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**The Androgen Receptor and Signal Transduction Pathways in
Hormone Refractory Prostate Cancer: Modifications to the Androgen
Receptor (part 1).**

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Abstract: Prostate cancer is the second most common male malignancy in the western world an increasing incidence in an ageing population. Treatment of advanced prostate cancer relies on androgen deprivation. Although the majority of patients initially respond favourably to androgen deprivation therapy, the mean time to relapse is 12-18 months. Currently there are few treatments available for men who have developed resistance to hormone therapy, due to the lack of understanding of the molecular mechanisms underlying development of this disease.

Recently, however, major advances have been made in understanding both androgen receptor (AR) dependent and independent pathways which promote development of hormone resistant prostate cancer. This review will focus on modifications to the AR and associated pathways. Molecular modifications to the androgen receptor itself, e.g. mutations and/or amplification, although involved in the development of hormone resistance cannot explain all cases. Phosphorylation of AR, via either Ras/MAP kinase or PI3K/Akt signal transduction pathways, have been shown to activate AR in both a ligand (androgen) dependent and independent fashion. During this review we will discuss the clinical evidence to support AR dependent pathways as mediators of hormone resistance.

Keywords: androgen receptor, hormone resistance, MAP kinase, PI3K and PKC.

INTRODUCTION

Prostate cancer is an extremely prevalent disease with 25,000 new cases diagnosed in the UK and 198,000 in the USA each year [1]. Despite progress in early diagnosis, due to increased awareness and PSA screening, prostate cancer remains the second leading cause of male cancer specific mortality in western countries [2].

Treatment of prostate cancer varies depending on the stage of the disease, treatment of advanced or metastatic disease relies on androgen deprivation therapy, the principle of which has remained unchanged for the past 55 years [3]. Androgen deprivation can be achieved surgically, by castration, or chemically, by suppression of androgen production using luteinising hormone-releasing hormone (LH-RH) agonists. This may be combined with an androgen receptor (AR) antagonist to achieve maximal androgen blockade. AR antagonists inhibit the action of adrenal steroids [3] and block the “flare” caused by the initial stimulatory action of LH-RH agonists on steroid production. Initial response rates to androgen deprivation therapy are high, however patients generally relapse within 18-24 months [3] with rising circulating PSA concentrations characteristic of renewed tumour growth. This is indicative of the development of hormone refractory prostate cancer, a process also termed androgen escape. At present, there are no effective treatment options for patients who develop androgen escape. **Although clinical trials with only antiandrogens therapy have been demonstrated to improve quality of life of the patient although it does not alter survival [4].** In addition alternative approaches such as chemotherapy and radiotherapy do not provide significant disease control, so treatment is generally palliative aimed at maintaining quality of life by decreasing bone pain and urinary tract obstruction [3]. The development of androgen escape is therefore one of the most significant events in the progression of prostate cancer, resulting in increased risk of

morbidity and reduced survival. The median survival of patients following development of androgen escape is 12 months [3]. The high rate of prostate cancer mortality is thus strongly linked to both development of androgen escape and a lack of alternate therapies.

The lack of novel and effective therapies to treat androgen escape is due to poor understanding of the molecular mechanisms underlying development of both the primary disease, and more particularly, those events which drive androgen escape. Previously the majority of research investigating the development of androgen escape focused on the AR, however, more recently research has focused on distinct molecular pathways which modulate AR function [5]. Identification of such pathways will provide novel therapeutic targets for treatment of patients with androgen escape [3].

ACTIVATION OF THE ANDROGEN RECEPTOR

AR ligands belong to a family of steroid hormones known as androgens and include both testosterone and dihydrotestosterone (DHT). Both testosterone and DHT can bind to the AR although DHT has a higher binding affinity (approximately 10 times) than testosterone, and is consequently the primary androgen bound by AR [5]. Testosterone is produced by testicular Leydig cells, and is converted to DHT by 5 α -reductase type 2 when it enters prostate epithelial cells.

Ligand free AR is sequestered in the cytoplasm bound to heat shock proteins (HSP70 & HSP90). Heat shock proteins function to stabilise the AR during folding, protecting it from degradation. DHT binds to the AR inducing a conformational change, which allows dissociation of heat shock proteins (HSP-70 and HSP-90). The AR then forms a homo-dimer and is phosphorylated at several sites including the PKC phosphorylation sites Ser⁸¹ and Ser⁶⁵⁰, plus Ser⁶⁶², Ser⁹³, Ser⁸⁰³, but excluding the

MAP kinase and AKT specific phosphorylation sites (Ser⁵¹⁵ and Ser²¹⁰, respectively)[6,7]. This phosphorylation of the receptor stabilises the ligand/receptor homodimer, marking the ligand-receptor complex for translocation to the nucleus. The AR-ligand complex then initiates gene transcription by binding to specific androgen response elements (AREs) in the promoter regions of target genes. Following DNA binding, the RNA polymerase machinery is recruited to the initiation site and transcription of AR regulated genes begins [5] (Fig. 1).

HORMONE RESISTANT PROSTATE CANCER

The function of androgen deprivation therapy is to prevent the activation of AR mediated gene transcription by removing or competing with the endogenously produced steroids. Failure of androgen deprivation therapy, observed in hormone resistant prostate cancer, was therefore logically linked to modification of the action of the AR. Early research into the failure of androgen deprivation therapy centred on investigations of molecular alterations to the AR. However, more recently, there has been a shift to suggest that post-translational modification of the AR, via phosphorylation at specific sites may influence the development of androgen escape. In the remainder of this review we summarise our current understanding of the molecular mechanisms underlying androgen escape with particular emphasis on evidence from clinical observations in human prostate cancers.

ANDROGEN RECEPTOR MUTATIONS

The first AR mutation was identified, in 1992, in LNCaP cells at T877A and was closely followed by H974Y in the hormone refractory prostate cancer xenograft CWR22. Both these mutations were in the AR ligand-binding domain (LBD) [8]. *In*

vitro research has demonstrated that AR mutations in the ligand binding domain alter ligand binding affinity and receptor transcriptional activity [9,10]. Activation of the mutated AR by non-androgenic steroids (corticosteroids) or indeed by androgen antagonists [5] mirror similar findings of tamoxifen resistance in breast cancer. Specific mutations of the AR are associated with hydroxyflutamide and bicalutamide resistance [11,12]. Taplin et al. demonstrated that AR mutations may occur in response to hydroxyflutamide therapy. Patients on combined androgen blockade with hydroxyflutamide as the AR antagonist, had mutations in the AR cause hydroxyflutamide to stimulate growth. However if therapy was changed to bicalutamide growth was again inhibited. However patients that were not on hydroxyflutamide did not develop these mutations [12]. AR mutations in the hinge region have also been identified that impact on the effectiveness of therapy and are thought to play a role in the emergence of hormone refractory disease [13]. However, AR mutations are found more commonly *in vitro* than *in vivo*, AR mutations are detected in 10-20% of prostate cancer specimens [5,11] with mutations more common in hormone escaped specimens compared to hormone sensitive specimens [5,14]. Mutations in the LBD are more common in primary tumours compared to mutations in the N-terminal domain of androgen escaped tumours [15]. N-terminal mutations may allow AR binding interaction with co-activators without activation by a ligand, therefore providing a possible mechanism of androgen escape, whereas mutations in the LBD have been documented *in vitro* to allow activation of the AR by corticosteroids or AR antagonists [15-17]. Interestingly AR mutations are more commonly found in distant metastases [18] than in local relapses, even following development of hormone resistance, this may reflect a role of AR mutations in

promoting disease spread or may simply be a consequence of an increasingly unstable genome due.

AR mutations, in clinical prostate cancer, occur prior to androgen escape but do not preclude response to hormonal therapy. In our opinion, mutations in the ligand-binding domain may be responsible for the development of androgen escape in a small sub-group of patients. The low frequency of such mutations is likely to preclude the development of therapies specifically targeting AR mutations although this group might benefit from the use of antisense oligonucleotides directed against AR to down regulate expression of the mutant receptor [5].

ANDROGEN RECEPTOR AMPLIFICATION AND PROTEIN OVER EXPRESSION

AR gene amplification has been associated with hormone escape [19] and recently amplification rates have been shown to rise significantly in the transition from hormone sensitive to hormone resistant disease (0-5% in hormone sensitive tissue and 20-30% in hormone resistant tissue) [20]. Evidence from a number of sources suggests that, unlike chromosomal duplication (aneusomy), gene amplification results in increase protein expression via gene dysregulation. We recently demonstrated that AR gene amplification was seen in 20% of hormone escaped prostate cancer and the majority of these cases had a corresponding increase in AR protein expression (80%). However the remainder of cases in our cohort who acquired AR gene amplification on the transition to hormone escape had no increase in AR protein expression. Therefore AR gene amplification does not always result in increased AR protein expression [20]. In the same study we identified a further 22% of cases that had increased AR expression, in the absence of AR amplification [20]. Although first impressions suggest that the significant event in development of

hormone escape is an increase in AR expression and not AR amplification, further analysis suggests this might not be the case. It is AR amplification and not AR protein expression levels that influences patient survival in our cohort (Fig 2). This may be due to gene amplification being able to detect those cases with marked protein over expression more accurately than immunohistochemistry, supporting the hypothesis that AR over expression is important in AIPC. In breast cancer HER2 studies it has been demonstrated that HER2 gene amplification is the better predictor of survival, and is linked to marked increases in protein expression (over 100 fold). A study earlier this year using xenograft models, demonstrated that increasing AR protein expression induced androgen escape independent of AR amplification [21]. However, this does not reflect the mechanism of AR up regulation in clinical prostate cancers. More significantly this study also demonstrated that high AR protein expression sensitises prostate cancer cells to low circulating levels of androgens and enables the AR to be activated. This suggests that by increasing the dose of anti androgen therapy it may be possible to inhibit progression to androgen escape. However animal experiments have demonstrated that increased AR expression levels may allow anti-androgens to function as weak agonists, resulting in activation of the AR[21].

In conclusion although the significant event in the development of androgen escape is an increase in AR protein expression, it appears that only cases with AR amplification can express AR protein at levels that are high enough to have functional relevance. Increased AR expression may explain the development of hormone escape prostate cancer in only 20-30% of cases i.e. those who develop AR amplification, requiring the existence of alternative mechanisms to explain the 70-80% of AIPC cases with no AR gene amplification. Conversely AR gene

amplification may provide a powerful diagnostic tool for targeting AR over expression.

ANDROGEN RECEPTOR ACTIVATION BY PHOSPHORYLATION

In 1990 van Laar et.al. were the first to report phosphorylation of the AR[22]. When AR is first synthesised it is in a non-phosphorylated state, however after about 15 min, even in the absence of a ligand it becomes phosphorylated at a few sites. When DHT binds to the AR it promotes recruitment of protein kinases resulting in phosphorylation of multiple serine residues (Ser^{80, 93 and 641})[22]. This phosphorylation protects the AR from proteolytic degradation and stabilises AR homodimers [22]. In addition to the protective and stabilisation role of AR phosphorylation, evidence is now emerging that demonstrates phosphorylation of the AR at specific sites can influence transactivation [6]. AR transcriptional activity correlates strongly with phosphorylation of specific serine residues (e.g. Ser^{213, 506 & 650})[23], only one of which is phosphorylated following DHT binding. Phosphorylation of the AR therefore appears to serve multiple functions, including both activation and stabilisation. It is currently unclear whether differing functions are linked to different phosphorylation sites or whether phosphorylation at certain sites is pluripotent. However, since phosphorylation of the receptor can be induced by multiple mechanisms, it is likely that AR phosphorylation is a multistage process.

In the physiological situation phosphorylation by MAP and Akt kinases sensitises the AR to low circulating levels of DHT such as those present during maximum androgen blockade [23]. This sensitisation allows low levels of androgens or indeed alternative steroids (e.g. oestrogen or anti androgens) to induce phosphorylation at specific sites required for translocation of the AR to the nucleus. Several proteins have previously been demonstrated to phosphorylate AR *in vitro*,

resulting in increased AR transactivation, including MAP kinase, Akt and Protein Kinase C (PKC)(Fig 3) [23,24]. It is unclear at the moment whether phosphorylation by these kinases occurs prior to DHT binding or if this is a separate event that occurs following translocation to the nucleus to increase AR transcriptional activity at sub-optimal DHT concentrations.

MAP kinase levels are raised in hormone refractory cell lines. Phosphorylation of the AR by MAP kinase, positively modulates expression of AR target genes, helps the recruitment of AR co-factors and increases prostate cancer cell growth [23]. Activated MAP kinase correlates with advanced stage and grade in prostate cancer and cell line studies demonstrate that MEK and MAP kinase are expressed and activated during prostate cancer progression [25]. Cell line studies also demonstrate that transfection with Ras results in increased expression and activation of MAP kinase resulting in the development of hormone escape [26]. It has been demonstrated *in vitro* using LNCaP cells that phosphorylation of AR at Ser⁵¹⁵ by MAP kinase results in hyper-sensitisation to low levels of the synthetic androgen R1881 [26]. It therefore may be possible that during maximum androgen blockade in the physiological situation, that AR phosphorylation by MAP kinase induces hypersensitivity to DHT and also increases recruitment of AR co-factors and AR transcription activity..

Akt also known as protein kinase B, is a downstream member of the PI3K/PTEN cascade, a pathway with an important role in cell growth, adhesion and migration in a variety of cell types [27]. Akt is frequently activated in cancer cells and provides not only growth and survival signals for prostate cancer cells, but also modulates AR activity via phosphorylation (Fig. 3)[27]. There are 3 members of the Akt family, Akt 1, 2 and 3, and there is evidence that the 3 Akt isoforms may play

different roles in the development of hormone resistant breast cancer via interactions with the oestrogen receptor. Akt may play a similar role in the development of androgen escape [28] as Akt has been demonstrated to phosphorylate the AR at Ser²¹⁰ and Ser⁷⁹⁰ and to modulate its transcriptional activity in prostate cancer cell lines (Fig. 3) [29]. During androgen ablation or anti androgen treatment the LNCaP cells undergo growth arrest and apoptosis, and up-regulation of Akt activity appears to compensate for this [30]. Androgen independent LNCaP cells have high basal Akt activity (more than 20 fold higher than sensitive cells)[27], suggesting that removal of androgens by androgen deprivation therapy may result in increased activation of the PI3K pathway and promote development of androgen escape [29]. This hypothesis is supported by data that demonstrates that up-regulation of the PI3K cascade upon loss of androgen signalling contributes to failure of androgen ablation therapy [31].

In vivo the PI3K/Akt pathway may be up-regulated due to loss of PTEN which acts by dephosphorylating inositol lipids at the D3 position of the inositol ring reducing Akt activation [32]. PTEN deletions or inactivating mutations, result in Akt activation, which can induce cellular transformation [32]. Inactivating PTEN mutations are commonly found in prostate cancer and loss of PTEN expression is associated with increased risk of recurrence[32]. We hypothesise that loss of PTEN function or expression, combined with amplification and increased expression of PI3K results in increased Akt expression and activation, resulting in increased prostate cancer cell survival and growth and promoting the development of androgen escape.

In summary, data gained predominantly from *in vitro* studies, suggest that over-expression and activation of Akt can trigger prostate cancer androgen escape via activation of AR. Akt expression in tumours is significantly higher than in BPH, and

the intensity of Akt expression correlates with PSA levels [28]. It has also been reported that only 10% of well-differentiated prostate tumours strongly express phosphorylated Akt compared to 92% of poorly differentiated tumours [29]. As advanced prostate cancer is often accompanied by androgen escape this suggests that Akt activity may be up-regulated in androgen insensitive clinical specimens [33]. These data are however, inconclusive, and to date no comparison has been made between androgen sensitive and insensitive tissue from the same patient.

PKC is a phospholipid-dependent cytoplasmic, serine/threonine kinase, which may be activated by TGF- β , phorbol esters e.g. phorbol 12-myristate 13-acetate (PMA) or intercellular activators such as calcium and diacylglycerol [34](Fig. 3). There are PKC phosphorylation consensus sites on the AR at Ser^{81, 650} [35] and PMA treatment has been reported to increase the phosphorylation of AR at Ser⁶⁵⁰ [35]. This suggests that activation of PKC signalling can affect AR phosphorylation. However there is again little clinical evidence, at present, to support a role of PKC in development of androgen escape and the PKC phosphorylation consensus sites on the AR are also phosphorylated when DHT binds [35]. It may therefore be the case that phosphorylation of the AR at Ser^{81, 650} may be required for activation of the receptor, via DHT binding.

In summary, a number of signalling pathways act, either synergistically or in isolation, to modify AR activity through phosphorylation at multiple distinct sites. AR phosphorylation both stabilises and modifies activity of the receptor and whilst there is strong evidence for both activities the specific role of many phosphorylation sites remains unclear at present. Whilst clinical androgen escape may be a result of sensitisation of the prostate cancer cell to low circulating levels of androgens present during maximum androgen blockade, achieved via either AR stabilisation or

activation through this mechanism, clinical evidence for these pathways being active *in vivo* remains sparse. However, sufficient evidence exists to suggest that hormone escape may be mediated via phosphorylation of the AR by a variety of kinases including MAP kinase and Akt. Further *in vivo* investigations are required to confirm this and to identify which of these pathways may provide specific molecular targets for future therapeutic intervention.

CONCLUSION

Whilst androgen ablation therapy is a highly successful for hormone sensitive prostate cancer, the rapid onset of hormone resistance in the majority of cases significantly limits the benefits derived from this therapy. Currently there is no effective standard treatment for androgen resistant prostate cancer. Previously research efforts have suggested that molecular modification of the AR gene was likely to underlie development of androgen escape. However, within the past 5 years we have witnessed a major shift in our understanding of the mechanisms underlying androgen escape. It is now apparent that the control of AR function involves post-translational modification, generally via phosphorylation by various kinase) cascades.

Currently evidence from the literature suggests that the dominant pathways involved in the development of androgen escape are the MAP kinase and PI3K pathways (Fig. 3). These pathways act to directly modify the AR, altering its sensitivity to both androgens and anti-androgens. The pluripotent nature of these pathways suggests that molecular targeting of specific kinases (e.g. AKT or MAP kinase) within these pathways could reverse hormone resistance via several mechanisms. Further research into the role of these pathways in clinical samples should be the immediate focus for future research.

However, despite the current shift away from molecular targeting of the AR it should be remembered that one in five hormone resistant prostate cancers show AR gene amplification, this may lead to screening of patients for AR amplification prior to implementation of therapies.

Perhaps the most significant advance in our understanding of the molecular genetics of hormone relapse is the recognition that, with multiple pathways being shown to be involved in this process, the hormone resistant tumour population must represent several molecular sub-groupings. In effect the AR amplified patient cohort (20%) identified the first molecular sub-typing of this disease. Data from molecular profiling of hormone resistant prostate cancer identifies separate groupings with activation of the Ras/Raf/MAP kinase and Akt/PI3K pathways. Each of these molecular sub-groups is likely to require a separate therapeutic approach. It therefore now seems likely that future therapy for prostate cancer could involve profiling of individual tumours in order to identify appropriate therapies. Matching the therapeutic agents to the molecular profile of the tumour has proven to be a highly effective strategy in other cancers. If this approach can be successfully applied to prostate cancer we may, at last, see significant therapeutic benefit for hormone relapsed prostate cancer sufferers.

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FIGURE LEGENDS

Figure 1 shows how testosterone (T) enters the prostate epithelial cell and is converted to dihydrotestosterone (DHT) which binds to and activates the androgen receptor (AR). HSP denotes heat shock proteins and P denotes phosphorylation.

Figure 2 shows kaplan-meier curves for patients with and without AR gene amplification (Figure 2 a) and AR protein expression (figure 2b).

Figure 3 shows how the MAP kinase, Akt and PKC pathways impacts prostate cancer growth. AR denotes androgen receptor, HSP denotes heat shock proteins, and P denotes phosphorylation.

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