulate Src in CaP. In addition to DOC-2/DAB2, tumor growth factor (TGF) β is reported to decrease both Src expression and its corresponding activity. This is shown by the accumulation of unphosphorylated form of SH2-containing protein (SHC) and a subsequent decrease in complex formation between SHC and growth factor receptor–bound protein 2 (Grb2) [54].

BrK/PTK6 BrK is an Src family member, and little is known about it in CaP. In patient samples, BrK is detected in the nuclei of normal luminal epithelial tissues and well-differentiated tumors, but not in poorly differentiated tumors [55]. Localization of BrK in CaP cell lines LNCaP, which is poorly tumorigenic, and PC-3, which is more aggressive, is primarily nuclear and cytoplasmic, respectively. Though PC-3 expressed more BrK than LNCaP did, BrK is less active in PC-3 cells. Thus, the localization of BrK may play a role in the differentiation of CaP and its aggressiveness.

FGR/Src-2 FGR is an Src kinase family member. It is a negative regulator of phosphatase and tensin homolog (PTEN) and a positive regulator of both Ras and Raf1, thus inhibiting apoptosis and stimulating cell growth, respectively [56]. Though little is known about FGR in CaP, FGR may be overexpressed in CaP, as shown by FGR DNA amplification in patient tumor tissues transitioning from androgen-

dependent to AI states [56]. Thus, FGR may play a role in CaP growth and survival.

Fyn Fyn is an Src family kinase member. It is involved in LNCaP mitogenesis following prolactin stimulation [57]. Though it is suggested that Fyn participates in prolactin-induced cell proliferation through K⁺ ion channels, further studies are necessary in order to elucidate the mechanism of Fyn-modulated prolactin-induced cell proliferation in CaP.

LcK LcK is an Src family kinase member. It is expressed in CWR22 xenograft cells [3]. Little else is known about the role of LcK in CaP.

Lyn Lyn is an Src family kinase member expressed in normal prostate, 95% of primary CaP, and AI PC-3 and DU145 cells [58]. Lyn knockout mice have abnormal prostate gland development. Treatment with KRX-123, a Lyn-specific inhibitor, results in the inhibition of cell growth in DU145 and PC-3 cell lines. DU145 explants in mice treated with KRX-123 were found to also undergo apoptosis. Thus, Lyn seems to play a role in the proliferation and the apoptosis of CaP.

Lyn may also be an important regulator of cell migration in CaP. DU145 cells treated with dasatinib, an Src family kinase inhibitor, have reduced migratory activity [2]. On the other hand, Lyn can bind with neutral endopeptidase (NEP) and act as a competitive inhibitor to the PI3K–FAK complex, resulting in decreased cell migration [59]. Lyn's role in CaP cell migration is therefore inconclusive.

In CaP, Lyn is down-regulated by TGF β and up-regulated by KAI1/CD82 [54,60]. Despite its elevated expression following KAI1/CD82 stimulation, however, Lyn's overall kinase activity was unchanged.

FAK Family

FAK As the predominate member of its namesake family of kinases, FAK is well studied in CaP. Several general reviews of FAK are available [61–71]. Though FAK may play roles in growth, apoptosis, and angiogenesis in CaP, FAK is known primarily for its role in cell motility and cytoskeletal rearrangement, as supported by *in vivo* and *in vitro* evidence. In clinical specimens, FAK expression and activation are uniformly higher in metastatic CaP than in normal and BPH tissues [72,73]. *In vitro* comparison between highly metastatic CaP cell lines and LNCaP, a cell line with lower metastatic potential, shows similar results, with increased expression and activation of FAK in the more aggressive cell lines [74]. FAK's association with molecular mediators of cell migration and adhesions are indicative of its function as well. Activated FAK complexes with β_1 and $\alpha(v)\beta_3$ integrins, molecules involved in cell adhesion [75–78]. As further evidence of FAK's function as a cell motility factor, inhibition of FAK with anti-FAK (pY397) antibody or FAK-related nonkinase (FRNK) resulted in significantly decreased cell migration [79].

Bombesin and IL-8 are both G protein–coupled receptors (GPCR) that activate FAK and stimulate cell migration

[34,79–81]. This is not surprising given FAK's reciprocal transactivation relationship with Src and both IL-8 and bombesin's abilities to activate Src. For bombesin to activate FAK, however, both PKC and an intact cytoskeleton are required [80,82]. Following its activation, FAK then phosphorylates p130CAS, leading to p130CAS–v-crk avian sarcoma virus CT10 oncogene homolog (CRKII) complex formation. Disruption of the p130CAS–CRKII complex by overexpressing KAI1/CD82 results in decreased cell motility [60].

Extracellularly, FAK is activated by integrins, ET1, bombesin, IL-8, and urokinase plasminogen activator (uPA), an invasion and metastasis factor in CaP [83,84]. Intracellularly, it is modulated by Src. It is important to note that Src and FAK activation often go hand in hand. They couple and reciprocally transactivate each other. There are, however, exceptions. FAK activation by autophosphorylation of tyrosine 397 is not Src-dependent; it is adhesion-dependent [74]. On the other hand, phosphorylation of tyrosine 861, which leads to increased FAK activity, is Src-dependent but not adhesion-dependent.

Though FAK is primarily a cell motility regulator, it is also involved in cell proliferation. Similar to cell migration, bombesin-induced FAK-mediated proliferation requires an intact cytoskeleton [80]. A signal downstream of FAK is ETK/BMX, an NRTK critical for bombesin-induced growth [27]. Following FAK activation of ETK/BMX, ETK/BMX subsequently activates AR, thereby inducing cell growth. Interestingly, not only can FAK indirectly activate AR, it can also be activated by membrane-associated AR in a PI3K-dependent manner [85].

In addition to migration and proliferation, FAK may also be involved in CaP angiogenesis and apoptosis. There is evidence that FAK induces VEGF transcription in an ERK1/2-dependent, Rap1-dependent, and Raf-dependent but Ras-independent manner [86]. Increased VEGF transcription may then lead to an increased level of its secreted protein and, thus, angiogenesis. In regard to apoptosis, treatment of cells with proapoptotic factors FTY720 and doxazosin both down-regulate FAK expression for reasons that are not currently known [87,88].

There are few known ways in which FAK is negatively regulated in CaP. Negative FAK regulators include *PTEN*, a tumor suppressor gene with dual phosphatase activity that is frequently deleted in aggressive CaP [89]. FAK may also be indirectly negatively regulated through the formation of the Lyn–PI3K–NEP complex instead of the PI3K–FAK complex [59].

Proline-rich tyrosine kinase 2/cell adhesion kinase β (PYK2/CAK β) PYK2/CAK β is a member of the FAK family of tyrosine kinases. A general review of PYK2 is available [90]. It is expressed in normal prostate epithelia and BPH, but its expression level decreases with increasing grade in CaP [91]. The gene is located on chromosome 8p21.1, a site of frequent deletion in CaP [92].

Though *in vivo* evidence suggests that PYK2 plays a tumor suppressive role in CaP, the *in vitro* evidence of this hypothesis is inconclusive. *In vitro* experiments show that

PYK2 is activated by LPA and tumor necrosis factor α . PYK2 plays a role in the activation of ERK1/2 following LPA stimulation and may thus stimulate cell proliferation [93]. In addition, cells expressing dominant negative PYK2 have decreased proliferation rates. On the other hand, PYK2 indirectly inhibits AR activation through the inactivation of an AR-associated protein, ARA55 [94]. Thus, PYK2's role in CaP may depend on the androgen sensitivity status of the cells in question and requires further investigation and clarification.

FeS Family

The FeS family of NRTKs consists of two members: FeS/FpS and FpS/FeS–related tyrosine kinase (FeR). Little is known about the FeS family in CaP. An examination of CaP cell lines PC-3, PC133, and PC135 failed to detect FeS transcript [95]. FeR expression, on the other hand, was found in CaP cell lines PC-3, DU145, and LNCaP and positively correlated with CaP *versus* normal and BPH tissue samples [96]. Consistent with patient sample data, cells transfected with antisense FeR grew at a slower rate and were unable to grow in an anchorage-independent fashion. In the dog model, a higher FeR expression was found in dividing *versus* resting prostate epithelial cells and in cells displaying basal cell hyperplasia and metaplasia following postcastration estrogen treatment [96]. Thus, FeR is likely a proliferation factor in CaP.

JaK Family

JaK1 The JaK family of kinases is well known for its role in signaling events in cells following cytokine stimulation and its association with the STAT family of kinases. Though JaK1 is present in some clinical CaP specimens, JaK1 is reported to be either negatively regulated or mutated in many CaP cell lines [4,97,98]. LNCaP is found to have both nonsense mutation and repressed JaK1 transcription whereas CWR22Rv1 and LAPC-4 have only nonsense mutations and no known transcriptional repression.

In DU145 cells, which have wild-type JaK1, there are reports that JaK1 associates with breast cancer susceptibility gene 1 (*BRCA1*) [99]. When *BRCA1* is overexpressed, JaK1 and STAT3 become activated. Subsequent inhibition of STAT3 activation results in decreased cell proliferation as well as in apoptosis. Interestingly, inhibition of JaK1 in wild-type DU145 does not result in apoptosis [100]. Thus, it may be possible that although JaK1 activation by *BRCA1* leads to increased JaK1 and STAT3 activation, STAT3 may in fact not be directly downstream of JaK1 in CaP, and their concurrent activation is coincidental.

JaK1 may also play a role in the inhibition of CaP migration and invasion following IL-10 stimulation [101]. Tissue inhibitor of metalloproteinases (TIMP) 1 is an anti-invasion factor. IL-10 is known to activate the JaK1–IL-10E1–TIMP-1 pathway in CaP [102].

JaK2 JaK2 is expressed in some CaP tissues [4]. Similar to JaK1, JaK2 is also activated by *BRCA1* in DU145 cells [99]. It

is interesting to note that although Jak1 inhibition does not result in apoptosis in wild-type DU145 cells, inhibition of Jak2 does [100]. Thus, STAT3 activation in DU145 may be dependent on Jak2 rather than on Jak1. Whether STAT3 is activated by Jak1 or Jak2 in CaP, however, seems to be cell line-dependent [103].

Jak2 may also be involved in cell proliferation in CaP. Tyrosine kinase inhibitor peptide (TKIP) directly inhibits Jak2 autophosphorylation, decreases STAT3 activation, and slows CaP proliferation [104]. Consistent with decreased cell proliferation and STAT3 activation, cyclin D1 level is decreased and cells are arrested in the G₁ phase of the cell cycle following TKIP treatment. Thus, Jak2 may be important for CaP growth through the STAT3 pathway. In addition to STAT3, Jak2 may be of particular importance in CaP through its regulation of STAT5, a factor that positively correlates with the histological grade of CaP [105,106].

Tyk2 Tyk2 is expressed in some CaP tissues [4]. Though Tyk2 may also be involved in CaP migration and invasion and similarly participates in the activation of IL-10E1 following IL-10 stimulation of CaP cells as Jak1, its temporal regulation profile is different from that of Jak1 [101,102].

Members of Other NRTK Families

Abl Abl is well known for its role in the etiology of CML following the formation of the Philadelphia chromosome (t(9;22)) and the breakpoint cluster region (Bcr)–Abl hybrid gene product. Less is known, however, about Abl in CaP. It is known that Abl is expressed in some CaP specimens and that Abl is necessary for retinoblastoma-mediated γ -radiation-induced apoptosis in DU145 cells [4,107]. There is indirect evidence that Abl may be important in CaP. Human spectrin SH domain-binding protein 1 (*Hssh3bp1*) is a gene that binds to Abl, possibly as a negative regulator [108]. A majority (9 of 17) of CaP tumor samples analyzed failed to express *Hssh3bp1*. Furthermore, *Hssh3bp1* is found on chromosome 10p, a region frequently deleted in CaP. Thus, Abl may be circumstantially implicated in CaP through its association with *Hssh3bp1*.

Imatinib mesylate (Gleevec; Novartis, East Hanover, NJ) is a Bcr–Abl inhibitor that is clinically used for the treatment of CML. It also has activity against Kit kinase and platelet-derived growth factor (PDGF) receptor. *In vitro*, Gleevec inhibits CaP cell growth with IC₅₀ in the 10- μ M range [109]. In mice models, however, Gleevec's efficacy against CaP growth is inconclusive with some, but not all, studies showing growth inhibition [110–113].

Similarly, preliminary results from clinical studies also paint a mixed picture. A phase I clinical trial of Gleevec in combination with docetaxel in AI CaP showed a prostate-specific antigen (PSA) decline in 14 of 21 patients, although it is unknown whether the decline can be attributed to Gleevec or docetaxel [114]. In another AI CaP study, Gleevec in combination with zoledronic acid (Zometa, Novartis) showed no clinical effect in 15 CaP patients [115]. Lastly, as monotherapy in 16 patients with androgen-sensitive CaP, Gleevec treatment resulted in nine patients with stable PSA levels

and seven patients with PSA progression [116]. Thus, clinical use of Gleevec as monotherapy in CaP may be ineffective. The efficacy of using Gleevec as an adjuvant therapy to other treatment modalities is presently unknown.

CSK CSK is a well known negative Src regulator [117]. Little is directly known about CSK in CaP other than that it complexes with FAK in metastatic tumors and PC-3 cells [73].

ETK/BMX Discovered in 1994, ETK/BMX belongs to the Tec family of NRTK [118]. In CaP, ETK is downstream of PI3K in the induction of the neuroendocrine differentiation of LNCaP cells following IL-6 stimulation [119]. It is also reported to function as an antiapoptotic factor. Overexpression of ETK confers resistance to apoptosis in CaP cells through its interaction with PI3K [120]. PI3K is not, however, required for ETK activation [27]. Another mechanism by which ETK may protect against apoptosis is through its interaction with p53 [121]. Interestingly, ETK also participates in the apoptotic cascade in CaP cells. Introduction of ETK's C-terminal fragment into PC-3 cells can lead to apoptosis following proteolytic cleavage of ETK by caspases [122].

ETK is also critical for cell proliferation following bombesin stimulation and AR activation in CaP [27]. ETK serves as a signal transducer between Src and FAK upstream and AR downstream. ETK alone, however, is insufficient for AR activation. ETK must be able to reciprocally transactivate with Pim1 before AR activation [123,124].

Other NRTKS SYK and TNK1 are other NRTKs that have been studied in CaP. Virtually nothing is known about their properties and functions in prostate cancer except that the promoter region of SYK is hypermethylated and TNK1 transcript is found in prostate tissues [125,126]. SYK expression may thus be down-regulated in CaP, whereas TNK1 protein expression level remains to be investigated.

Conclusion

Much is known regarding specific NRTKs in CaP (Src, FAK, Jak1/2, and ETK), whereas less is known about the other NRTKs. Perhaps it is not a coincidence that the well-studied Src, FAK, Jak1/2, and ETK kinases are involved in processes indispensable to the pathology of CaP: cell growth, migration, invasion, angiogenesis, and apoptosis. It is therefore imperative that we learn more about these NRTKs through future studies. Although Src, FAK, Jak1/2, and ETK are important in CaP biology, we should not neglect the other NRTKs that may also play important roles in CaP and should also investigate the lesser known NRTKs.

Looking at the current literature of NRTKs in CaP, there emerges a picture of Src being an ubiquitous player in multiple biological processes interacting with numerous players in multiple signaling pathways. Src transduces signals from upstream receptors such as IL-8, EGF, IGF-1, neurotensin, ET1, and HGF/SF to downstream molecules such as FAK, ETK, JAK1/2, STAT3, Ras, ERK1/2, Akt, HIF-1 α , and, of particular significance in CaP biology, AR (Figure 4). Given

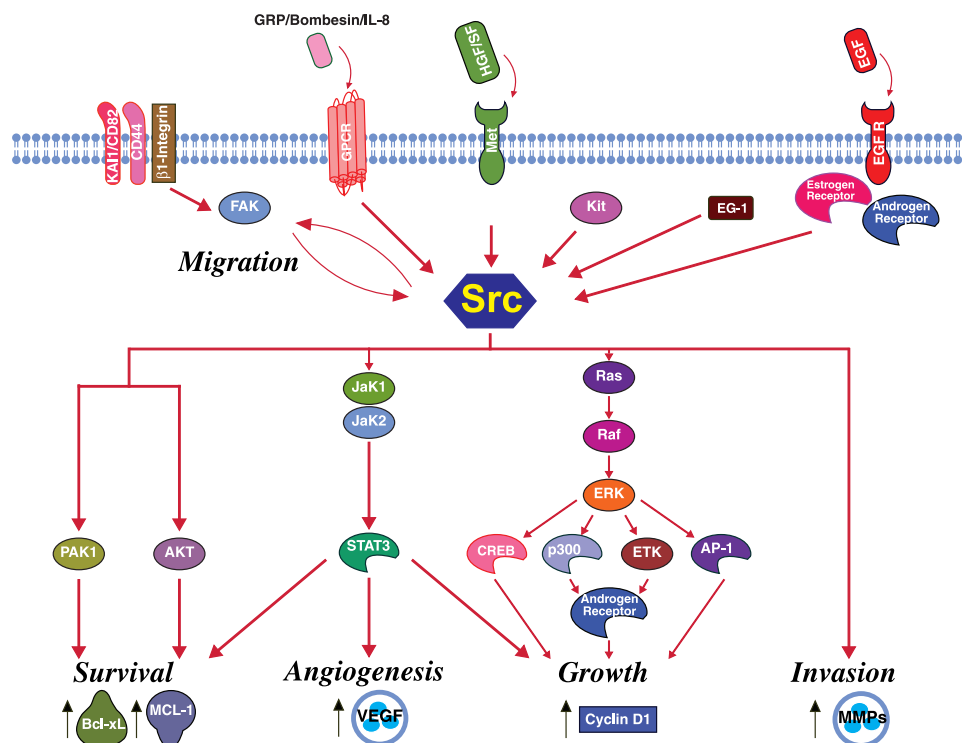


Figure 4. Known Src pathways in prostate cancer. The close proximity of molecules not connected with arrows denote physical association. Red arrows denote activation. Black arrows denote change in levels of molecule. Figure templates were provided by BioCarta (San Diego, CA).

the preponderance of evidence in multiple biological processes linking Src to CaP, Src is likely an important point of pathway convergence in CaP. Perhaps it is not surprising then that Src is currently the only NRTK target in clinical trials for CaP, whereas no NRTK-specific therapy is available for general clinical use in CaP. What remains unclear, however, is Src's relative importance in particular stages of CaP: oncogenesis, growth, survival, AI growth, angiogenesis, and metastasis. Nevertheless, with cancer treatments moving toward targeting specific pathways, it is important that we continue investigating signaling pathways so that we can develop novel therapies through continued research.

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Biologic agents as adjunctive therapy for prostate cancer: a rationale for use with androgen deprivation

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SUMMARY

The prevalence of prostate cancer emphasizes the need for improved therapeutic options, particularly for metastatic disease. Current treatment includes medical or surgical castration, which initially induces apoptosis of prostate cancer cells, but ultimately an androgen-independent subpopulation emerges. In addition to a transient therapeutic effect, androgen-deprivation therapy (ADT) can initiate biochemical events that may contribute to the development of and progression to an androgen-independent state. This transition involves multiple signal transduction pathways that are accompanied by many biochemical changes resulting from ADT. These molecular events themselves are therapeutic targets and serve as a rationale for adjunctive treatment at the time of ADT.

KEYWORDS androgen-deprivation, androgen-independent, castration, hormone refractory prostate cancer, prostate cancer

REVIEW CRITERIA

A PubMed search of the English language literature from 1990 to 2006 for pertinent articles was conducted using the MeSH term "Prostate neoplasms" in conjunction with terms such as "biochemistry," "mechanism," "androgen independent," "metastatic," and "treatment." The bibliographies of retrieved articles were scrutinized for additional articles. Identified concepts, biochemical factors, and treatment strategies were searched again using MeSH terms. Particularly relevant articles were input into Web of Science to retrieve the latest citations and articles.

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INTRODUCTION

Prostate cancer remains the most common noncutaneous malignancy in the US and is predicted to be the third leading cause of cancer mortality in 2006.¹ Currently, androgen-deprivation therapy (ADT) is the mainstay of therapy for metastatic disease and is principally palliative in nature. ADT removes androgen stimulation, initially inducing apoptotic involution of prostatic tissue. The disease, however, eventually progresses to an androgen-independent state, which is associated with a life-expectancy of approximately 15–20 months.² Despite research efforts and recent advances using chemotherapeutic agents, minimal progress has been made in the treatment of advanced prostate cancer in the last 50 years, and the life expectancy of patients with metastatic disease has changed very little. ADT, while extending length and quality of life for many patients, induces biological changes in prostate cancer cells that promote an androgen-independent state.

When prostate cancer becomes androgen independent, methods of growth stimulation other than those mediated by androgens dominate, allowing progression of the disease. The androgen receptor (AR) continues to be expressed and even amplified in androgen-independent prostate cancer, and androgen responsive genes are re-expressed when cancer progresses to the androgen-independent state.³ As such, much research has focused on the AR in order to understand the method of continued AR-stimulated transcription in the absence of a natural ligand. Many possible pathways have been explored. AR amplification, mutation, and hypersensitivity might allow continued signaling with low androgen levels. Alternate pathways might activate the AR by phosphorylation in an androgen-independent manner. Finally, pathways that regulate apoptosis and cell proliferation, but without direct AR effects, might oppose the effects of ADT. The current understanding of possible mechanisms involved in the development of

androgen-independent prostate cancer has been the subject of two reviews.^{4,5}

This paper focuses on the mechanisms activated or upregulated with ADT that currently show potential as targets for treatment for advanced prostate cancer and have a theoretical rationale for castration-adjunctive therapy. We summarize selected pathways and therapies, dividing our discussion into AR-dependent and AR-independent categories (Figure 1).

ANDROGEN-RECEPTOR-DEPENDENT PATHWAYS

Androgen-receptor cofactors

Activity of the AR is modulated by many cofactors that can increase or decrease its transcriptional activity.⁶ Coactivators permit receptor activation with the very low levels of androgen typically seen in the castrate setting, or facilitate greater AR activity with aberrant stimulation from a number of pathways (outlined below).⁴ Several families of cofactors have been shown to change expression levels in response to ADT (Table 1), indicating a mechanism for the development of androgen-independent disease. Feldman and Feldman⁴ speculate that loss of corepressors might be another mechanism for AR activation. We are not aware of any current therapy aimed at increasing corepressors or decreasing coactivators of the AR. The consistency of the castration-induced changes and strength of the effects of these proteins in preclinical studies, however, indicate that they might be targets for novel therapies in the future.⁷

Growth factors

Growth factors are the major paracrine signaling mechanism used by the prostatic stroma and neuroendocrine cells. In healthy prostate glands, growth factors regulate growth and cellular maturation. In the setting of androgen-independent tumors, growth factors influence an alternate pathway that activates AR-mediated signaling. In general, growth factors and neuropeptides bind cell membrane receptors, which either polymerize to activate intracellular tyrosine kinase domains, or activate associated G-proteins that in turn activate nonreceptor tyrosine kinases (Figure 2).

One of the best characterized growth factors is the epidermal growth factor (EGF) family including transforming growth factor α (TGF α), EGF, amphiregulin and others. These bind the

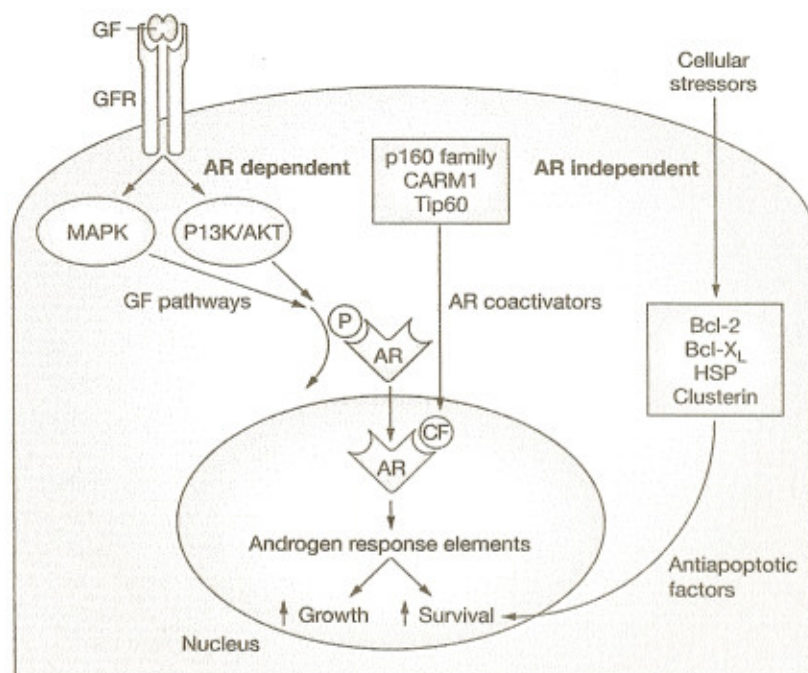


Figure 1 Three possible pathways leading to androgen-independent prostate cancer. Growth factor signaling cascades may activate the AR by phosphorylation in the absence of ligand. Coactivators modify transcriptional activity of the AR. When activated, the AR enters the nucleus, binds to androgen response elements, and transcribes specific genes promoting survival and proliferation. Antiapoptotic factors increase in response to cellular stressors, also promoting survival. Abbreviations: AR, androgen receptor; CARM1, coactivator-associated arginine methyltransferase 1; CF, cofactor; GF, growth factor; GFR, growth factor receptor; HSP, heat-shock protein; P, phosphate group; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; Tip60, Tat interacting protein 60 kDa.

EGF receptor (EGFR), activating the intracellular tyrosine kinase domain with subsequent effects mediated by multiple pathways. Two of the most important involve mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K). Both of these pathways can activate the AR by phosphorylation independently of a natural ligand, in addition to other effects that promote cell proliferation and oppose apoptosis in an androgen-independent manner. Consistent with this mechanism, activation of MAPK was shown to be increased following ADT.⁸

Expression of the EGFR and its subtype HER2/neu in prostate cancer is the subject of controversy. Lorenzo *et al.*⁹ reviewed 14 studies, which variously showed expression of HER2/neu to be present in 0–100% of prostate tumors. There is, however, greater uniformity in evidence supporting HER2/neu upregulation by ADT.

Table 1 Castration-induced changes in androgen receptor cofactors possibly involved in androgen receptor-dependent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
ARA70	AR coactivator	Initial decrease; increased with AI progression	Future: blockade may decrease AI AR signaling	54
CARM1 ^a	AR coactivator ^a	Increased ^a	Future target ^a	55
p160 family ^a	Family of AR coactivators including Src-1, TIF-2 and RAC-3 ^a	Increased ^a	Future: blockade may decrease AI AR signaling ^a	7
p300 (CBP)	AR coactivator	Unknown	Future: blockade may decrease AI AR signaling	56
Tip60 ^a	AR coactivator ^a	Increased ^a	Future: blockade may decrease AI AR signaling ^a	57

^aStudies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; ARA70, androgen receptor associated protein 70; CaP, prostate cancer; CARM1, coactivator-associated arginine methyltransferase 1; CBP, CREB binding protein; RAC-3, nuclear receptor coactivator 3; Tip60, Tat interacting protein 60kDa; TIF-2, transcription intermediary factor 2.

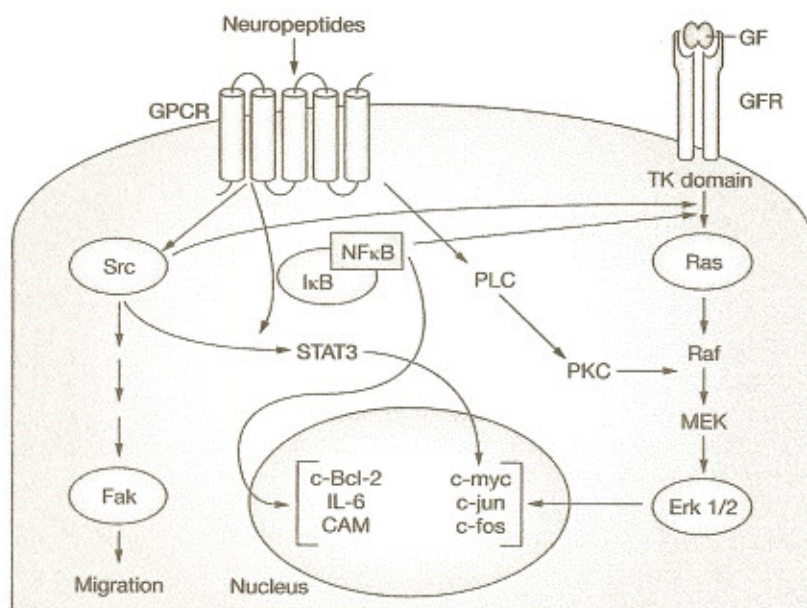


Figure 2 Comparison of growth factor and neuropeptide signaling pathways emphasizing cross-talk between them. Growth factors activate receptors with intracellular TK domains, subsequently activating a signaling cascade through Ras leading to modification of transcription. Neuropeptides activate GPCR, activating Src and NFκB by separation from its binding protein IκB. Both of these may activate growth factor receptor TK domains as well as affecting transcription of various factors. The GPCR may also activate other signaling cascades through Src and PLC/PKC. Abbreviations: CAM, cell adhesion molecule; GF, growth factor; GFR, growth factor receptor; GPCR, G-protein coupled receptor; IκB, inhibitor of κB; IL-6, interleukin 6; NFκB, nuclear factor κB; PKC, protein kinase C; PLC, phospholipase C; TK, tyrosine kinase.

Three of four studies reported that there were significant increases in this receptor following administration of ADT, and the study that did not demonstrate this effect reported a significant increase in EGFR overall compared to pre-ADT levels. Phase II trials of the monoclonal antibody (mAb) against HER2/neu, trastuzumab, have, however, been limited due to low levels of expression of the receptor in the screened patients. Screening produced generally disappointing results in the few patients tested.^{10,11} Other trials of mAbs against the extracellular or intracellular domains of the EGFR are ongoing.^{12,13} Other therapeutic options that target the EGFR include small-molecule inhibitors of the intracellular tyrosine kinase domain, such as gefitinib and erlotinib. Unfortunately, trials using these drugs as monotherapies have not demonstrated a clear benefit to patients.¹⁴

The insulin-like growth factor (IGF) pathway functions in a similar way to the pathways outlined earlier. In contrast to EGF, no consistent changes in IGF or its receptors are noted in men who have undergone castration,⁵ but two patients with advanced disease did show increased IGF-1 receptor levels.¹⁵ Of more interest is the finding that the levels of several IGF binding proteins (IGFBP) change in response to ADT; IGFBP2 and IGFBP5 both increase acutely and IGFBP3 decreases within weeks of initiation of ADT.^{16–18} All of these changes might increase the effect of IGF signaling through the PI3K pathway.

Many other growth factors, neuropeptides and cytokines have been shown to activate signaling pathways in a similar manner to IGF and EGF, resulting in activation of the AR (Tables 2–5). The downregulation of neutral endopeptidase at castration is thought to provide an important source for increasing levels of growth factors and neuropeptides, as the enzyme is the primary means of degrading these cellular signals.¹⁹ Almost all of the specific growth factors and receptors have antagonists that are undergoing early clinical trials. These were recently reviewed by van der Poel.¹²

The multiplicity of growth factors and neuropeptides leads to significant cross-talk between the cell signaling pathways (Figure 2). The many pathways lead to the same effectors, which might make downstream targets, that impact on multiple pathways, more likely to exhibit significant inhibitory effects. One such target is the Src family kinases, which are

Table 2 Castration-induced changes in growth factors possibly involved in androgen receptor-dependent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
EGF family	Ligand for receptor tyrosine kinases initiating multiple signaling cascades ^a	Some are increased	See EGF receptor blockers	58
FGF family ^a	Binds receptor with generally trophic effects ^a	FGF-8 increased ^a	TNP-470 inhibits binding, no effect in early trials ^a	58, 59
HGF ^a	Coactivator of AR ^a	Increased ^a	Future inhibitor may decrease AR effects in AI CaP ^a	60
IGF-1 ^a	Ligand for receptor tyrosine kinases initiating multiple signaling cascades ^a	No change ^a	Future inhibitor may decrease AR effects in AI CaP ^a	5, 58
IGFBP-2, IGFBP-5 ^a	Increases IGF signal transduction ^a	Increased ^a	ASOs show activity ^a	16, 17, 61
IGFBP-3 ^a	Decreases IGF signal transduction ^a	Decreased ^a	Prognostic indicator ^a	18
TGF- β 1 ^a	Ligand for receptor tyrosine kinases initiating multiple signaling cascades ^a	Increased in some ^a	Prognostic factor ^a	35
VEGF ^a	Angiogenesis and cell motility ^a	Initial decrease; NE cells continue to produce ^a	mAb to VEGF (bevacizumab) specifically inhibits tyrosine kinase activity ^a	12, 36, 62, 63

^aStudies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; ASO, antisense oligonucleotide; CaP, prostate cancer; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGFBP, IGF binding protein; mAb, monoclonal antibody; NE, neuroendocrine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Table 3 Castration-induced changes in receptors possibly involved in androgen receptor-dependent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
EGFR ^a	Receptor with intracellular tyrosine kinase domain activating multiple pathways ^a	Increased ^a	Multiple mAb and tyrosine kinase inhibitors ^a	9, 12, 64
ErbB2/HER-2/neu	Receptor with intracellular tyrosine kinase domain activating multiple pathways	ADT selects for HER-2/neu expressing cells	Multiple mAb and tyrosine kinase inhibitors	9, 12
FGFR-2 ^a	Binds ligand with effects on angiogenesis and wound healing ^a	Decreased ^a	NR	65
IGF-1R ^a	Binds ligand initiating multiple signaling cascades ^a	Increased in 2 patients ^a	Small molecule inhibitors show some activity in other cancers ^a	15
TGF- β 1R 1 ^a and TGF- β 1R 2 ^a	Binds ligand with complex cell signaling effects ^a	Increased in some cancers ^a	Prognostic factor ^a	35
Serotonin receptor 2B, ^a serotonin receptor 4 ^a	Active in autocrine and paracrine signaling supporting AI growth ^a	Receptor 4 increased ^a	Serotonin receptor antagonists inhibit CaP cell growth; AI cells more sensitive ^a	66

^aStudies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; CaP, prostate cancer; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; IGF-1R, insulin-like growth factor receptor 1; mAb, monoclonal antibody; NR, not reported; TGF β 1R, transforming growth factor receptor- β 1.

Table 4 Castration-induced changes in neuropeptides possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
Calcitonin	Angiogenesis, invasiveness stimulation through PKA, AKT and others	Produced by NE cells unaffected by ADT	Blockade may allow apoptosis in response to chemotherapy	68, 76
Endothelin-1	Angiogenesis, osteoblastic lesion formation, neuropeptide for multiple receptors	Increased in CaP in general; endothelin receptors increased by ADT	Endothelin-A receptor antagonist, atrasentan, in phase II/III studies ^a	12, 21, 38, 69
ProGRP ^a	Proneuropeptide ^a	Follows level of NED ^a	Marker for NED ^a	70
GRP/bombesin	Stimulates AI growth; multiple effects through NFκB	Follows level of NED	Marker for NED; prognostic utility	20, 67, 68, 70, 71
NEP (CD10) ^a	Cleaves many neuropeptides controlling extracellular concentrations ^a	Decreased ^a	Increasing NEP expression through gene constructs possible future treatment ^a	19
Neurotensin	Neuropeptide stimulates androgen independent growth	Increased in NE cells	Marker for NED	20, 21, 72
PTHrP	Multiple bioactive proteins from initial transcript; generally trophic neuropeptide	Increased	Anti-PTHrP mAb inhibits hypercalcemia of malignancy	67, 72
Serotonin ^a	Binds multiple receptors supporting AI growth ^a	Increased ^a	Serotonin antagonists ^a	66, 67, 73
Somatostatin ^a	Inhibits angiogenesis, proliferation, stimulates apoptosis ^a	Receptors are upregulated in CaP in general; produced by NE cells ^a	Somatostatin analogs ^a	73
VIP	Activates ERK 1/2, PI3K and other pathways supporting AI growth	Unknown	NR	74

^aStudies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; CaP, prostate cancer; ERK, extracellular-signal-regulated protein kinase; GRP, gastrin releasing peptide; mAb, monoclonal antibody; NE, neuroendocrine; NED, neuroendocrine differentiation; NEP, neutral endopeptidase; NFκB, nuclear factor κB; NR, not reported; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PTHrP, parathyroid hormone related protein; VIP, vasoactive intestinal peptide.

nonreceptor tyrosine kinases that participate in a variety of intracellular pathways stimulated by neuropeptides.²⁰ In addition, Src kinases can activate STAT3, a transcription factor that subsequently activates cyclin D1, vascular endothelial growth factor (VEGF), and c-myc.²¹ The activities represented by these pathways are clearly involved in the progression of androgen-independent prostate cancer. Two selective non-receptor tyrosine kinase inhibitors, MBS-354825 (dasatinib) and AZD0530, are both undergoing phase II investigation sponsored by the National Cancer Institute for use in patients with androgen-independent prostate cancer.^{22,23} Inhibitory molecules, such as the nonreceptor tyrosine kinases, have shown therapeutic promise *in vitro*²⁴ and might prove

to be good treatment options for men with androgen-independent prostate cancer.

ANDROGEN-RECEPTOR-INDEPENDENT PATHWAYS

Antiapoptotic pathways

Multiple pathways lead to apoptosis and several cellular proteins involved in apoptosis show changes following castration (Table 6). The Bcl family of proteins includes both proapoptotic and antiapoptotic members, the balance of which is critical for maintaining cellular homeostasis. The antiapoptotic Bcl-2 is consistently upregulated by castration and probably has an important role in allowing androgen-independent progression of prostate cancer.^{25–27} The antisense oligonucleotide (ASO) against

Table 5 Castration-induced changes in interleukins possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
IL-1 α/β ^a	Increase bone resorption ^a	Serum levels decreased ^a	NR	75
IL-3 ^a	Increase bone resorption ^a	Serum levels unchanged ^a	NR	75
IL-4 ^a	Modulate inflammation ^a	Serum levels increase when CaP becomes AI ^a	Marker ^a	76
IL-6 ^a	Activates multiple signaling cascades supporting growth, differentiation and cell survival ^a	Serum levels increase when CaP becomes AI ^a	Anti-IL-6 mAbs in early development ^a	73, 76
IL-8	Angiogenesis, metastasis, AI growth through Src and FAK	May be increased if TGF- β is increased	NR	77
IL-10 ^a	Modulates inflammation ^a	Serum levels increase when CaP becomes AI ^a	Marker ^a	76
M-CSF ^a	Increase bone resorption ^a	Unchanged ^a	Future: blockade may delay metastasis ^a	75
Oncostatin M	Related to IL-6; activates AR	Decreased	Future: blocking pathway might reduce AI-AR stimulation	61
TNF- α ^a	Inhibits apoptosis through NF κ B ^a	Unchanged ^a	Future: blockade may promote apoptosis and inhibit metastasis ^a	75, 78

^aStudies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; CaP, prostate cancer; FAK, focal adhesion kinase; IL, interleukin; M-CSF, macrophage colony stimulating factor; mAb, monoclonal antibody; NF κ B, nuclear factor κ B; NR, not reported; TGF, transforming growth factor; TNF, tumor necrosis factor.

Table 6 Castration-induced changes in apoptosis regulators possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
Bcl-2 ^a	Family of factors both proapoptotic and antiapoptotic; balance determines cell survival ^a	Increased ^a	ASO oblimersen sodium is undergoing trials ^a	25–27, 64
Clusterin ^a	Nuclear chaperone inhibits apoptosis ^a	Increased ^a	ASOs show promise in early trials ^a	31, 32
HSP27, HSP60, HSP70, HSP90 ^a	Nuclear chaperones bind denatured proteins; prevent apoptosis ^a	Most increased ^a	ASO and siRNA to HSP27 in early trials; geldanamycin and other ansamycins inhibit HSP90 ^a	30, 61
IAPs	Apoptotic inhibitor	Survivin and others increased in NE cells	ASOs have shown promise; must target multiple IAPs	79
p53 ^a	Cell cycle regulator; proapoptotic ^a	Increased ^a	NR	34, 64, 80
p21 (WAF1, CIP1) ^a	Effector for p53; halts cell cycle in G1 ^a	Increased ^a	Prognostic indicator ^a	34

^aStudies in human subjects available for review. Abbreviations: ASO, antisense oligonucleotide; CaP, prostate cancer; HSP, heat shock protein; IAP, inhibitor-of-apoptosis protein; NE, neuroendocrine; NR, not reported; siRNA, small interfering RNA.

Bcl-2 has shown activity, both *in vitro* and *in vivo*, delaying the progression to androgen-independent disease.²⁸ Recent phase II results in androgen-independent prostate cancer showed tolerability in combination with docetaxel and an impressive 80% response rate in the subset of patients achieving high serum concentrations of the oligonucleotide.²⁹

Another cellular pathway leading to apoptosis is induced by the accumulation of denatured proteins in the cell. Cellular stress leads to this condition and increasing numbers of dysfunctional proteins may precipitate, signaling caspase activation. The term 'heat shock protein' (HSP) encompasses a large family of related proteins that function as 'chaperones' to stabilize the

Table 7 Castration-induced changes in cell wall factors possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
Caveolin	Component of plasma membrane pits involved in cell signaling	Decreased	NR	81
FAK	Cell motility/metastasis; signaling pathways	Possibly increased if NE GF pathways increased	Genistein alters FAK activity, may reduce metastasis	46, 77
Fibronectin	Cell adhesion through interaction with integrin	Increased 60× <i>in vitro</i>	Future: blocking upregulation to decrease metastatic potential	43
ILK	Protein kinase linking cell adhesion to signaling; negatively regulated by PTEN	None	Future: inhibit in tumors with PTEN mutation	82
MMP-2, MMP-9	Cleaves basement membrane constituents; possible role in invasion/metastasis	Inconsistent	Many MMP inhibitors may have clinical utility in combination; prognostic utility	83
Mucin	Family of glycoproteins modulating cell adhesion	Most are increased	Vaccines targeting MUC-1 peptide ^a	44, 45
Syndecan-1 (CD-138) ^a	Binds matrix proteins and growth factors; involved with adhesion and differentiation ^a	Increased ^a	Possible role in immunotherapy ^a	64, 84
TIMP-1, TIMP-2	Inhibits MMPs	Inconsistent; opposite MMP	Analogues could decrease MMP expression; regulation of endogenous levels possible	85, 86

^aStudies in human subjects available for review. Abbreviations: CaP, prostate cancer; FAK, focal adhesion kinase; GF, growth factor; ILK, integrin-linked kinase; MMP, matrix metalloproteinase; MUC-1, urinary mucin 1; NE, neuroendocrine; NR, not reported; PTEN, phosphatase and tensin homolog; TIMP, tissue inhibitor of matrix metalloproteinase.

denatured proteins that result from cellular stress. The cell thereby becomes more resistant to apoptosis induced by multiple factors such as heat, radiation, and toxic drugs. As would be expected, many of the HSPs change expression in response to the stress of castration (Table 6). HSP27 has been extensively studied and might represent another important pathway of resistance to apoptosis in the androgen-independent state. In addition to its functions as a chaperone, HSP27 also directly interferes with caspase activation, modulates cellular oxidative stress, and stabilizes the cytoskeleton. The upregulation of HSP27 can be targeted by ASOs or small interfering RNA, and both of these therapies are entering phase I/II trials.³⁰

Clusterin is a nuclear protein that also functions as a chaperone. One isoform of clusterin shows greater potency than even heat shock proteins to 'stabilize' denatured proteins. Levels of clusterin increase significantly following castration and might be an important signal inhibiting apoptosis.^{31,32} An ASO against the clusterin gene successfully decreases expression, causing an increase in apoptosis in cells exposed to multiple chemotherapeutic drugs, radiation,

and oxidative stress. A phase I trial showed the clusterin ASO OGX-011 to have acceptable toxicity at effective plasma concentrations.³³ Phase II trials are underway to further define the role of ASOs in prostate cancer therapy.³⁰

The antiapoptotic changes discussed above can be counteracted by the p53 protein, which inhibits the cell cycle when DNA damage has occurred. After the dividing cell is held in the G1 stage, apoptosis is induced by a mechanism (not yet fully understood) involving both the p53 protein and its effector p21. Both these proteins are upregulated by castration. It has, however, been suggested that one or both might be mutated and nonfunctional.³⁴ This would upset the balance of proapoptotic and antiapoptotic signals within the cell, allowing resistance to ADT to develop and thereby facilitating the emergence of androgen-independent prostate cancer.

Miscellaneous pathways

The intricate pathways of cellular homeostasis show wide-ranging effects following castration. The brief discussion of the following three pathways highlights their potential importance to castration-adjunctive therapy.

Table 8 Castration-induced changes in signal transduction factors possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
AKT ^a	Antiapoptotic ^a	Possible increase if NE cells switch to GF pathways ^a	Testing of specific inhibitors ongoing ^a	4, 83
DHT ^a	Binds AR activating transcriptional activity ^a	Decreased ^a	ADT standard of care ^a	87
MAPK ^a	Protein kinase; activates multiple pathways ^a	Increased in 2 patients, associated with advanced disease ^a	Future: blockade may decrease AI AR stimulation ^a	8
Nkx3.1	Protein-protein interactions modify cell signaling; transcription factor	Initial decrease; increased with AI progression	Future: replacement may modify effects of PTEN mutation	88
PI3K	Protein kinase; activates multiple pathways	Possible increase if GF pathways increase activity	Wortmannin derivatives and peptidomimetics may be helpful in blocking this pathway	89
PKC ^a	Protein kinase; activates multiple pathways ^a	Variable ^a	ASO in early trials; prognostic factor ^a	90
PTEN ^a	Proapoptotic through inactivation of AKT pathway ^a	Lost to mutation in most cancers ^a	Competitive inhibitors under development; see AKT	4
Src	Involved in multiple pathways activating AR	Increased if GF pathways increase activity	Dasatinib, AZD0530 inhibits Src family kinases	21, 24, 77, 91, 92

^aStudies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; AR, androgen receptor; ASO, antisense oligonucleotide; CaP, prostate cancer; DHT, dihydrotestosterone; GF, growth factor; MAPK, mitogen-activated protein kinase; NE, neuroendocrine; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTEN, phosphatase and tensin homolog.

Angiogenesis is necessary for tumor growth, and some of the factors known to be upregulated in androgen-independent prostate cancer stimulate angiogenesis (Table 2). For example, TGF β is upregulated following castration,³⁵ and VEGF is produced by androgen-independent neuroendocrine cells, which are relatively unaffected by ADT.³⁶ Furthermore, VEGF levels are an independent prognostic factor in androgen-independent prostate cancer.³⁷ Broad spectrum antiangiogenic drugs such as suramin analogs and thalidomide have shown activity, singly and in combination, in early trials.³⁸ Targeted treatment with the mAb against VEGF, bevacizumab, showed some activity in combination with chemotherapy.³⁹ Ongoing trial results are awaited.

Multiple factors regulating cellular adhesion change expression level in response to ADT (Tables 7 and 8), and those that do not change may still be attractive targets for treatment to inhibit invasion or metastasis. Antimetastatic therapies might have the added advantage of modifying cellular signaling in addition to their intended cellular adhesion effects. For example, fibronectin interacts with integrins to affect cellular adhesion. These interactions also activate intracellular domains through focal

adhesion kinase, however (Table 7), and subsequently by MAPK or PI3K activation (Table 8) leading to multiple effects as outlined above.^{40,41} Stewart *et al.*⁴² provide an excellent review of changes in the extracellular matrix associated with prostate cancer progression. The data specifically related to castration is more limited. *In vitro*, fibronectin expression is increased over 60-fold in androgen-independent clones⁴³ and mucin expression also undergoes significant changes.⁴⁴ Current treatment strategies based on these observations are under development. The expression of mucin type 1 by neoplastic cells has inspired attempts at vaccine creation.⁴⁵ In addition, part of the putative activity of genistein in opposing neoplastic progression might be caused by alteration of signaling involving focal adhesion kinase.⁴⁶

A factor that encompasses multiple effects is nuclear factor κ B (NF κ B), a nuclear transcription factor (Table 9). Multiple pathways can phosphorylate inhibitor of κ B (I κ B), the cytosolic binding protein for NF κ B, causing the release of NF κ B, which quickly translocates to the nucleus. Once in the nucleus, NF κ B facilitates transcription of multiple genes associated with inflammation (e.g interleukins 1 and 6 and tumor necrosis factor- α [Table 5]), cell adhesion

Table 9 Castration-induced changes in transcription factors possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
AR ^a	Androgen binding induces dimerization, activating transcription factor activity for many genes ^a	Increased ^a	Future: blocking downstream signals completely ^a	3, 73
c-Fos ^a	Combines with c-Jun to make AP-1 transcription factor ^a	Variable ^a	Prognostic factor ^a	90
c-Jun ^a	Dimerizes or combines with c-Fos to make AP-1 transcription factor ^a	Variable ^a	Prognostic factor ^a	90
c-Myc	Transcription factor; supports AI growth; inhibits apoptosis	May be increased	Phosphorodiamidate morpholino oligomers against c-Myc in early trials ^a	82, 73
NFκB	Causes transcription of multiple factors involved in angiogenesis, metastasis, and antiapoptosis	Increased	Proteasome inhibitor bortezomib opposes degradation of IκB ^a	71

^aStudies in human subjects available for review. Abbreviations: AI, androgen independent; AP-1, activating protein 1; AR, androgen receptor; CaP, prostate cancer; IκB, inhibitor of κB; NFκB, nuclear factor κB.

Table 10 Castration-induced changes in miscellaneous factors possibly involved in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
Arachidonic acid	Increased by EGF/neurotensin signaling	Possible upregulation if NE cells switch to GF pathways	COX-2 inhibitors show modest activity in early trials	94
CD-117 (c-kit)	NR	Induced in 1 metastatic sample	NR	64
COX-2	Enzyme produces prostaglandins from arachidonic acid	Increased	COX-2 inhibitors show modest activity in early trials	83, 94, 95
Id-1	Upregulates EGFR; inhibits transcription factors	Increased	Future target	96
Insulin receptor	Controls glucose balance	Increased	NR	61
RPTPα	Role in neuronal differentiation; activates Src	Increased	Marker for NED and correlation with ERK1/2 activation	72
SREBPs ^a	Regulate transcription of enzymes involved in lipogenesis ^a	Increased ^a	NR	97
SREBP cleavage protein ^a	Cleaves SREBPs ^a	Initial decrease; increased upon AI progression ^a	NR	97

^aStudies in human subjects available for review. Abbreviations: AI, androgen independent; CaP, prostate cancer; COX, cyclo-oxygenase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated protein kinase; GF, growth factor; Id-1, inhibitor of DNA binding 1; NE, neuroendocrine; NED, neuroendocrine differentiation; NR, not reported; RPTPα, receptor-like tyrosine phosphatase α; SREBP, sterol regulatory element-binding proteins.

(e.g. vascular cell-adhesion molecule, inter-cellular cell-adhesion molecule and E-selectin), stress response (e.g. cyclooxygenase 2 [Table 10] and nitric oxide synthase), and factors inhibiting apoptosis (e.g. Bcl-2 family and inhibitor-of-apoptosis proteins [Table 6]).⁴⁷ These pathways are intimately involved in proliferation, angiogenesis, invasiveness, metastasis, and apoptosis inhibition.⁴⁸ Recently, bortezomib, an inhibitor of the proteasome responsible for degrading IκB, has shown efficacy and tolerability in prostate cancer and might be a promising treatment in combination with other agents.^{49,50}

CURRENT TREATMENTS FOR ANDROGEN-INDEPENDENT PROSTATE CANCER

Docetaxel was recently shown to modestly improve survival in androgen-independent prostate cancer.^{51,52} Most of the biologic agents discussed earlier in this review are currently undergoing testing in combination with docetaxel at the time of androgen-independent disease progression. This strategy shows some promise, as many of the biologic agents show modest activity alone, seeming to function better as chemosensitizers or radiosensitizers.¹² By contrast, very few studies exist that examine

Table 11 Castration-induced changes in prostate cancer markers in androgen receptor-independent prostate cancer progression.

Factor	CaP related physiology	Castration related changes	Potential clinical role	Selected references
CgA ^a	Marker ^a	Increase in plasma levels ^a	Marker of NED ^a	98
CgB	Marker	5–10 fold increase from NE cells	Better marker than CgA in certain NED cancers	72
Ki67 (MIB-1) ^a	Nuclear antigen; marker of cell division ^a	Decreased or no change ^a	Marker for cell division ^a	34, 64
NSE ^a	Marker ^a	Variable ^a	Prognostic marker ^a	72
PSA	Marker	Decreased overall owing to cell death; decreased expression by NE cells	Prognostic marker	72
PSP94 ^a	Unknown ^a	Persists during ADT ^a	Marker of AI CaP ^a	99
Tissue glutaminase ^a	Marker for apoptosis ^a	Initial increase followed by decrease ^a	Apoptosis marker ^a	100

^aStudies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; CaP, prostate cancer; CgA, chromogranin A; CgB, chromogranin B; MIB-1, ubiquitin ligase MIB-1 (mindbomb homolog 1); NE, neuroendocrine; NED, neuroendocrine differentiation; NSE, neuron specific enolase; PSA, prostate-specific antigen; PSP94, prostate-specific protein-94.

the adjunctive use of these biologic agents at the time of castration. We believe that the changes occurring at the time of castration set in motion events leading to androgen-independent prostate cancer, thus indicating a role for adjunctive therapy at that time. The rationale for adjunctive treatment for prostate cancer at the time of castration is based on several lines of evidence.

First, microenvironmental changes in the cellular milieu are likely to have a major role in the initiation of clinical disease.⁵³ ADT induces further changes (listed in Tables 1–11), which, through poorly defined biochemical pathways, promote the emergence of androgen-independent prostate cancer. Future treatment strategies might target these microenvironmental cellular changes and attempt to reverse or ameliorate them through gene silencing (ASOs, small interfering RNA) or direct inactivation/activation of opposing factors (mAbs). Second, the apoptotic response to the act of castration is never complete, and efforts to enhance this response will decrease residual disease and possibly delay disease progression. Subsets of cells can have resistance to apoptosis through mutational changes in effectors (e.g. p53/p21) or through the chance occurrence of elevated precastration levels of antiapoptotic factors (e.g. Bcl family proteins or HSPs). Efforts, initiated at the time of castration, to increase the effectiveness of treatment and approximate a maximal apoptotic response should be attempted. Third, the smaller tumor

burden associated with earlier treatment of prostate cancer potentially allows a greater chance for effective intervention.²⁸ Finally, the biologic therapies discussed are attractive in that they generally have relatively low toxicity profiles, making them suitable adjunctive treatments for prostate cancer.

Of the many castration-induced changes listed in the tables presented in this review, mechanisms suggested for future study include those that are consistently seen *in vivo* and that have well-characterized and effective inhibitors available. Proteasome inhibition, targeted therapies such as PI3K inhibitors or Bcl-2 ASOs, and small molecule tyrosine kinase inhibitors all represent possible strategies for treating prostate cancer in the adjunctive setting. In addition, angiogenesis inhibitors and cellular adhesion modifications might decrease growth and metastasis as well as having direct intracellular effects on a variety of pathways.

CONCLUSION

Increasing the effectiveness of ADT and/or delaying the onset of androgen-independent prostate cancer represents a major treatment strategy for this disease. The biochemical changes demonstrated by prostate cancer cells undergoing castration suggest therapeutic methods for adjunctive treatment. Specific therapies currently exist that target many of these pathways. These should ultimately be tested in the adjunctive setting.

KEY POINTS

- Although not completely understood, the progression of prostate cancer to an androgen-independent state probably involves multiple biochemical pathways
- Therapeutic androgen ablation is likely to be an initial factor driving this biochemical cascade of events
- Novel biologic agents are now available enabling modification of some of the pathways involved in the development of androgen-independent prostate cancer
- Current clinical data shows some efficacy for biologic agents when used with chemotherapy in the setting of androgen-independent disease
- Future trials should also test biologic agents at the time of androgen ablation to attempt to maximize the initial apoptotic response and delay the onset of androgen independence

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Competing interests

The authors declared they have no competing interests.

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REVIEW

Inhibition of Akt pathways in the treatment of prostate cancer

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Akt is a serine/threonine kinase mediating multiple intracellular pathways involved in prostate cancer (CaP) biology. Increased understanding of the molecular mechanisms of Akt activation and signaling have led to the development of an increasing number of Akt inhibitors. These biologic agents demonstrate activity against a wide range of cancers in preclinical studies. Clinical studies of Akt inhibition in CaP are in progress, including agents such as celecoxib, perifosine and genistein. How best to integrate Akt inhibitors with standard CaP therapy or select patients most likely to benefit is the subject of ongoing research.

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Introduction

Akt, or protein kinase B, is a serine/threonine kinase that plays an important role in intracellular signaling cascades. A variety of neoplasms show perturbations in the biochemical pathways affected by Akt. Prostate cancer (CaP) specifically shows biochemical abnormalities related to Akt that may be of importance in sustaining tumor growth by preventing apoptosis and promoting proliferation and angiogenesis.

CaP is the most common noncutaneous malignancy in American males and is predicted to be the third leading cause of cancer deaths for 2006.¹ While local therapy for CaP is relatively effective, androgen deprivation therapy remains the mainstay of treatment for disseminated disease and is principally palliative in nature. Introduced in the 1940s,² androgen deprivation removes androgen stimulation, initially inducing apoptosis in CaP. However, the disease eventually progresses to an androgen independent (AI) state with an associated life expectancy of only 15–20 months. Androgen deprivation, while extending length and quality of life for many patients, also induces tumor-specific biochemical changes of many intracellular factors including Akt. These changes may promote progression to an AI state.³

Novel treatments for AI CaP are needed. Increasing understanding of the many biochemical changes associated with neoplastic progression and androgen independence has led to the identification of novel targets for therapeutic intervention. In this review, we discuss

pathways relating directly to Akt, focusing on those showing the greatest relevance to current and possible future therapeutic strategies.

Pathways affected by Akt

Akt form and function

Akt was originally identified as an oncogene within the AKT8 retrovirus. This retrovirus was isolated from the AKR strain of mice that have a high incidence of leukemia and lymphoma.⁴ Subsequent genetic analysis demonstrated that Akt is an important intracellular signaling moiety highly conserved across species. A member of the AGC kinase family, it is very similar to protein kinase A and protein kinase C. When first discovered, it was therefore named 'protein kinase B' and is sometimes called RAC (related to A and C).

In humans, Akt is a family of three homologous members out of which Akt1 and Akt2 are more widely distributed than Akt3.⁵ Akt has three domains with specific functions. The N-terminal domain is a pleckstrin homology (PH) domain, which can bind phosphoinositides (PI) in the cellular membrane. The C-terminal domain is a regulatory domain and the central portion of the protein is the catalytic domain.⁶ Complete activation of the catalytic activity of Akt requires phosphorylation of a threonine residue at 308 and a serine residue at 473. It is possible that Akt shows partial activation with phosphorylation at the threonine 308 position.⁷

Akt activation

Akt activation occurs in response to multiple extracellular signals acting through tyrosine kinase and G-protein coupled receptors (see Figure 1). These receptor types activate phosphoinositol-3-kinase (PI3K) class IA

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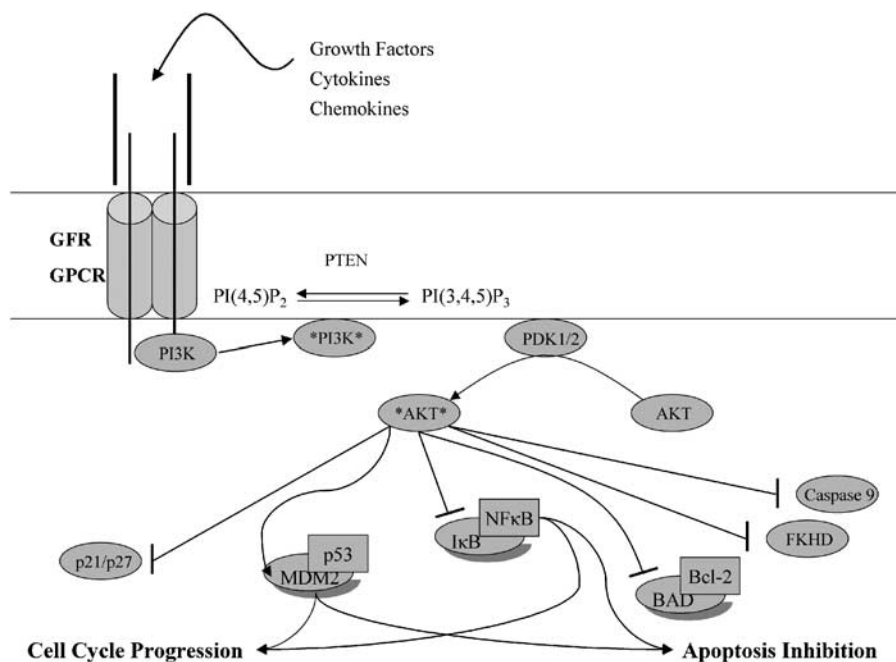


Figure 1 Simplified diagram of Akt activation and selected downstream effector pathways. Multiple extracellular signaling factors activate intracellular receptor domains. Activated PI3K promotes the 3' phosphorylation of PI(4,5)P₂. The resulting PI(3,4,5)P₃ recruits Akt and PDK1 to the cell membrane through interactions with their PH domains. Akt is activated by phosphorylation resulting in multiple downstream effects. Abbreviations: GFR, growth factor receptor; GPCR, G-protein coupled receptor; NFκB, nuclear factor kappa B; PDK, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide-3-kinase; PI(3,4,5)P₃, phosphatidylinositol-3,4,5-trisphosphate; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

and IB, respectively. In turn, PI3K phosphorylates the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), which then acts as the second-messenger phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) for a variety of pathways. This event promotes recruitment of Akt from the cytoplasm to the cellular membrane where phosphoinositide-dependent kinase-1 (PDK1) phosphorylates the threonine 308 position of Akt. Phosphorylation of the serine 473 position also occurs, although the kinase responsible has not been definitively identified. This step appears to be tightly regulated and may require multiple intracellular messengers including integrin-linked kinase, PDK2 and others.⁷

Upstream inhibitors

An intricate web of inhibitory factors opposing the actions of PI3K and subsequent second messengers control regulation of Akt activation. Most notably, 'phosphatase and tensin homolog deleted on chromosome 10' (PTEN) directly opposes PI3K by removing the 3'-phosphate from PI(3,4,5)P₃. A recently discovered class of phosphatases called SHIP phosphatases also may play a role in controlling levels of PI(3,4,5)P₃, and thus subsequent Akt activation. However it appears that as SHIP removes the 5-phosphate from PI(3,4,5)P₃, the resultant PI(3,4)P₂ may still recruit Akt to the plasma membrane. Therefore, PTEN is probably the more important inhibitor.⁵ Other recently discovered phosphatases include C-terminal modulator protein and PH domain leucine-rich repeat protein phosphatase, both of which may be significant in Akt regulation.⁸

Direct deactivation of Akt is also possible. Protein phosphatases (PP1 and PP2a) both govern the regulatory activity of many intracellular messengers including Akt through dephosphorylation. The complex activity of these regulatory proteins is controlled through cell wall constituents including palmitate, integrin and caveolin.⁵ Heat shock protein 90 may oppose the actions of these phosphatases, thereby promoting Akt activity.⁹

Akt downstream effects

Following activation, Akt moves from the cellular membrane to the cytoplasm where it exercises broad control over a variety of intracellular pathways generally supporting survival, proliferation, and other activities necessary for neoplastic disease progression.

Apoptosis and the cell cycle. Cancer cells escape normal biochemical systems regulating the balance between apoptosis and survival. Akt generally acts to promote survival through inhibition of proapoptotic factors and activation of anti-apoptotic factors. For example, the Bcl-2 family of proteins consists of both proapoptotic and anti-apoptotic factors, the balance of which is critical for maintaining cellular homeostasis. Through phosphorylation, Akt inhibits the activity of proapoptotic members such as BAD, BAX and BID while activating anti-apoptotic members such as Bcl-xL.¹⁰⁻¹²

Another family of apoptotic regulators is the forkhead family. In general, Akt phosphorylates various members of this family causing translocation from the nucleus to

the cytoplasm, thus inhibiting the transcription of proapoptotic genes.⁷

Other biochemical pathways affected include apoptosis-signal-regulating kinase cyclic AMP response element binding protein, and the oncoprotein MDM2. Effects through these include inhibition of jun N-terminal kinase and p53. In addition, Akt may block apoptosis after it has been initiated. For example, Akt can prevent the activation of caspase-9 despite mitochondrial cytochrome *c* release.⁵

Cancer cells also escape normal cellular controls over the cell cycle, generally resulting in increased, deregulated proliferation. Akt activation may promote this process through multiple pathways. Three central regulators of the cell cycle affected by Akt are cyclin D, p21 and p27. Cyclin D is necessary for cyclin-dependent kinase (CDK) activity regulating entry into the cell cycle. p21 and p27 are CDK inhibitors, which oppose cell-cycle progression. Akt phosphorylates and inactivates p21 and p27 thereby eliminating a critical negative regulator of CDK activity and promoting progression through the cell cycle.⁵

Other/multiple pathways

Other necessary cellular characteristics for neoplastic growth include angiogenesis, invasion and metastasis. Biochemical pathways affected by Akt may accomplish these effects with significant changes noted when Akt signaling intensity changes. In addition, three pathways discussed here have multiple or complex effects.

Akt activation may lead to increased angiogenesis through phosphorylation of endothelial nitric oxide synthase and subsequent production of nitric oxide.^{13,14} In addition, Akt is a key activator of the mammalian target of rapamycin (mTOR) which, through stabilization of the hypoxia inducible factor, induces expression of pro-angiogenic genes such as vascular endothelial growth factor. mTOR also promotes cell survival and proliferation through other pathways. Examples of these include activation of p70 ribosomal S6 kinase and inhibition of 4E-BP1, thus promoting ribosomal translation in general, and increased expression of cyclin D, which promotes cellular cycling and proliferation.⁵

One cellular pathway affected by Akt with multiple effects is the nuclear factor kappa-B (NFκB) pathway. Akt causes the NFκB binding protein, IκB, to release NFκB, which then translocates to the nucleus where it transcribes multiple genes involved with proliferation, inflammation, cell adhesion, stress response and anti-apoptosis.¹⁵ NFκB also increases expression of matrix metalloproteinases, which are frequently elevated in CaP specimens and may play a role in promoting invasion and metastasis.

p53 is a tumor suppressor showing aberrant regulation or mutation in many neoplasms. Akt may play a role in regulating its activity through activation of its binding protein, MDM2. Phosphorylation of MDM2 causes translocation to the nucleus where it inactivates p53 resulting in cell-cycle progression and inhibition of apoptosis.¹⁶

Of special interest in CaP is the observation that Akt can directly phosphorylate the androgen receptor on

serine residues at positions 210 and 790. The results of this are controversial with some authors reporting activation while others report suppression of androgen receptor signaling.^{17,18} The reason for this may relate to different cell passage numbers, which is an interesting concept in the context of a rapidly dividing neoplasm.¹⁹

Alterations of Akt activity in CaP

Increased Akt activity in CaP may be caused by genetic overexpression of Akt or altered expression of its upstream-positive and upstream-negative regulators. In CaP, several such mechanisms are probably active.

Akt overexpression has been demonstrated in CaP.²⁰ However, the most consistent finding in this disease is the silencing of PTEN and subsequent increase in Akt signaling.⁸ PTEN may be lost by deletion, mutation or epigenetic mechanisms.⁵ Up to half of the patients CaP tissue specimens show inactivation of PTEN with increasing incidence of this finding in metastatic deposits and AI disease, emphasizing its possible importance in tumor progression.^{21–23} Other genetic overexpression or underexpression of factors upstream of Akt have also been demonstrated in CaP. Various growth factor receptors including the fibroblast growth factor, epidermal growth factor, and insulin-like growth factor are overexpressed in some CaP leading to increased Akt signaling.⁵ In addition, PI3K may be overexpressed in CaP,⁵ and may be important in the progression to AI disease.²⁴

Regardless of the molecular mechanisms responsible, the excessive activation of Akt is a poor prognostic factor in CaP. In one report, phosphorylation of Akt was superior to measurements of cellular proliferation and even Gleason grade for predicting biochemical recurrence following radical prostatectomy.²⁵

Treatments for CaP may also upregulate Akt pathways through activation of cellular stress responses. In CaP specifically, androgen withdrawal may lead to biochemical changes ultimately supporting the emergence of AI disease. Although Akt itself may not be directly involved in causing this transition, some of these pathways signal through Akt.^{3,26} For example, emergence of a neuroendocrine phenotype in CaP may be important in disease progression. Such neuroendocrine cells may convert CaP cells to a dependence on survival signals through G-protein coupled receptors and growth factor receptors upstream of Akt, bypassing the usual androgen receptor signaling.²⁶

These data cumulatively provide a rationale for Akt inhibition as a therapeutic paradigm in CaP.

Feedback mechanisms

In light of the pathways and effects of Akt activation discussed above, it seems that Akt inhibition would naturally lead to positive therapeutic benefits in CaP and other neoplasms. Unfortunately, feedback mechanisms inherent in this complex biologic system may cause paradoxical responses to inhibition at various levels of Akt pathways. Two recently discovered feedback mechanisms demonstrate that inhibition of mTOR may, in fact, increase signaling through the Akt pathway.²⁷ Although not yet demonstrated *in vivo*, the existence of such complexity demonstrates a clear need for future

4 clinical trials to carefully measure the biologic effects of new therapies at the molecular level. Another factor complicating treatment using Akt inhibitors is one of the most important pathway for normal cellular physiology. It is not yet clear that developing treatment will demonstrate significant efficacy with acceptable levels of toxicity. These facts may lead to the use of Akt inhibition as adjunctive treatment rather than monotherapy.

Akt inhibition as a therapeutic strategy

Data demonstrating the importance of increased Akt signaling pathways in supporting prostatic growth and the neoplastic progression of CaP have stimulated efforts to modulate these pathways through direct and indirect Akt inhibition. In view of the increased activation of Akt during some treatments for CaP, inhibition of Akt may be an important strategy for adjunctive therapy. Multiple inhibitors have been developed using a variety of mechanisms. Inhibition of PDK1 prevents activation of Akt and several effective agents are available (see Table 1). Direct inhibition of Akt may target any of the three domains discussed above using competitive, allosteric, pseudosubstrate or other mechanisms (see Table 2). Preclinical data on many Akt inhibitors are available and are reviewed in detail elsewhere.^{28–31} Additional data regarding the Akt inhibitory properties of several nutraceuticals is emerging and may prove important in the future. Examples include quercetin,³² diallyl trisulfide,³³ curcumin³⁴ and silymarin.³⁵ This review will be limited to agents for which clinical data are now available.

Selective inhibitors of Akt

Celecoxib

Celecoxib is a potent inhibitor of the inducible enzyme cyclooxygenase-2 (COX-2). By selective inhibition of COX-2 and avoidance of interference with the constitutively active COX-1, it was thought that celecoxib and other selective COX-2 inhibitors might be an effective treatment for inflammatory conditions while avoiding the gastric complications of long-term COX-1 inhibition. Although subsequent testing revealed an association with adverse cardiac outcomes leading to cessation of some ongoing trials, the drug remains on the market.

Celecoxib is currently of interest as preclinical experiments demonstrate significant proapoptotic effects in CaP cell lines. The biochemical activity of the drug is due to prevention of Akt phosphorylation by inhibiting the

action of PDK1^{27,36} and this activity is independent of the COX-2 inhibitory effects.³⁷ In addition, COX-2 inhibitors may have other cellular functions potentiating the apoptotic response.³⁸ A therapeutic window for celecoxib might exist as the COX-2 enzyme is preferentially expressed in cancer tissue in response to tumor promoters, cytokines and growth factors.³⁶ However, some experiments show expression of induced COX-2 in CaP to be low if present, especially compared to other epithelial malignancies.³⁹ Although controversy exists on this point, COX-1 and -2 expression might be higher in the prostate in general regardless of disease processes.⁴⁰

Outcomes data up to 20 years ago indicated a cancer chemopreventive effect for anti-inflammatory medications.⁴¹ Large epidemiologic studies have examined this effect in celecoxib in a variety of cancers.⁴² Specifically, the rationale for CaP chemoprevention using COX-2 inhibition was reviewed by Basler and Piazza.⁴³ Although no current clinical chemoprevention data are available, the use of celecoxib as adjunctive therapy merits attention.

A phase II study by Pruthi *et al.*⁴⁴ of celecoxib monotherapy to modify prostate-specific antigen (PSA) doubling time (PSADT) in patients with biochemical relapse following definitive therapy has been reported. Forty patients were enrolled, nineteen of whom had a PSADT of less than 6 months. Following treatment, 36 of 40 patients showed a declining PSADT, and 11 of 40 had their PSA decline with an additional 8 of 40 showing stable PSA values. A following randomized, placebo-controlled trial of this effect was terminated early based on the question of celecoxib safety. An *ad hoc* analysis of existing data on 78 randomized patients revealed a greater than 200% increase in PSADT in 40% of patients receiving celecoxib compared to 20% receiving placebo ($P = 0.08$).⁴⁵

Recent phase II studies demonstrate the use of celecoxib in combination with docetaxel and zoledronate.^{46,47} Both the studies demonstrated biochemical and objective tumor responses. Another randomized, blinded trial of celecoxib as neoadjuvant therapy before prostatectomy showed activity in the disease. Significant effects on cellular signaling, oxidative stress and cell-cycle regulation were apparent upon blinded in comparison of the pathology specimens.⁴⁸

In summary, preclinical data suggest a role for celecoxib in the treatment of CaP. Its apoptotic effects are mediated through inhibition of Akt phosphorylation by antagonism of PDK1. A therapeutic window may allow efficacy and development of derivatives will further refine the specificity of this medication.²⁷

Table 1 Select PDK-1 inhibitors

Name	IC ₅₀ /L	Comment	Selected citations
Celecoxib	3.5–48 μ M	COX-2 inhibitor	36,37,72,73
DMC	38 μ M	Celecoxib analog w/o COX-2 activity	73
OSU-03012/3	3 μ M	Celecoxib derivatives	37
UCN-01	33 nM	7-hydroxy staurosporine analog, Phase I/II studies available	55,74
BX-795, -912, -320	11–30 nM	Aminopyridines	75

Abbreviations: COX-2, cyclooxygenase-2; PDK, phosphoinositide-dependent kinase.

Table 2 Select Akt inhibitors by class

Name	IC ₅₀	Comments	Selected citations
<i>ATP competitive inhibitors</i>			
Balanol analogs	4–5 nM	Rationally designed	76
H-89	2.5 μ M	Protein kinase A inhibitor	77
NL-71-101	3.7 μ M	Developed from H-89	78
<i>Lipid-based/phosphatidylinositol analog inhibitors</i>			
PIA 5/6/23/24/25	<5 μ M	Ether lipid analogs, prevent translocation of Akt	79–81
Perifosine	5 μ M	Prevents Akt translocation, phase II data available	50
PX-316	1.7 μ M	Binds to PH domain of Akt	82
PX-866	16.8 nM	Inhibits PI signaling	
<i>Pseudosubstrate Inhibitors</i>			
AKTide-2T	12 μ M		83,84
FOXO3 hybrid	1.1 μ M	Hybrid with AKTide-2T	30
FOXO3 hybrid modification	0.11 μ M	Replaced Ser w Ala	30
<i>Allosteric inhibitors of AKT kinase domain</i>			
Compound 12	AKT1 = 4.6 μ M AKT2 = >250 μ M	First isozyme specific AKT inhibitor	85,86
Compound 13	AKT1 = 2.1 μ M AKT2 = 21 μ M	Dual activity	85,86
Compounds 14-29		Iterative improvements with greater specificity	85,86
<i>Akt antibodies</i>			
GST-anti-Akt1-MTS		Cell-permeable antibody, blocks catalytic site	87
<i>Interaction with PH domain of AKT</i>			
Triciribine/API-2		May interact with PH domain (?) Prior phase II trials at high doses showed high toxicity	88,89
TCN-P		Triciribine monophosphate	90
Akt-in		Synthesized peptide	84
<i>Unknown/multiple mechanism(s)</i>			
KP372-1			91
N10-substituted phenoxazines	1–2 μ M	May bind ATP-binding site or act as allosteric inhibitors	92
Genistein		Inhibits multiple intracellular kinases	56,59,63

Abbreviation: PH, pleckstrin homology.

Perifosine

Phospholipid analogues have been in use as medications for some time. Miltefosine demonstrated activity against many cancers and is still approved in Europe for use in cutaneous lymphoma and cutaneous breast cancer metastases. High rates of gastrointestinal toxicity and low bioavailability led to efforts to discover further modifications of phospholipid analogues with enhanced pharmaceutical potential.

Perifosine is a substituted alkylphosphocholine with oral bioavailability. In preclinical experiments, it causes cell-cycle arrest in G₁/S or G₂/M, probably through effects mediated by p21 upregulation.⁴⁹ These effects appear to occur through inhibition of Akt activation, although the mechanism is incompletely understood. As a phospholipid analogue, perifosine incorporates into the cell wall where it prevents Akt phosphorylation in a PDK1-independent manner.⁵⁰ This apparently involves interference with the normal association of PH domains with 3-PI moieties.

Phase I trials established the tolerability of perifosine with most frequent side effects being nausea, vomiting and diarrhea.^{51,52} Two recent phase II trials of perifosine in CaP have been reported. A California Cancer

Consortium trial in 25 patients with biochemical recurrence following definitive therapy demonstrated biological and chemical activity, with 23% of patients having a decrease in PSA, although none were >50%.⁵³ A National Cancer Institute study of 19 men with metastatic AI CaP and average PSA of 180 ng/ml showed no objective or PSA responses, and four patients with PSA stabilization for 12 weeks.⁵⁴

Although perifosine is apparently relatively ineffective as monotherapy, the strong preclinical rationale combined with preclinical results showing synergism with other Akt inhibitory drugs suggest the need for more trials examining its role in an adjunctive setting.⁵⁵ Phase I trials are already underway for perifosine combined with docetaxel, paclitaxel, gemcitabine and radiation therapy. Future phase II trials at our institution will examine the combination of perifosine with other inhibitors of the Akt pathway in CaP.

Genistein

Genistein is a naturally occurring isoflavone found in soy-based products. Gastric and intestinal hydrolytic reactions convert it to a well-absorbed aglyconic form. In

addition, some Asian forms of fermented soy, such as miso, natto and tempeh, are rich in isoflavone aglycones. Speculation regarding the CaP-inhibitory effects of soy isoflavones exists because of significant epidemiologic differences in CaP incidence mirroring the consumption of, among other things, soy products.⁵⁶ Preclinical data demonstrate truly remarkable biochemical characteristics of genistein. In many different cell lines and xenografts, the tyrosine kinase inhibitory characteristics of genistein induce apoptosis, cell-cycle arrest, hinders proliferation, prevents angiogenesis, and blocks androgen and estrogen-stimulated transcription.^{56–60} In addition, studies demonstrate genistein may oppose many cellular survival mechanisms induced by radiation, chemotherapy or androgen deprivation, suggesting the usefulness of this agent as adjunctive therapy.^{61,62}

Subsequent analysis of these favorable biochemical changes reveal that many of them are mediated through Akt pathways (see Figure 1). For example, NF κ B, an upstream regulator of the apoptotic Bcl-2 family and the inhibitors of cyclin dependent kinases, p21 and p27, is activated by many cellular stimuli. Li and Sarkar⁶³ demonstrated that NF κ B activation in PC3 cells is mediated by Akt. In prostate cell lines, experiments at our institution confirm that genistein inhibits activation of Akt, thereby inducing apoptosis similar to other inhibitors of Akt (see Figure 2).⁶⁴ Inhibitors such as LY294002, although completely blocking Akt phosphorylation through PI3K inhibition, are too toxic for human use. Genistein may provide a non-toxic alternative while maintaining sufficient activity to effect a clinical response.

Some of the cellular effects of genistein seen *in vitro* cannot be initiated at attainable concentrations in humans, even when consuming large amounts of soy. Concentrated aglycone-rich food supplements, such as genistein combined polysaccharide (GCP), may allow higher plasma levels of isoflavones.⁵⁸ Recently, a prospective randomized study by Rannikko *et al.*⁶⁵ found that prostate tissue concentrates phytoestrogens includ-

ing genistein, thus suggesting that plasma concentrations underestimate tissue concentrations by over 50%.

Given the promising preclinical data, studies are ongoing examining the activity of genistein *in vivo* against CaP. Phase I studies demonstrate general tolerability.⁶⁶ A phase II pilot study at our institution examined the ability of GCP to decrease PSA in men with histologically proven CaP. Sixty-two patients with elevated PSA were enrolled. Patients were categorized by prior treatment. Nine, seventeen and six had received prior prostatectomy, radiation therapy, or both, respectively. Fourteen were receiving intermittent hormone ablation and were currently off-cycle. Finally, 16 patients were on an active surveillance protocol. All patients received GCP supplements to be taken orally three times daily for 6 months. Three patients discontinued because of grade 2 diarrhea and seven patients discontinued for personal reasons. Of the 52 remaining patients, 8 had PSA reductions and 1 of them greater than 50%. Interestingly, all of these patients were in the watchful waiting subgroup. In analysis of the responding patients vs others, there did not appear to be any correlation with Gleason score. In addition, estrogenic effects of genistein were probably not the causative factor as testosterone was increased in five responders, decreased in one, and unchanged in two.⁶⁷

On the basis of these results, further randomized studies are currently ongoing at our institution. In addition to examination of GCP as monotherapy in patients on watchful waiting protocols, we plan to study GCP in conjunction with androgen deprivation therapy in patients with biochemical relapse following definitive therapy.

Patient selection

Biologic therapies modifying cellular signaling pathways generally show modest responses in most cases. This suggests the need to test such therapies in the adjunctive setting, possibly increasing the response to radiation, chemotherapy or androgen deprivation.³ For ethical reasons, many existing studies will use Akt inhibitors in conjunction with docetaxel-based chemotherapy. This is a rational strategy as chemotherapy upregulates survival pathways that involve Akt activity.^{68,69}

Further efforts to predict patient response to biologic agents by pretreatment measurements of activated pathways through direct tissue or serum analysis may allow individualized treatment. One method involves profiling the entire proteome in patients' serum using matrix assisted laser desorption/ionization-time-of-flight mass spectrometry. Multiple bioinformatics analyses of CaP patient serum and normal controls allows for proteomic 'fingerprints' with high discriminatory power. Once such profiles are produced, patients on clinical studies may be compared, allowing characterization of treatment effects on proteome profiles. Examination of the entire proteome may allow more efficient sorting of possible tumor markers. Eventually, exact identification of identified tumor markers is carried out by 2-D electrophoresis or liquid chromatography.^{70,71} In the future, biopsy samples analyzed for expression of such markers may allow *ex vivo* modeling of diseased pathways. Subsequent study

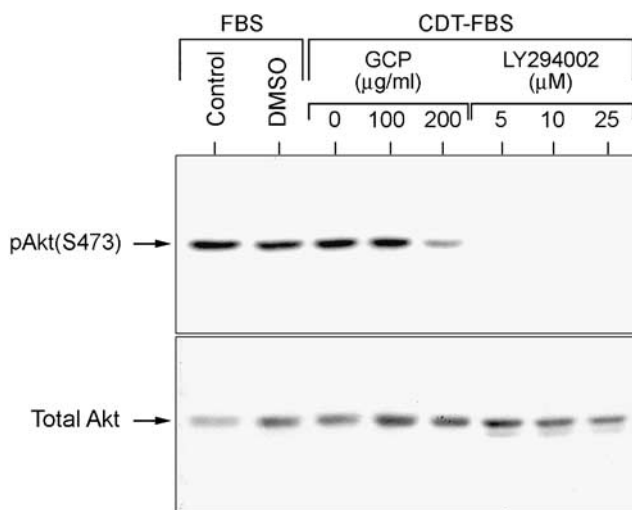


Figure 2 Western blot demonstrating the inhibition of Akt phosphorylation in the presence of GCP compared to LY294002 (Courtesy of Dr Clifford Tepper). GCP, genistein combined polysaccharide; DMSO, dimethylsulfoxide.

will eventually lead to individualized and targeted biologic therapy.

Conclusions

Akt inhibition is a rational therapy in CaP treatment. Upregulation of this pathway is involved in initial neoplastic changes in some patients. An increasing number of patients show overexpression or overactivity of Akt as metastatic and AI diseases develop. Preclinical studies demonstrate the importance of this pathway in CaP and the possibility of targeting this pathway with any of an increasing number of inhibitors. In addition to preventing activation by blocking upstream signaling, strategies include allosteric inhibition, small molecule competitive inhibitors, pseudosubstrate inhibitors and others with multiple or unknown activity.

Clinical studies with agents known to act through Akt inhibition show some promise. Further studies examining vertical inhibition strategies to block Akt pathways more completely should be performed. More importantly, efforts to extend the activity of current therapeutic options through combination with Akt inhibitors and more accurate methods to select those patients most likely to benefit from Akt inhibition are needed.

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Prostate cancer and markers of bone metabolism: diagnostic, prognostic, and therapeutic implications

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Abstract Knowledge of bone metastases complicating advanced prostate cancer (CaP) is increasingly relevant in patient selection for novel therapies. Current nuclear bone scintigraphy imaging has limited specificity for prostate metastases. As serum bone markers do correlate with bony lesions, they may play multiple roles in patients with advanced CaP. Currently, these markers play a role in prognostic nomograms for CaP. Recent studies suggest an expanding role for bone markers in the diagnosis and selection of patients for novel therapies. In the future, therapeutic roles for some of these marker pathways will emerge, eventually allowing greater individualization of patient care.

Keywords Androgen-independent prostate cancer · Bone markers · Bone metastases · Osteoprotegerin · Prostate cancer

Introduction

In advanced prostate cancer (CaP), metastatic deposits in bone are common and are the most frequent source of pain and morbidity [1]. “Skeletal related events” or SREs include vertebral compression fractures, complete vertebral collapse, spinal cord or spinal nerve entrapment, pathologic fractures, bone pain, and significant serum calcium abnormalities. These may be directly related to metastatic deposits in the bone, or be secondary to medical or surgical castration, which forms the foundation of current treatment strategies for metastatic CaP [2]. Osteoporosis due to castration is the more common cause, with only 7–16% of fractures being caused directly by metastatic lesions [3]. In addition, an association exists between the diagnosis of CaP and baseline osteopenia and osteoporosis prior to treatment or the development of metastatic disease [4]. Overall, yearly incidence of SREs is about 12% in patients with metastatic androgen-independent CaP [5].

Bone metabolism overview

Normal bone metabolism is distinguished by two opposing activities, which are coupled in both space and time and subject to tight control. The formation of new bone by osteoblasts and the resorption of old bone by osteoclasts are both constitutively active and the balance of these activities ultimately determines bone mass.

Bony metastatic lesions in CaP are classically thought of as osteoblastic or sclerotic lesions caused by a relative increase in bone formation. Though this is true for the majority of lesions, recent studies have indicated a therapeutic role for osteoclast inhibition in the prevention of SREs. Specifically, zoledronic acid has been shown to be the only

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effective bisphosphonate for decreasing SREs in patients with advanced CaP [6]. This activity by an osteoclast inhibitor in what are typically osteoblastic lesions emphasizes the close coupling of the action of these two cells.

Current diagnostic strategies for metastatic CaP in bone rely on nuclear bone scintigraphy scans. Unfortunately, these are expensive, require special equipment, are relatively cumbersome and expose the patient to radiation. As these scans detect increased bone formation, they are highly sensitive for detecting metastatic CaP lesions. However, they lack specificity and do not accurately measure treatment response as increased bone formation may correlate with healing areas of lytic lesions [7]. The need for more specificity in diagnostic algorithms for CaP bone metastases suggests the possibility of using bone markers, either alone, or in combination with imaging studies to increase the accuracy of diagnosis.

Bone markers are typically biologically inactive peptide fragments or small proteins cleaved from larger proteins and released into the blood during the formation or resorption of bone. A list of bone markers used in CaP appears in Table 1. Only those with most clinical relevance will be discussed here.

Table 1 Selected markers of bone metabolism relevant to CaP

	Markers	Measured in
Markers of formation	tALP	Serum
	bALP	Serum
	PINP/PIIINP	Serum
	OC	Serum
	PICP	Serum
Markers of resorption	BSP	Serum
	ICTP	Serum
	CTx	Serum
	NTx	Serum
	TRAP	Serum
	Deoxypyridinoline	Serum/Urine
	Pyridinoline	Serum/Urine
	Hydroxyproline	Urine
	N-terminal telopeptide of type I collagen	Serum/Urine
	Calcium:Creatinine ratio	Urine
Markers of osteoclastogenesis	OPG	Serum
	RANKL	Serum

bALP Bone alkaline phosphatase; *BSP* bone sialoprotein; *CaP* prostate cancer; *CTx* carboxy-terminal telopeptide; *ICTP* pyridinoline cross-linked carboxy-terminal telopeptide; *NTx* amino-terminal telopeptide; *OC* osteocalcin; *OPG* osteoprotegerin; *PICP* procollagen I carboxy-terminal propeptide; *PIIINP* procollagen III amino-terminal propeptide; *PINP* procollagen I amino-terminal propeptide; *RANKL* receptor activator of NF κ B ligand; *tALP* total alkaline phosphatase; *TRAP* tartrate resistant acid phosphatase

Formation markers

Among the markers of bone formation are alkaline phosphatase, procollagen peptide fragments (which are cleaved at the time of bone collagen formation), and osteocalcin (OC). Total alkaline phosphatase (tALP) has also traditionally been used as a marker for bone formation because of its wide availability as part of the comprehensive metabolic panel. However, it lacks true specificity for bone due to its presence in biliary, hepatic, and intestinal tissues. The bone isoenzyme form of alkaline phosphatase (bALP), a tetrameric protein located in the plasma membrane of osteoblasts, has been used to overcome this deficiency. The procollagen peptide fragments, on the other hand, are by-products of the extracellular cleavage of pro-collagen during bone formation and are specific for bone formation. These fragments are released into the circulation and are detected clinically using antibodies directed against the amino- (PINP) and carboxy- (PICP) terminal ends of procollagen I, or against the amino-terminal end of procollagen III (PIIINP) [8]. Finally, OC is a vitamin K-dependent, non-collagenous low molecular weight protein produced and released by osteoblasts. OC, also known as Gla protein, is widely deposited in the bony matrix and is the most abundant organic component of bone after collagen. Its presence in human sera is believed to be an index of osteoblastic activity and has been proposed as a useful marker for treatment response in metastatic CaP [9].

Resorption markers

Among the markers for bone resorption are the pyridinium compounds pyridinoline and deoxypyridinoline, both of which are found in Type I collagen and are amino acid derivatives. It has long been known that the tensile strength of bone is largely due to cross-linking of these derivatives in Type I collagen [10]. The deoxypyridinoline crosslink is felt to be specific to bone since it is only found in Type I collagen. On the other hand, pyridinoline is also present in Type II collagen, a component of articular cartilage. During bone resorption, these pyridinium cross-links are released into the circulation. Once released by the resorptive process, the pyridinium cross-links are not metabolized further; therefore, they represent degradative end products of mature collagen [11]. Another resorption marker is the collagen degradative product telopeptide, on which pyridinium cross-links are attached [12]. These include the amino- or carboxy-terminal telopeptide assay called N-telopeptide (NTx) and C-telopeptide (CTx) fragments, respectively. The commercial assay employs an antibody against the alpha-2 chain of Type I bone collagen fibrils. A related marker of a slightly larger telopeptide of the carboxy terminal is abbreviated ICTP [13]. Though some of these

markers may also be measured in urine, serum collection allows simultaneous measurement of other markers, is convenient in the setting of clinical trials, and may be more reproducible.

Osteoclastogenesis markers

A new class of bone markers may more closely reflect the biochemistry of the metastatic site by giving insights into cellular signaling. Osteoprotegerin (OPG), receptor activator of NF κ B (RANK), and receptor activator of NF κ B ligand (RANKL) are thought to belong to an important cytokine system controlling the process of osteoclastogenesis. Preclinical data confirms the importance of the relative concentration of these cellular signaling molecules in controlling the balance of bone formation and breakdown [14]. These markers may not only serve as a surrogate for osteoclastic activity but may prove to be important in future therapeutic interventions.

Bone markers: potential diagnostic applications

Because nearly all the bone markers discussed above show strong correlation with bone scan results in multiple studies, the use of bone markers as diagnostic instruments has been proposed. Various studies have shown promising results for ICTP [15], PINP [16, 17], CTx [17] and OPG [18]. Most studied markers demonstrate a wide range of results, which negatively impacts sensitivity and specificity. In addition, other conditions such as arthritis or bone healing secondary to trauma may further decrease specificity. Therefore, though mean bone marker concentration is of diagnostic significance for a population, individual patient counseling is difficult.

As mentioned previously, bone scintigraphy scans have high sensitivity but low specificity. Any effort to increase the specificity of diagnostic testing requires a highly specific “gold standard” for comparison. In this setting, only biopsy of each metastatic deposit would qualify as such a gold standard and this is obviously not feasible. Increase in lesion size on imaging of untreated patients may provide a surrogate marker but is also rather unhelpful, as most patients will be treated. Lesion shrinkage may not be seen on imaging with treatment as bone scans cannot discriminate between bone formation activity of metastatic disease and the healing process following response to treatment.

The strong rationale supporting bone markers as a diagnostic modality encourages efforts to find a surrogate marker verifying adequate specificity. One strategy is to acquire an ability to predict future metastatic deposits on imaging. If bone markers are able to do this, it may provide the needed proof of concept. At least one small study has demonstrated that increases in bone markers precede evi-

dence of bony metastatic lesions on bone scan by about 3 months [19]. This study measured urinary pyridinolines normalized by creatinine and should be repeated using other markers to confirm the concept. In the future, algorithms using bone scintigraphy in combination with prostate specific antigen (PSA) and various bone markers will maximize sensitivity and specificity in the diagnosis of CaP.

Bone markers: prognostic implications

Compared to proving diagnostic specificity, it is easier to demonstrate the ability of bone markers to stratify patients into prognostic categories. The use of bone markers in prognostic nomograms is well known. At least since 1992, tALP has been included in many nomograms predicting outcomes for different patient populations with CaP [20–23]. As discussed above, other bone markers demonstrate the ability to predict future bone metastases. This suggests they might have a prognostic role in predicting overall survival.

Correlative studies confirm the prognostic capabilities of some bone metabolism markers. Brown et al. [24] retrospectively examined bALP and NTx in large patient cohorts from phase III studies examining zoledronic acid in metastatic CaP and other neoplasms [25, 26]. In patients in the placebo arms of the trials, both NTx and bALP were statistically significant predictors of outcome, though NTx was superior [24]. Because of the results of the prospective phase III trial, zoledronic acid treatment is likely to be given to many patients with metastatic hormone refractory CaP [25]. As zoledronic acid significantly affects NTx levels, a second study by Cook et al. [27] examined all CaP patients using only baseline NTx and bALP levels. They found both markers were significantly associated with overall survival and progression-free survival, but only bALP was independently associated with overall survival on multivariate analysis. Dividing patients into quartiles based on bALP correlated strongly with overall survival and thus may be a useful prognostic tool for clinicians and patients.

Our group prospectively evaluated the prognostic and predictive significance of selected markers of bone metabolism in the context of a randomized phase II clinical trial of a matrix metalloproteinase inhibitor in hormone refractory CaP [28]. Markers of bone formation (OC, PINP and PIIINP) and resorption (NTx, pyridinoline and deoxypyridinoline) in serum were measured using commercial enzyme immunoassays. Marker values were dichotomized at the median and correlated with overall survival and progression-free survival by log-rank testing. Of eighty patients enrolled, 69 had evaluable baseline serum specimens.

We found that lower levels of tALP, NTx, deoxypyridinoline, OC, PINP, and PIIINP were all significant predictors of improved median and progression-free survival. In our study population, the significant prognostic value of these markers was not affected by bisphosphonate treatment. On multivariate analysis, log PINP and PIIINP remained significant as part of a model including hemoglobin and log PSA [29].

These retrospective and prospective clinical results should be prospectively validated in the phase III setting. Toward that end, we have initiated a molecular correlative study of the prognostic and possible predictive value of bone markers in patients with bone-metastatic hormone refractory CaP in conjunction with Southwest Oncology Group trial S0421. This phase III, placebo-controlled trial of docetaxel/prednisone with or without Atrasentan will randomize 706 patients with hormone refractory CaP and bone metastases, and provides an ideal setting to study bone markers. Earlier trials indicate Atrasentan's modest therapeutic effect is most significant in patients with bone metastases [30]. Serial measurements of bone markers will hopefully allow creation of validated prognostic nomograms. In addition, discovery of bone marker parameters predicting a beneficial response to Atrasentan will in the future allow appropriate patient selection for therapy.

In addition to predicting survival, bone markers may be able to predict response to treatment with androgen ablation. As discussed below, studies show a clear correlation of bone markers with treatment response on imaging and clinical improvement. With further refinement, new markers more closely reflecting actual bone biochemistry may predict not only overall survival for patient populations, but may allow for individualized treatment.

Bone markers: disease activity monitoring

As early as 1992, studies have examined the ability of bone markers to monitor the metastatic CaP response to androgen deprivation [9]. Clear correlation of bone marker changes on serial measurements with clinical response, whether based on imaging or PSA, has been conclusively demonstrated. Urinary pyridinolines [19], PICP [31], ICTP [15] and PINP [32] have all shown significant correlations. In addition, Koizumi et al suggest that the ratios of markers of bone formation may be helpful in following response to treatment [32].

In the future, bone markers may play a role in monitoring disease in patients with or without clear metastatic disease on bone scan. Though further studies are needed before this occurs, a strong rationale exists for using bone markers in this setting. As mentioned above, elevations in bone markers may precede evidence of bony metastatic lesions by up to three months. Thus, the usefulness of these

markers for monitoring disease is suggested, even in patients with no corroborating imaging.

Bone markers: therapeutic

The novel bone marker OPG, in contrast to most other markers, is a biologically active member of the tumor necrosis factor (TNF) superfamily [33]. As a decoy receptor for RANKL, it inhibits downstream signaling activating osteoclasts [14]. It may prevent apoptosis by inhibiting TNF related apoptosis inducing ligand or TRAIL [34]. Pre-clinical studies also suggest a role for OPG in promoting angiogenesis [35]. The variety of effects apparently related to this cytokine system suggest it not only provides a marker of diagnosis and prognosis, but may suggest therapeutic interventions [14, 34, 35].

A fully human monoclonal antibody against RANKL has recently entered clinical trials. Denosumab may be considered an OPG analogue in that it binds and inactivates RANKL. A phase III randomized, double blind, multicenter comparison of denosumab with the current reference standard, zoledronic acid, in patients with metastatic CaP began in April 2006. Results are eagerly anticipated.

The superiority of OPG as a marker, discussed below, may be due to the fact that it represents not only a byproduct of bone metabolism, but also an active participant in the microenvironment affecting metastatic growth and related bone turnover.

Bone marker comparisons

Few head to head comparisons of the various bone markers have been performed and those studies listed in Table 2 may sometimes yield contradictory results. This may be due to different patient populations and the fact that different bone markers measure different stages of bone metabolism and are affected differently by androgen deprivation [13, 16]. Nevertheless, some conclusions can be drawn. tALP and bALP have been the most studied and consistently demonstrate equivalent, and sometimes superior prognostic value compared to other markers [13, 27]. Of the other markers of bone formation, PINP may be preferable to use as a marker [16, 17, 29]. OC is clearly inferior to other markers [5, 13, 18].

Deciding which of the markers of bone resorption is most accurate is more difficult. One study comparing multiple bone markers suggests that OPG is most helpful for prognostic use [18]. As a marker of osteoclastogenesis, it may serve as a marker for osteoclast action while giving additional prognostic information based on tumor biochemistry. However, OPG is not, strictly speaking, a marker of bone resorption. Future studies should prospectively assess OPG in comparison to CTx, NTx, ICTP, and the pyridinolines.

Table 2 Selected clinical studies of bone markers in prostate cancer

Year of publication	Markers	Conclusions	Reference
1992	OC	No prognostic significance to baseline levels, questionable significant to changes with treatment	[9]
1996	Urinary pyridinoline, urinary deoxypyridinoline, tALP, OC	Urine markers correlate with response to treatment and predicted new lesions on bone scan	[19]
1997	PICP, ICTP	PICP correlated more closely with bone scan, but both decrease with treatment	[31]
1999	ICTP	ICTP effective diagnostic tool. Serial measurements useful for following disease	[15]
1999	bALP, ICTP, serum Calcium, parathyroid hormone, urinary Calcium:Creatinine ratio, urinary deoxypyridinoline	Resorption markers correlate best with pain scores	[36]
2000	tALP, urinary pyridoline/deoxypyridoline, urinary hydroxyproline, urine Calcium:Creatinine ratio, PICP, bone Gla protein, ICTP	Deoxypyridoline can predict SREs with high specificity. tALP as good as bALP because liver metastases are rare	[5]
2001	bALP, OC, PICP, ICTP, CTx, NTx, pyridinoline, deoxypyridinoline	Castration caused significant changes in bALP, NTx, CTx, and the pyridinolines. For initial diagnosis in the hormone-naïve, bALP, and deoxypyridinoline were best. Following hormone therapy, bALP and ICTP were superior	[13]
2001	PINP, PICP, bALP, OC, ICTP	All markers were higher in patients with metastases except OC. PINP correlated best with disease extent	[16]
2002	PINP, bALP, OC, ratios of all three	Ratios of markers may be useful	[32]
2003	BALP, PINP, urinary NTx, urinary CTx, serum CTx, ICTP	PINP and serum CTx demonstrate 100% sensitivity and specificity	[17]
2004	OPG	OPG may be effective for following disease	[34]
2004	tALP, bALP, PINP, OC, BSP, CTx, NTx, TRAP, RANKL, OPG	OPG was an independent predictor of CaP death	[18]
2005	Urinary NTx, tALP	Both markers predict outcomes on univariate, but not multivariate analysis	[37]
2005	OC, PINP, PIIINP, NTx, pyridinoline, deoxypyridinoline	Baseline levels of PINP and PIIINP were independent predictors of survival. Bisphosphonate use did not affect this	[29]
2005	Urinary NTx, bALP	NTx effective for predicting SREs and disease progression in patients on zoledronic acid	[38]
2005	Urinary NTx, bALP	NTx better than bALP for predicting disease progression in patients on placebo	[24]
2006	Urinary NTx, bALP	bALP independent predictor of outcomes, better than NTx in CaP	[27]

Markers in bold indicate serial measurements were performed

bALP Bone alkaline phosphatase; *BSP* bone sialoprotein; *CaP* prostate cancer; *CTx* carboxy-terminal telopeptide; *ICTP* pyridinoline cross-linked carboxy-terminal telopeptide; *NTx* amino-terminal telopeptide; *OC* osteocalcin; *OPG* osteoprotegerin; *PICP* procollagen I carboxy-terminal propeptide; *PIIINP* procollagen III amino-terminal propeptide; *PINP* procollagen I amino-terminal propeptide; *RANKL* receptor activator of NF- κ B ligand; *tALP* total alkaline phosphatase; *TRAP* tartrate resistant acid phosphatase

Conclusions

Markers of bone metabolism play a role in current prognostic nomograms for CaP. In the future, their role will increase, especially in the areas of earlier diagnosis of bony metastatic disease and the monitoring of therapeutic interventions. Bone markers will extend the specificity of current diagnostic imaging techniques and panels of bone markers may reach sufficient accuracy to be used alone. Serial measurements of bone markers will allow greater insight into the action of novel therapies currently under development for CaP. Finally, bone markers of metabolic activity, such as OPG, will suggest future strategies that will allow individualization of oncologic therapy.

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